Partial Characterization of the Sexual Agglutination Factor from *Hansenula wingei* Y-2340 Type 5 Cells†

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ABSTRACT: The sexual agglutination factor on *Hansenula wingei* Y-2340 type 5 cells (5-agglutinin) was purified by affinity chromatography and gel filtration after its release from the cell surface by subtilisin digestion. The preparation had a molecular weight of 9.6×10^5, and it was composed of 85% carbohydrate (mostly mannose), 10% protein, and 5% phosphate. The protein part contained 55% serine and 6–8% threonine, and 85% of these two amino acids was destroyed on treatment of 5-agglutinin with 0.1 N NaOH at 23° for 24 hr, conditions that promote β elimination of substituted serine and threonine units. During this treatment, 90% of the carbohydrate attached to the agglutinin was released as mannooligosaccharides with 1–15 sugar units, the principal fragment being the octasaccharide. Thus, 5-agglutinin is a novel glycoprotein in which 60% of the amino acids are substituted by carbohydrate, a structure very different from that of the total cell wall mannan–protein of *H. wingei* [P. H. Yen and C. E. Ballou (1974), *Biochemistry 13*, 2420] in which most of the carbohydrate is present as long branched polysaccharide chains attached to asparagine. In spite of these differences, the 5-agglutinin and the whole cell wall mannan gave similar results in their acetolysis patterns, methylation analysis, periodate consumption, and immunological studies, all suggesting that the linkages and configurations in the carbohydrate fragments were very similar. 5-Agglutinin was inactivated by digestion with Pronase or with a bacterial exo-α-mannanase, about one-third of the mannose being removed by the latter enzyme. Both Pronase and mannanase inactivation were prevented by the addition of a competing substrate. 5-Agglutinin was also inactivated by alkali probably as a result of the β-elimination reaction, by hot acid probably from hydrolysis of phosphodiester bonds, and by sodium periodate probably owing to oxidation of the mannose. Treatment of 5-agglutinin with dithiothreitol released a small binding fragment [N. W. Taylor and W. L. Orton (1968), *Arch. Biochem. Biophys. 126*, 912] with an amino acid composition very different from the original 5-agglutinin or the large inactive fragment that was also produced in the reaction. The small binding site component was calculated to contain 28 amino acids and 60 mannose units, giving a molecular weight of about 12,500. Attempts to dissociate the central inactive core into subunits were unsuccessful, although the mannanase digestion did produce fragments of a size suggesting that some dissociation occurred concomitant with the removal of the mannose. In general, our results suggest that the specificity of the binding sites resides in the structure of the protein part, but that both the protein and the carbohydrate contribute to maintenance of the biological activity of the agglutination factor.

Specific cell–cell interaction is a universal phenomenon and has been implicated in development (Okazaki and Holtzer, 1965), reassociation of sponge cells (Moscona, 1963), fertilization (Tyler, 1965), and conjugation in various microorganisms (Curtiss, 1969; Cohen and Siegel, 1963; Wiese, 1961) including yeasts (Brock, 1959). Of these, the sexual agglutination reaction in the yeast *Hansenula wingei* is the most thoroughly investigated as far as the structural basis for the interaction is concerned, and complementary macromolecules have been isolated from the cells of opposite mating type, 5 and 21 cells (Crandall and Brock, 1968). Both factors were purified by their ability to bind specifically to the cells of the other mating type.

The factor released from 21 cells by trypsinization was a glycoprotein with 35% carbohydrate, and it had a sedimentation constant of 2.9 S (Crandall and Brock, 1968). Although this factor did not agglutinate 5 cells, it did inhibit the agglutination reaction, and was apparently monovalent. It was inactivated by alkali, heat, high ionic strength, and protein denaturants, a suggestion that the specificity resided in the protein component.

A substance that specifically agglutinated 21 cells was released from 5 cells by subtilisin digestion (Taylor and Orton, 1967). This 5-agglutinin was a mannan–protein which contained 4% protein. Its molecular weight varied among preparations, ranging from 5.7×10^5 (Taylor and Orton, 1967) to 1.3×10^6 (Taylor and Orton, 1970). The 5-agglutinin activity was destroyed by pronase and by disulfide-cleaving reagents (Taylor, 1964; Brock, 1965). On reduction with thiols, approximately six 1.7S fragments of molecular weight 12,000 were released from 5-agglutinin of molecular weight 9.4×10^5 (Taylor and Orton, 1968). After fractionation, the large reduced component was not adsorbed by 21 cells, while the 1.7S fragments retained a weak specific binding activity (Taylor and Orton, 1971). It was proposed (Taylor and Orton, 1971) that the 5-agglutinin molecule consisted of a large central component to which six 1.7S "active site" fragments were joined by disulfide bonds. These active sites presumably were located at each apex of a regular octahedron and acted independently but more or less additively.

In this paper, further characterization of the 5-agglutinin from *H. wingei* 5 cells is described, and its structure is compared with that of the whole cell wall mannan. Studies on the chemical nature of the active site component of the 5-agglutinin and on the central core are also reported. The 5-agglutinin is shown to differ dramatically in structure from the total cell wall mannan, and the results suggest that the mannan–protein

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complex in the yeast cell wall may exist in many structurally and functionally differentiated forms. The results also indicate that, although the major component of the 5-agglutinin is carbohydrate, the specific binding activity resides in the protein part and the carbohydrate moiety serves to stabilize the binding sites. A preliminary report has been published (Yen and Ballou, 1973).

Experimental Section

Materials and Methods. The materials and general procedures are essentially the same as in the preceding paper (Yen and Ballou, 1974). Carrier free $H_2{}^{35}SO_4$ came from Schwarz/Mann Corp., Pronase (grade B) from Calbiochem, and protease (subtilisin BPN′) type VII from Sigma. Bio-Gel A 1.5m (200–400 mesh), Cellex-P, and Cellex-PAB were obtained from Bio-Rad Corp.

Molecular weights were measured in 0.03 M Tris buffer (pH 7.6) by high-speed sedimentation equilibrium according to Yphantis (1964) in a Beckman/Spinco Model E analytical ultracentrifuge with the Rayleigh interference optical system. A partial specific volume of 0.635 was used for 5-agglutinin, calculated assuming a composition of 90% carbohydrate ($\bar{v}$ = 0.625, Gray and Ballou, 1971) and 10% protein ($\bar{v}$ = 0.73).

For preparation of ^{35}S-labeled 5-agglutinin, cells were grown in the synthetic medium described by Taylor and Orton (1970), and 500 μCi of $H_2{}^{35}SO_4$ was added per liter.

Acetolysis was done according to Yen and Ballou (1974). The acetolysis pattern of a small quantity of material was obtained by reduction of the fragments with sodium borotritide. Acetolysis fragments from 5 mg of mannan were dissolved in 1 ml of 0.05 M NH_4HCO_3. To this solution was added 10 mg of sodium borohydride together with a small crystal of sodium borotritide in 1 ml of 0.05 M NH_4HCO_3. The reaction was carried out at 60° for 90 min and was stopped by the addition of Dowex 50(H^+). The resin was removed by filtration and the filtrate was evaporated to dryness. Boric acid in the sample was removed by repeated addition and evaporation of methanol. The residue was dissolved in 0.5 ml of water and fractionated on a Bio-Gel P-4 column (2 × 200 cm).

Agglutination Activity Assay. In the semiquantitative assay, serial dilution of the 5-agglutinin solution was made using the microtiter system manufactured by Cooke Engineering Co. Magnesium buffer (1% $MgSO_4 \cdot 7H_2O$ in 0.01 M KH_2PO_4, pH 5.5) was used as the diluent. To each of the dilutions was added one drop of a cell suspension containing 30 mg dry weight of heat-activated (100°, 30 min) 21 cells (21-H cells) per ml in the above buffer. The plate was shaken gently and left at room temperature (23°) for 30 min before being examined under a microscope. The highest dilution which showed detectable agglutination was taken as the activity of the solution.

In the quantitative assay, the binding of [^{35}S]-5-agglutinin to 21-H cells was measured. To an aliquot of [^{35}S]-5-agglutinin solution was added 0.5 ml of a 21-H suspension, 50 mg/ml in 0.1 M formate buffer (pH 4.0). After 30 min at 4°, the tubes were centrifuged and an aliquot of the supernatant was taken for counting radioactivity. For a nonspecific binding control, 5-H or diploid-H cells were used in place of 21-H cells.

Preparation of 5-Agglutinin. H. wingei 5 cells were grown in a 200-l. fermenter (Yen and Ballou, 1974), giving about 5 kg of wet cells which were stored frozen. A 50-ml suspension of cells, A_{650} of 100, was treated with subtilisin according to Taylor and Orton (1967) for release of 5-agglutinin, except that the digestion was done twice for 1 hr each time. The cells were removed and the crude 5-agglutinin in the extract was adsorbed at pH 4 to a 6 × 35 cm affinity column containing 40 g of 21 cells bound to cellulose (Taylor and Orton, 1968). The 5-agglutinin was eluted by shifting to a pH 1.8 buffer, and the combined 5-agglutinin peaks from three such affinity column isolations were purified on a 2 × 200 cm Bio-Gel A 5m column in 0.1 M KH_2PO_4. The yield of pure 5-agglutinin was about 50 mg.

Enzymes. In the studies of exo-α-mannanase digestion of 5-agglutinin, a solution containing 5 mg of [^{35}S]-5-agglutinin, 20 mg of bovine serum albumin, and 10^{-4} M $CaCl_2$ in 7.5 ml of 0.1 M phosphate buffer (pH 6.8) was incubated with 2.5 ml of exo-α-mannanase (59 units/ml) at 37°. After 3 days, the solution was concentrated by lyophilization and the residue was fractionated on an A 5m column (2 × 200 cm) at 4° with 0.1 M KH_2PO_4 as the eluent.

For Pronase digestion of 5-agglutinin, 5 mg of [^{35}S]-5-agglutinin was incubated with 12.5 mg of Pronase in 40 ml of 0.1 M phosphate buffer (pH 6.8) at 37°. After 90 min, the solution was concentrated and applied to an A 5m column at 4°. For exhaustive Pronase digestion, the incubation was carried out for 24 hr.

In the studies of the effects of various enzymes on the activity of 5-agglutinin, 25 μl of [^{35}S]-5-agglutinin (agglutination activity 256, 1200 cpm) and 25 μl of enzyme solution, 5 mg/ml, were incubated at 37° in 0.1 M phosphate buffer (pH 6.8). At different times, 0.5 ml of a 21 cell suspension, 50 mg of dry cells/ml in 0.1 M formate buffer (pH 4.0), was added to one of the incubation mixtures. After 30 min at 4°, the tubes were centrifuged and the radioactivity in the supernatant was counted. Diploid cells were used in place of 21 cells for a nonspecific binding control. The per cent inactivation was calculated as the cpm remaining in the supernatant of a sample after adsorption by 21 cells divided by the cpm remaining in the supernatant of the nonspecific binding control, multiplied by 100.

Periodate Oxidation. In a tube, 0.5 ml of 1 mM sodium periodate, 0.5 ml of 0.1 M sodium acetate (pH 4.0), and 0.5 ml of [^{35}S]-5-agglutinin (or Y-5 mannan), 0.2 mg/ml, were mixed and kept at 4° or at room temperature (23°) in the dark. Samples were removed at intervals and analyzed for periodate consumption and 5-agglutinin activity. In the 5-agglutinin activity control, water was added instead of periodate, while the blank contained sodium acetate buffer in place of the mannan.

Reduction of 5-Agglutinin. Partial reduction of 5-agglutinin with dithiothreitol was done according to Konigsberg (1972). To 2 ml of 0.002 M EDTA in 0.15 M Tris buffer (pH 8.5), 17.2 mg of [^{35}S]-5-agglutinin and 2.4 μmol of dithiothreitol were added. The reaction mixture was left at 25° for 2 hr. The free sulfhydryl group was then alkylated with 16 μmol of recrystallized iodoacetamide. After incubation in the dark for 20 min, the excess reagent was removed by dialysis against water in the dark at 4°.

Results

Investigation of the Properties of 5-Agglutinin. Preparation and Characterization of 5-Agglutinin. About 50 mg of pure 5-agglutinin was obtained from 50 g of wet 5 cell paste. Although most of the carbohydrate in the subtilisin extract passed through the affinity column, protein was retarded to a different extent (Figure 1). This probably resulted from the negative charge on the yeast cells. Protein contamination in the active peak eluted from the affinity column was removed on a Bio-Gel A 5m or by a Cellex-P column. On the Bio-Gel A 5m

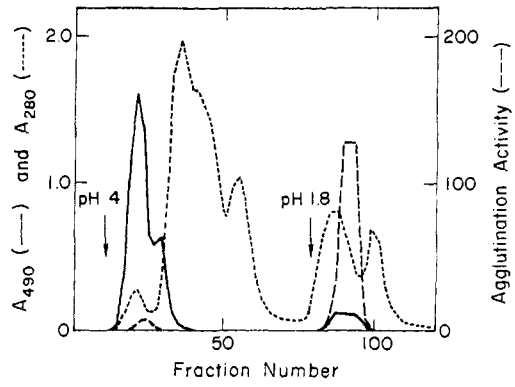

FIGURE 1: Elution pattern of crude 5-agglutinin preparation from an affinity column in which 21-H cells were covalently linked to cellulose. 5-Agglutinin was bound to the column at pH 4.0 and was eluted at pH 1.8. Fractions were assayed for carbohydrate (—), protein (- - - -), and agglutination activity (— —).

column (Figure 2), most of the carbohydrate and activity came out in a single peak near the void volume, whereas the protein contaminant was well separated from the 5-agglutinin. When 5-agglutinin from the affinity column was applied to a Cellex-P ion exchange column in 0.01 M citrate buffer (pH 3.5), about 18% of the carbohydrate and 5-agglutinin activity were retained on the column, indicating heterogeneity of charge. This activity was eluted at the beginning of a gradient of 0.01 M citrate (pH 3.5) to 0.05 M citrate (pH 3.5) in the presence of 0.4 M NaCl.

The purified 5-agglutinin gave one asymmetrical peak on sedimentation velocity runs, indicating some heterogeneity of size. Essentially all (99%) of a [^{35}S]-5-agglutinin preparation was absorbed by 21-H cells after the A 5m column purification step. Thus, although the preparations showed microheterogeneity with regard to size and charge, all of the material was biologically active in its binding property.

Chemical Composition and Physical Properties. 5-Agglutinin gave mannose and a trace of glucose, glucosamine, and mannose 6-phosphate on acid hydrolysis. Table I lists the compositions of several preparations, the average being about 85% carbohydrate, 10% protein, and 5% phosphate. Thus, 5-agglutinin, like the cell wall mannan, is a phosphomannan. Both the mannose to phosphate ratio and the protein content, which was calculated from the amino acid composition, varied among the preparations. However, the amino acid compositions of these preparations were essentially the same, and one of them is shown in Table II. The most striking feature

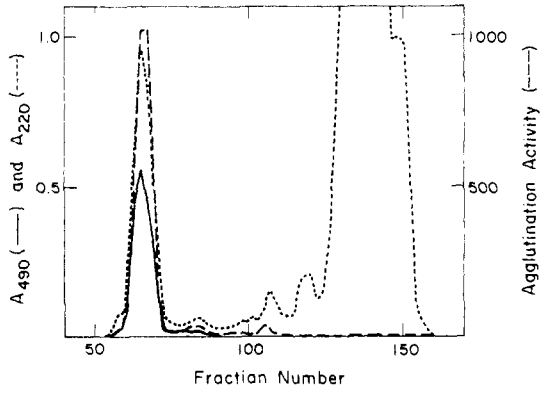

FIGURE 2: Gel filtration on a 2 × 200 cm column of Bio-Gel A 5m of 5-agglutinin isolated from the affinity column. Fractions were assayed for carbohydrate (—), protein (OD_{220}, - - - -), and agglutination activity (— —).

TABLE I: Chemical Composition of 5-Agglutinin.

Component	Preparation Composition (%)[a]			
	I	II	III	IV
Carbohydrate	87.0	81.5	84.0	85.4
Protein	9.2	9.5	8.8	12.2
Phosphate (PO_4)	3.7	9.1	7.2	2.4

[a] Preparations I and II were both peak I from a Bio-Gel A 5m column (see Figure 2); preparations II and IV were peak I and peak II, respectively, from a Cellex-P column.

is that more than half of the residues of 5-agglutinin were serine, and together with threonine, these hydroxy amino acids contributed nearly two-thirds of the total. This is quite different from Y-5 mannan in which one-third of the residues were hydroxy amino acids (Yen and Ballou, 1974).

The protein content of 5-agglutinin found in this study (10%) is considerably higher than that reported by Taylor and Orton (1967), who used the Lowry method probably with bovine serum albumin as the standard. Because 5-agglutinin has a very low content of aromatic amino acids, the Lowry method would give a deceptively low value. Although glycoproteins often have a high content of hydroxy amino acid, the amount of serine and threonine in 5-agglutinin is exceptional. Recently, a glycopeptide from the coat of mouse TA3 cells was reported to have a similarly high serine and threonine content (Codington *et al.*, 1972).

Sedimentation coefficient and molecular weight determinations on one 5-agglutinin preparation showed a strong concentration dependence, with increased values as the concentration decreased. At zero concentration a $s^0_{20,w}$ value of 15.4 and a molecular weight of 9.61×10^5 were found.

Acetolysis. For acetolysis studies of small amounts of substance, the products were reduced with sodium borotritide

TABLE II: Amino Acid Composition of 5-Agglutinin Before and After β Elimination.

Amino Acid[a]	Before	After
Aspartic acid	2.91	3.88
Threonine	8.89	3.10
Serine	52.40	7.14
Glutamic acid	7.16	9.75
Proline	1.96	2.54
Glycine	1.68	2.50
Alanine	5.20	45.53
Half-cystine	0.45	0.00
α-Aminobutyric acid	0.00	1.68
Valine	6.51	8.05
Methionine	0.00	0.00
Isoleucine	3.52	4.13
Leucine	3.57	4.46
Tyrosine	1.09	1.42
Phenylalanine	0.69	0.86
Histidine	0.16	0.30
Lysine	0.39	0.27
Arginine	1.21	1.22
Glucosamine	2.07	2.63

[a] Residues per 100 residues.

TABLE III: Methylation Analysis of Y-5 Mannan and 5-Agglutinin.

	Molar Ratios of the Partially Methylated Alditol Acetates							
	Tetra-*O*-methyl	Tri-*O*-methyl				Di-*O*-methyl		
Substance	2,3,4,6	3,4,6	2,4,6	2,3,4	Total	3,4	2,4	Total
Y-5 Mannan	1.00	1.32	1.32	0.74	3.38	0.34	0.56	0.90
5-Agglutinin	1.00	0.95	1.50	0.52	2.97	0.40	0.79	1.19

before gel filtration. However, oligosaccharides which differ in chain length might be labeled differently owing to differences in the isotope effect. Therefore, one portion of the acetolysis product was applied to the P-4 column and carbohydrate in the fractions was assayed directly. Another portion of the same product was reduced with sodium borotritide before P-4 column chromatography, and radioactivity in the fractions was assayed. The molar ratios of the oligosaccharides obtained by these two methods were essentially the same, indicating that all of the oligosaccharides were labeled to the same extent under the conditions employed. Thus, reduction of the acetolysis products with sodium borotritide offered a sensitive way to determine the acetolysis pattern with small amounts of material.

Gel filtration of the reduced neutral acetolysis products of 5-agglutinin and Y-5 mannan gave the same five peaks (Figure 3). When chromatographed on paper with solvent system B, the corresponding peaks had identical migration rates. However, the peak ratios differed slightly, which implied structural differences in the carbohydrate portions of these two macromolecules.

Methylation, Periodate Oxidation, and Mannanase Digestion. Methylation analysis of 5-agglutinin gave results similar to that of Y-5 mannan (Table III). The data show that the mannose units were linked 1→2, 1→3, and 1→6; and the presence of both 3,4- and 2,4-di-*O*-methyl derivatives indicates that mannose units at the branch points were substituted at positions 6 and 3 as well as at positions 6 and 2. The ratio of the sum of the tri-*O*-methyl derivatives to the tetra-*O*-methyl derivative was about 3, giving an average chain length of 5. Thus, in 5-agglutinin, as in Y-5 mannan, the carbohydrate portion was highly branched.

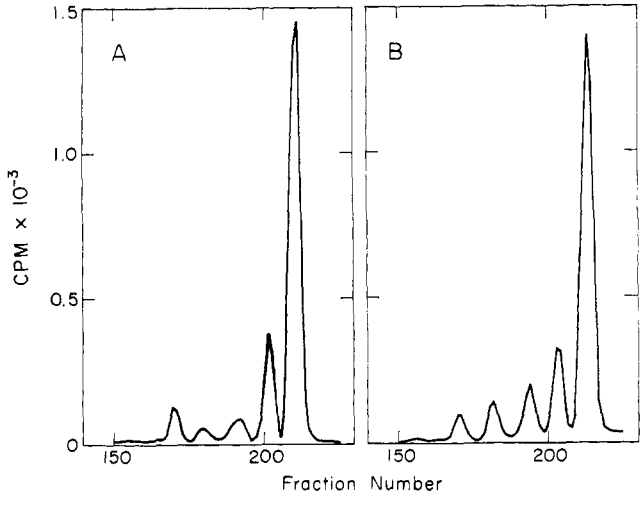

FIGURE 3: Gel filtration on a 2 × 200 cm column of Bio-Gel P-4 of the reduced acetolysis products of (A) Y-5 mannan and (B) 5-agglutinin. The tracing represents molar ratios based on radioactivity incorporated by reduction with $NaBT_4$.

Periodate oxidation of 5-agglutinin at room temperature for 6 days resulted in consumption of 0.72 mol of periodate/mannose unit, a result similar to that for Y-5 mannan. However, 38% of the carbohydrate in 5-agglutinin was released by exo-α-mannanase digestion in contrast to 22% for Y-5 mannan. Thus, more of the mannose in 5-agglutinin was accessible to the enzyme, although not to periodate oxidation.

β Elimination. After β elimination of 5-agglutinin, only 13% of the carbohydrate remained associated with the peptide chain which was recovered from a Bio-Gel A 1.5m column (Figure 4). Chromatography of the main carbohydrate peak (peak II) on a P-4 column gave a mixture of oligosaccharides ranging in size from 1 to about 15 sugar units (Figure 5). The nature of the radioactive peak at fraction 160 is not known, and it was also observed in the β-elimination products of Y-5 mannan. When chromatographed on paper with solvent system B, peaks II, III, IV, and V migrated with the corresponding reduced acetolysis fragments from Y-5 mannan, mannobiose, mannotriose, mannotetraose, and mannopentaose, respectively. The acid hydrolysates of peaks III, VI, IX, and XI all gave one major spot of radioactivity which migrated with mannitol on paper chromatography. Acetolysis of peak VIII gave five radioactive peaks which cochromatographed with the five reduced acetolysis fragments obtained from intact mannan. Thus, the β elimination oligosaccharides apparently were built up from the same five acetolysis fragments found in Y-5 mannan, and all had mannose at the reducing end.

The amino acid compositions of 5-agglutinin, before and after β elimination, are shown in Table II. During the reaction, 87% of the serine was destroyed, of which 89% was converted to alanine by reduction. The threonine content decreased by 65%, of which 29% was converted to α-aminobutyric acid. Thus, in contrast to Y-5 mannan, 5-agglutinin

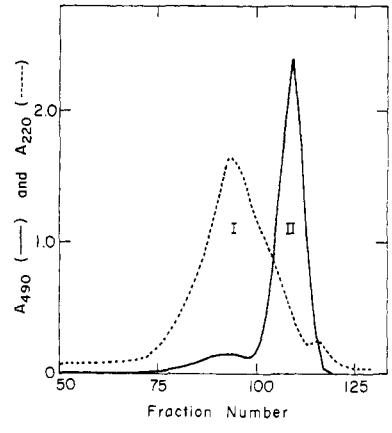

FIGURE 4: Gel filtration of 5-agglutinin after β elimination on a 2 × 100 cm column of Bio-Gel A 1.5m eluted with 0.1 N KH_2PO_4. Fractions were assayed for carbohydrate (——) and protein (OD_{220}, - - - -).

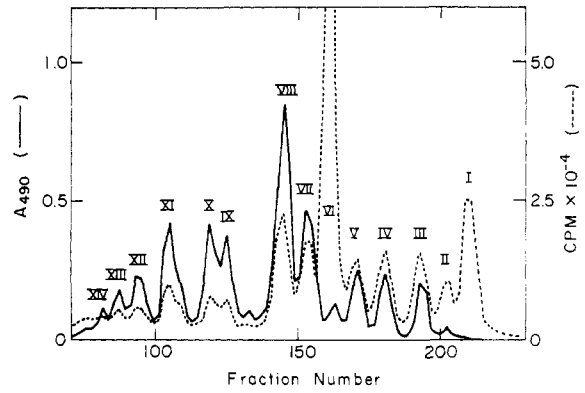

FIGURE 5: Gel filtration of peak II from Figure 4 on a 3 × 200 cm column of Bio-Gel P-4 eluted with 0.1 N NH_4HCO_3. Fractions were assayed for carbohydrate (——), and ^3H radioactivity (- - - -). The large peak of radioactivity at fraction VI was not identified. Peak I is mannitol and the other peaks are reduced mannooligosaccharides.

had most of its carbohydrate as short oligosaccharides linked to the peptide chain through the *O*-glycosidic linkage, and half of the amino acid residues were involved in such linkage. That portion of carbohydrate which remained associated with the peptide chain after β elimination probably was attached to the protein through the *N*-acylglycosylamine linkage, although this was not directly established.

Immunochemistry. 5-Agglutinin and Y-5 mannan gave similar precipitin curves with Y-5 antiserum (Figure 6). Oligosaccharides obtained from Y-5 mannan by acetolysis inhibited no more than 30% of the precipitin reaction between Y-5 antiserum and 5-agglutinin (Figure 7). As in the precipitin reaction with the Y-5 mannan, mannotriose was the best inhibitor. After mild acid treatment, 5-agglutinin gave only 30% of the original precipitin reaction, again a result similar to that found for Y-5 mannan. Thus, 5-agglutinin and Y-5 mannan contain very similar antigenic determinants, with 30% of the reaction being directed against αMan(1→3)α-Man(1→3)Man, and 70% of the antigenic reactivity being associated with some unidentified acid-labile structure.

Investigation of the Active Binding Site of 5-Agglutinin. To determine whether the carbohydrate or protein part, or both, were involved in the activity of 5-agglutinin, the effects of

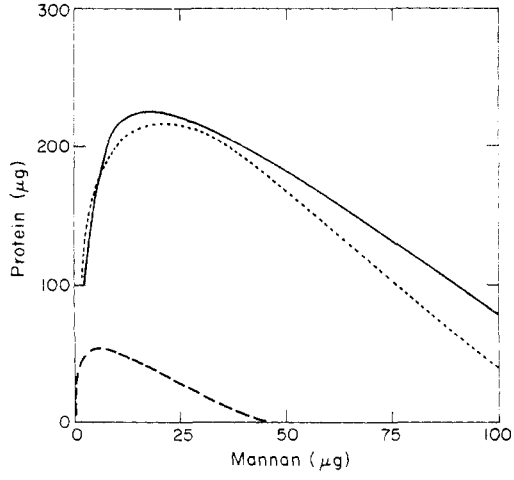

FIGURE 6: Precipitin curves of *H. wingei* Y-5 antiserum with Y-5 mannan (——) and with 5-agglutinin before (- - - -) and after (— —) mild acid hydrolysis. For each assay, 15 μl of antiserum was used.

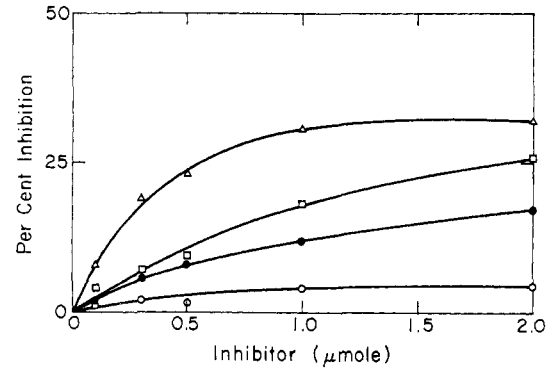

FIGURE 7: Inhibition of the precipitin reaction between *H. wingei* Y-5 antiserum and 5-agglutinin. Inhibitors are mannobiose (○), mannotriose (△), mannotetraose (□), and mannopentaose (●), all obtained by acetolysis of *H. wingei* Y-5 mannan.

various treatments were investigated. However, the study was complicated because the binding activity of 5-agglutinin did not always correlate with the agglutination activity. A treatment which destroyed the binding activity also destroyed the agglutination activity, but the reverse was not always true. This is reasonable because a treatment could disrupt the 5-agglutinin into monovalent subunits with loss of agglutination activity but retention of binding activity. Therefore, [^{35}S]-5-agglutinin was used, and both the agglutination activity and the binding activity were assayed. As shown in Table IV, chymotrypsin, lysozyme, subtilisin, trypsin, and Glusulase, all at concentrations as high as 2.5 mg/ml, had little effect on the activity; while Pronase and exo-α-mannanase destroyed most of it. Heating in water at 100° for 30 min did not inactivate the 5-agglutinin, whereas inactivation did occur in alkali (0.1 N NaOH or 1 N NaOH) either at 100° or at room temperature and in 0.1 N HCl at 100°. Some preparations of 5-agglutinin were more stable to acid than others for no obvious reason. None of the treatments in Table IV affected the agglutination

TABLE IV: Effects of Various Treatments on the Binding and Agglutination Activities of 5-Agglutinin.

Treatment	% Inactivation[a]
Chymotrypsin	0
Lysozyme	0
Subtilisin	0
Trypsin	0
Glusulase	7
Pronase	64, 83, 46 (98)
Exo-α-mannanase	62, 72, 91 (97)
100°, 30 min	
Water	2 (0), 3 (0)
0.01 N HCl	10 (75)
0.1 N HCl	40 (90)
0.1 N NaOH	57 (97), 54 (97)
1 N NaOH	47 (75), 62 (50)
Room temperature, 30 min	
Water	0 (0)
0.1 N HCl	0 (0)
1 N HCl	11 (0), 5 (50)
0.1 N NaOH	37 (50), 53 (87)
1 N NaOH	74 (75), 79 (97)

[a] Agglutination activities are shown in parentheses.

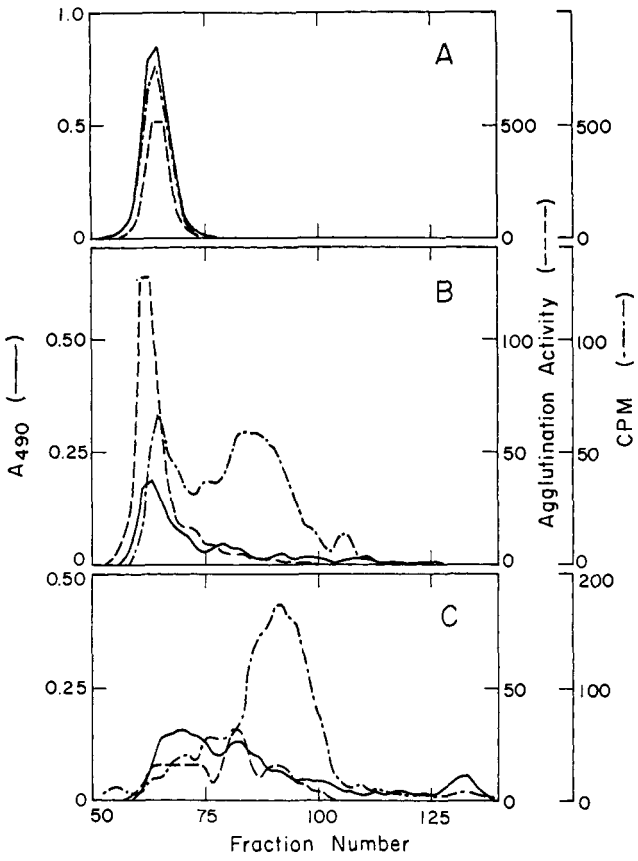

FIGURE 8: Gel filtration of 5-agglutinin on a 2 × 200 cm column of Bio-Gel A 5m (A) before and (B) after treatment with 0.01 N HCl at 100° for 30 min, or (C) after treatment with 0.1 N HCl at 100° for 30 min. Fractions were assayed for carbohydrate (—), agglutination activity (— —), and ^{35}S radioactivity (—·—).

activity without affecting the binding activity, and in all cases the agglutination activity was reduced to a greater extent than the binding activity.

Acid Treatment. Treatment with 0.01 N HCl at 100° for 30 min destroyed 70% of the antigenic activity of 5-agglutinin, and it had a similar effect on the agglutination activity (Table IV). Most of the carbohydrate and remaining agglutination activity came out of the Bio-Gel A 5m column at the position of the original 5-agglutinin, but much of the ^{35}S radioactivity was associated with lower molecular weight material (Figure 8B). With 0.1 N HCl, the molecular weight of 5-agglutinin decreased significantly (Figure 8C), and what agglutination activity remained was associated with the now polydisperse carbohydrate peak, while the molecular weight of the ^{35}S peak decreased further.

The results of mild acid treatment suggest that the agglutinating activity of 5-agglutinin may be indirectly related to the acid-labile antigenic determinant. The most likely consequence of the acid treatment is a cleavage of phosphodiester linkages. If the 5-agglutinin is cross-linked by such linkages, the acid hydrolysis could lead to a reduction in size of the molecule and a concomitant decrease in its precipitability by specific antibody without a complete loss of agglutination activity.

Pronase and Exo-α-mannanase Inactivation of 5-Agglutinin. Both Pronase and exo-α-mannanase inactivated 5-agglutinin, although high enzyme concentrations (2.5 mg/ml) were required. The Pronase inactivation was inhibited by bovine serum albumin, but not by the mannotetraose from *S. cerevisiae* mannan. On the other hand, exo-α-mannanase inactivation was inhibited by the mannotetraose but not by

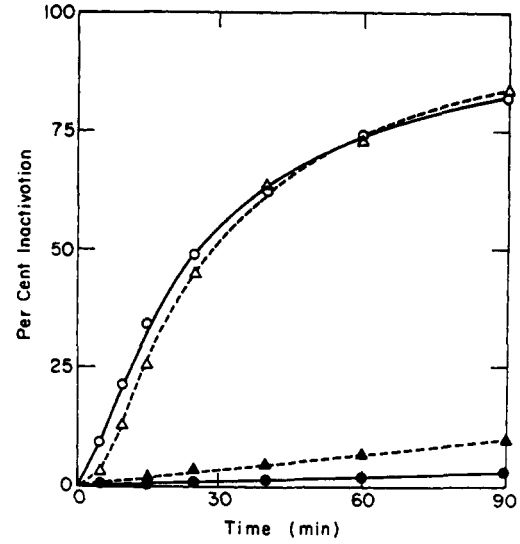

FIGURE 9: Effect of Pronase and exo-α-mannanase digestion on the binding of 5-agglutinin to 21 cells: (○) inactivation by Pronase and (△) by mannanase. The addition of 500 μg of bovine serum albumin to the Pronase digests (●) or of 20 μg of mannotetraose from yeast mannan (Lee and Ballou, 1965) to the mannanase digests (▲) almost completely prevented inactivation. Serum albumin did not inhibit mannanase inactivation and the mannotetraose did not affect Pronase inactivation.

serum albumin. Thus, the inactivation was due to the specified enzymes and not to contaminating activities. The inactivation curves were sigmoid in both cases, but much more so for the mannanase inactivation (Figure 9). This suggests either that one break in a molecule made the rest of the molecule more susceptible to the enzyme, or that the enzyme digested each molecule at a constant rate and only after a certain amount of degradation had occurred was the binding activity affected.

When 5-agglutinin was 65% inactivated by Pronase, only a small fraction of the carbohydrate had been released (Figure 10A), and the large residue retained 30% of the binding activity. However, half of the ^{35}S radioactivity appeared as material of lower molecular weight that no longer bound to 21-H cells. Following exhaustive Pronase digestion, no binding activity was detected and the size of the molecule declined still further (Figure 10B).

Since ^{35}S was associated only with cysteine residues in 5-agglutinin, the inability of the radioactive Pronase-digestion products to bind 21-H cells did not prove that the binding activity had been destroyed. It was possible that the binding sites did not contain cysteine. Therefore, the total product from exhaustive digestion of unlabeled 5-agglutinin was tested as an inhibitor of the binding of [^{35}S]-5-agglutinin to 21-H cells. No inhibition was found. This indicates that Pronase really destroyed the binding sites of 5-agglutinin, and that the specificity of these sites probably does not reside in the carbohydrate component.

Whereas digestion of 5-agglutinin with a large amount of exo-α-mannanase led to complete inactivation (Table IV), digestion with mannanase under controlled conditions (see Experimental Section) removed 38% of the carbohydrate with loss of only 35% of the binding activity. On gel filtration of the mannanase-digestion products (Figure 10C), 75% of the ^{35}S radioactivity was eluted as a single peak of lower molecular weight material that no longer bound to 21-H cells. Some intact molecules with binding activity remained, but only a fraction of that observed before the gel filtration step. Since 65% of the radioactivity could be bound to 21-H cells at the

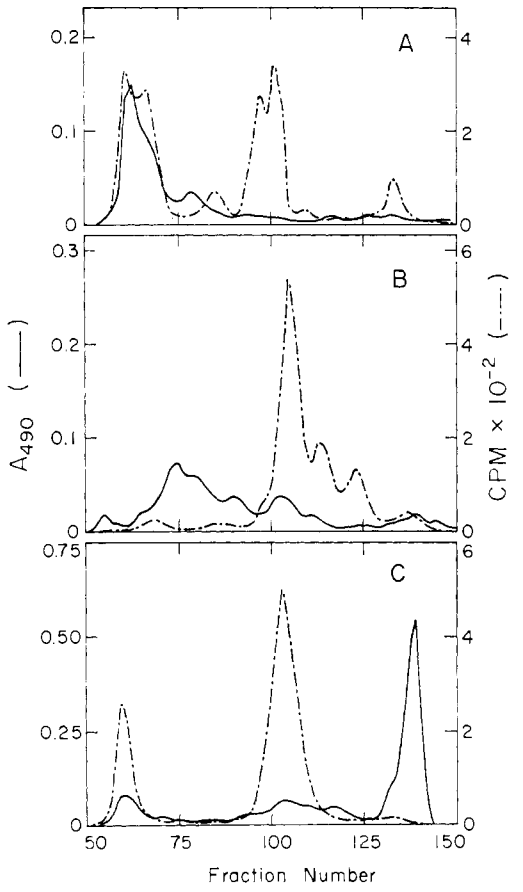

FIGURE 10: Gel filtration of 5-agglutinin after Pronase digestion for (A) 2 hr or (B) 24 hr, and (C) exo-α-mannanase digestion for 3 days. A 2 × 200 cm column of Bio-Gel A 5m was used and was eluted with 0.1 N KH_2PO_4. Fractions were assayed for carbohydrate (—) and ^{35}S radioactivity (—·—).

end of the mannanase digestion, but much less following gel filtration of this product, we infer that the lower molecular weight material lost its binding activity during gel filtration and that the 5-agglutinin became more susceptible to inactivation after part of its carbohydrate was removed.

Periodate Oxidation. It was reported that periodate at 0° in the dark reacted specifically with carbohydrate and had no effect on the agglutinability of 5 cells (Brock, 1965). We studied the effect of periodate on purified 5-agglutinin and found that the consumption per mannose unit was 0.72 mol at room temperature and 0.59 mol at 4° after 6 days of reaction (Figure 11). In both cases, inactivation of 5-agglutinin became apparent only after about 0.2 mol of periodate was consumed per mannose unit. Again, this result suggests that the carbohydrate moiety in 5-agglutinin functions to stabilize the molecule, and that only after a certain amount of the carbohydrate was destroyed was an effect on activity observable.

Reduction of 5-Agglutinin. 5-Agglutinin is known to be inactivated by sulfhydryl reagents (Taylor, 1964). When 5-agglutinin was reduced with dithiothreitol and the product alkylated with iodoacetamide and then fractionated on a Bio-Gel A 5m column, four protein-containing peaks were obtained (Figure 12). The main reduced component I contained most of the protein and carbohydrate, and its position in the column indicated that its size did not differ significantly from the original 5-agglutinin. The amino acid compositions of 5-agglutinin and of the four reduced components are shown in Table V. The composition of fraction I was similar to that of the original 5-agglutinin, except that the serine content was

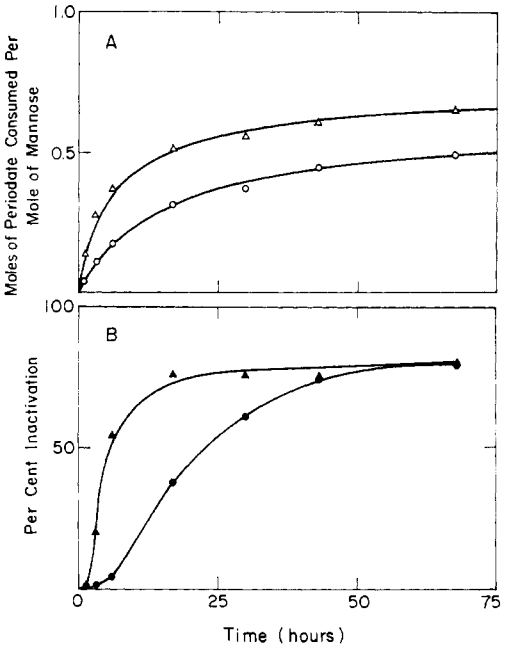

FIGURE 11: Periodate oxidation of 5-agglutinin: (A) periodate consumption per mannose unit at 22° (△) and 4° (○); (B) inactivation of 5-agglutinin at 22° (▲) and at 4° (●).

slightly increased. The amino acid compositions of fractions II, III, and IV were similar to each other, but were significantly different from that of the original 5-agglutinin or Y-5 mannan. As in Y-5 mannan, serine and threonine made up about 40% of the amino acids in these small components, but there was more threonine than serine.

When [^{35}S]-5-agglutinin was reduced, about equal amounts of ^{35}S radioactivity were associated with fractions I and III (Figure 12), a result similar to that of Taylor and Orton (1971). Fraction I did not show any binding activity, while III still bound weakly to 21-H cells. Assuming that III was the binding site of 5-agglutinin and contained one cysteine residue per particle, as proposed by Taylor and Orton (1971), the number of amino acids was calculated from the amino acid composition (Table VI). This indicated that fraction III consisted of 28 amino acids, of which 12 were hydroxy and five were acidic. No glucosamine was found. From the protein content of III, calculated from the amino acid composition to be 22%, the molecular weight was estimated to be 12,500, which would allow for about 60 mannose units. This agrees with the value 12,000 (Taylor and Orton, 1968) obtained by the approach-to-equilibrium sedimentation method.

Discussion

The structures of *Hansenula wingei* 5-agglutinin and Y-5 mannan are similar in several respects. Both are phosphomannans with less than 15% protein. They give the same five neutral oligosaccharides on acetolysis, although in slightly different proportions, and consume about the same amount of periodate per mannose unit. The mannose units in both are connected by $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$, and $\alpha(1\rightarrow6)$ linkages, and the carbohydrate chains are highly branched. The antigenic groups appear to be the same, mild acid treatment destroys about 70% of the precipitin activity in both, and the precipitin reactions are inhibited to the same extent by the acetolysis products. However, the protein and hydroxy amino acid contents were twice as high in 5-agglutinin as in Y-5 mannan.

TABLE V: Amino Acid Composition of 5-Agglutinin Before and After Reduction with Dithiothreitol.

Amino Acid[a]	5-Agglutinin	Reduced Fractions[b] I	II	III	IV
Carboxylmethyl-cysteine	0.0	0.1[c]	0.4	3.5	0.0
Aspartic acid	2.8	1.9	6.3	10.1	5.6
Threonine	9.2	7.1	27.6	26.8	23.4
Serine	55.3	66.5	16.6	17.4	14.6
Glutamic acid	7.0	5.2	6.7	7.1	9.6
Proline	2.0	1.5	2.3	2.2	0.8
Glycine	1.6	1.2	3.5	2.7	3.0
Alanine	5.1	3.5	6.6	7.0	8.1
Half-cystine	0.3	0.5	1.6	0.3	0.0
Valine	6.3	4.8	9.3	8.3	10.6
Methionine	0.0	0.0	0.0	0.0	0.0
Isoleucine	3.5	2.8	5.3	3.7	3.9
Leucine	3.5	2.4	6.2	5.9	8.3
Tyrosine	0.9	0.6	2.0	0.8	5.9
Phenylalanine	0.4	0.3	0.3	0.1	0.0
Histidine	0.2	0.1	0.4	0.1	0.9
Lysine	0.2	0.2	0.6	0.1	1.1
Arginine	1.2	0.9	4.3	3.8	4.1
Glucosamine	0.4[d]	0.2	0.0	0.0	0.0

[a] Residues per 100 residues. [b] Fraction I is the largest component formed by reduction of 5-agglutinin, and fractions II, III, and IV are the three smaller components (see Figure 12). [c] The central core contains 10% protein or about 900 amino acid residues, and it should yield 0.66 carboxymethylcysteine residue per 100 amino acid residues from the 6 mol of cysteine it is assumed to contain. The analysis indicates that carboxymethylation of the large polysaccharide–protein residue was incomplete because 0.1 residue of carboxymethylcysteine and 0.5 residue of cysteine were found. Since free cysteine is easily destroyed during analysis, it is possible that this agreement is fortuitous. [d] The reason for the discrepancy between this glucosamine value and that in Table II is unknown, but the fact that only 10% of the carbohydrate in 5-agglutinin is attached by an alkali-stable linkage suggests that this lower value may be more nearly correct. Alternatively, if *H. wingei* mannan contains *N*-acetylglucosamine on some of the side chains as does *Kluyveromyces lactis* mannan (Raschke and Ballou, 1972) different preparations of 5-agglutinin could well differ in hexosamine content. A variable and slight contamination by chitin is another possible source of *N*-acetylglucosamine.

Exo-α-mannanase removed 38% of the carbohydrate from 5-agglutinin but only 20% from Y-5 mannan.

The most significant difference between these two mannan–proteins was revealed by the β-elimination reaction which breaks the glycosidic linkages between carbohydrate and the hydroxy amino acids in the protein. In Y-5 mannan, only 9% of the carbohydrate was released as short oligosaccharides from serine and threonine. The rest is present as polysaccharide chains, with 100–200 mannose units, apparently attached to the protein through the *N*-acylglycosylamine linkage. About 30% of the amino acid residues in Y-5 mannan are substituted by carbohydrate. In 5-agglutinin, the situation is quite different. Only 13% of the carbohydrate remained associated with protein after β elimination. The remaining 87% was released

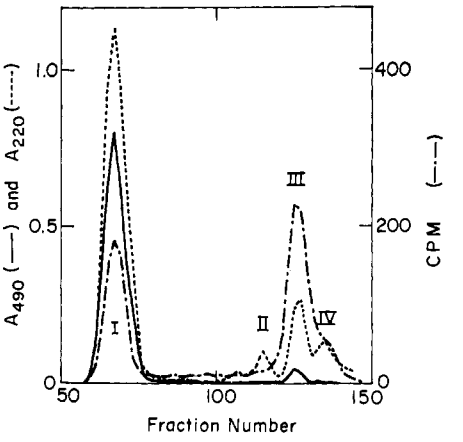

FIGURE 12: Gel filtration of the reduced 5-agglutinin on a 2 × 200 cm column of Bio-Gel A 5m. Fractions were assayed for carbohydrate (——), protein (OD_{220}, - - - -), and ^{35}S radioactivity (- · - · -). The radioactive experiment was done separately and the shape does not correspond exactly with the sulfur contents of peaks II and IV as given in Table V. The reason for this discrepancy is not known.

as small mannooligosaccharides, from 1 to 15 sugar units in length, with mannose at the reducing end. Mannooctaose was the major component, and on acetolysis it yielded the same five fragments obtained from Y-5 mannan. Thus, it must have been a mixture of isomeric octasaccharides made up from different combinations of mono- to pentasaccharide units. About 60% of the amino acid residues (serine and threonine) are substituted by carbohydrate. Thus, although Y-5 mannan and 5-agglutinin are 85–90% mannose and are constructed from the same five oligosaccharides, the overall structures are quite different, as shown in the speculative models in Figure 13.

Brock (1959) first reported that periodate oxidation at 37° inactivated 5 cells, and suggested that a polysaccharide was responsible for their agglutinability. However, this idea was later brought into question by the finding that at 37° periodate reacted with several amino acid residues in proteins. At 0° in the dark, the periodate reacted specifically with carbohydrate

TABLE VI: Calculated Composition of the Active Site of 5-Agglutinin.[a]

Composition	No.
Aspartic acid	3
Threonine	7
Serine	5
Glutamic acid	2
Proline	1
Glycine	1
Alanine	2
Cysteine	1
Valine	2
Isoleucine	1
Leucine	2
Arginine	1
Mannose	60
Molecular weight	12,500

[a] This calculation was made on the assumption that fragment III in Figure 12 contains a single cysteine per molecule and that the nonprotein component, 78% of the weight of the fragment, is mannose.

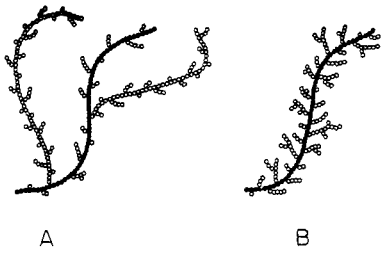

FIGURE 13: Postulated structures of (A) Y-5 mannan in which most of the carbohydrate exists as long polysaccharide chains, and (B) 5-agglutinin in which most of the carbohydrate is in the form of short oligosaccharides: (○) mannose and (●) amino acids.

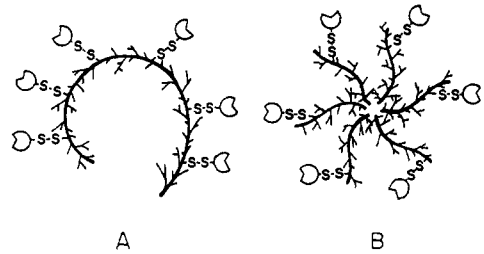

FIGURE 14: Two possible structures of the 5-agglutinin molecule. In (A) the central core is composed of one polypeptide chain, and in (B) it is composed of six polypeptide chains. The thick lines represent the polypeptide chains and the thin lines represent the carbohydrate chains. ⊖denotes the active site component which is a glycopeptide similar in structure to the central core.

and had no effect on the activity of 5 cells (Brock, 1965). Moreover, boric acid, which complexes carbohydrate, reacted with the cells but did not inhibit agglutination (Brock, 1959). On the other hand, 5-agglutinin was inactivated by Pronase and by disulfide cleaving reagents (Taylor, 1964). Thus, the activity of 5-agglutinin seemed to involve the protein component. In the present study, the inability of the residue from Pronase-digested 5-agglutinin to inhibit the binding of [^{35}S]-5-agglutinin to 21 cells reduces further the probability that the activity of 5-agglutinin resides in the sugar structure.

Although the carbohydrate component is not directly involved in forming the binding sites of 5-agglutinin, it is important for the binding activity, since exo-α-mannanase digestion and controlled periodate oxidation do inactivate 5-agglutinin. The discrepancy between the results of periodate oxidation from Brock's laboratory and ours might have arisen because he did the experiment on whole 5 cells (Brock, 1959). The structural mannan molecules on the yeast cell wall would consume most of the periodate, and a few inactivated 5-agglutinin molecules should not affect the agglutinability of the cells. The sigmoid curves for the inactivation by mannanase and periodate suggest that the carbohydrate might function to stabilize the binding sites, and the unusual stability of 5-agglutinin to heat could be due to the carbohydrate. The sugar chains might also serve to protect the molecule against proteolytic enzymes, because chymotrypsin, subtilisin, and trypsin had no effect on the binding activity, whereas a very high concentration of Pronase had to be used to inactivate it. On the cell surface, the carbohydrate might also serve to orient the 5-agglutinin among the other mannan molecules.

The model for 5-agglutinin, proposed by Taylor and Orton (1971), consists of a large central core to which six small "active site" fragments are joined by disulfide bonds. This model was based on the finding that reduction with dithiothreitol inactivated 5-agglutinin and the molecule was broken into two components of different size. When both components were dialyzed together, 25–75% of the original agglutination activity was regained. This experiment was repeated in the present study. 5-Agglutinin lost both the agglutination activity and the binding activity on reduction with dithiothreitol, with the agglutination activity decreasing more rapidly than the binding activity. On reoxidation, about 40% of the binding activity was regained. A long Bio-Gel A 5m column was used to separate the reduced components, and this appeared to give better resolution than the Sephadex G-100 column used by Taylor and Orton (1971). Instead of one small reduced component, three peaks of low molecular weight were found. Fraction III retained weak binding activity and is assumed to represent the binding site of the molecule. The absence of glucosamine in this fraction suggests that all of its carbohydrate was linked to the peptide through O-glycosidic linkages. We estimate that the glycopeptide contains 28 amino acids and about 60 mannose units, giving a molecular weight of 12,500. The amino acid composition of this active binding fragment was quite different from that of the intact 5-agglutinin. The presence of three small reduced components instead of one is difficult to explain. One possibility is that fractions II and IV came from contaminants. This seems unlikely because they were present in three different preparations, and their compositions were similar to each other but different from the cell wall mannan which was the most likely contaminant.

Heating at 100° with 0.01 N HCl for 30 min destroyed about 75% of the activity of 5-agglutinin and led to considerable disruption of the molecule. It was reported previously (Hsiao, 1973) that this treatment did not inactivate 5-agglutinin, but repetition of this experiment has given variable results. Some preparations are inactivated while others seem to be unusually stable. The reason for this discrepancy is not known. This treatment would split phosphodiester bonds and it is probable that such linkages occur in the agglutinin because of the phosphate content and the known structure of phosphomannans (Thieme and Ballou, 1971). The loss of agglutinating activity is paralleled by a loss of precipitability by specific antibody. Both could result from a general reduction in size of the agglutinin rather than from specific damage to the binding sites. The acid treatment also released a large amount of small ^{35}S-containing material that separated from the main carbohydrate-containing material on gel filtration, a result not too different from that for the dithiothreitol reduction. This could be a result of disulfide interchange (Ryle and Sanger, 1955) and the smaller material might be cross-linked inactive binding fragments.

Whether the central core in Taylor's model consists of a single peptide chain to which the six 1.7S components are linked through disulfide bridges (Figure 14A) or is composed of several polypeptide chains each linked to one 1.7S component (Figure 14B) is not known. The idea that the central core of 5-agglutinin might be composed of subunits was suggested by the finding that exo-α-mannanase digestion yielded a product that was eluted from the Bio-Gel A 5m column at the position of a globular protein with molecular weight 1.2×10^5. Since glycoproteins usually have an apparent volume greater than a globular protein of the same molecular weight, 1.2×10^5 represents the maximum molecular weight for the mannanase-resistant residue. If the action of mannanase were simply to remove 38% of the carbohydrate from 5-agglutinin, the residue should have a molecular weight of about 6.3×10^5. It is unlikely that the lower observed value could be caused by a proteolytic contaminant in the mannanase preparation, because the digestion was performed in the presence of a large amount of bovine serum albumin. Whereas this mannanase digestion appeared to lead to dissociation of the

central core, other attempts to disrupt the central core into subunits were not successful. Gel filtration of the reduced fraction I in the presence of 6 M guanidine-HCl gave one peak near the void volume on a Bio-Gel A 5m column. Treatment of the reduced fraction I with alkali, under conditions which break ester linkages, also had no effect on its size.

5-Agglutinin as it exists in the yeast cell wall is probably in a form much larger than that we have studied. The material [5-agglutinin(H)] isolated by Taylor (1965) using the snail digestion enzyme had a particle weight greater than 10^8. When subjected to subtilisin digestion, 5-agglutinin(H) was converted to 5-agglutinin(S), similar to that obtained directly from cells by subtilisin (Taylor and Orton, 1967). Thus, in the cell wall, several 5-agglutinin(S) molecules may be linked together to form a large 5-agglutinin(H) particle. The mechanism by which subtilisin leads to release of 5-agglutinin has not been investigated. If it cleaves the agglutinin peptide chain at the point of attachment to a hold-fast fragment anchored in the mannan–glucan matrix of the cell wall, this presumed fragment may have a structure and composition more like that of the total Y-5 mannan-protein (Yen and Ballou, 1974). It is possible that the reduced fraction IV is the fragment of a chain that is involved in cross-linking the 5-agglutinin(S) units in a manner analogous to the cross-linking of immunoglobulins (Morrison and Koshland, 1972).

Acknowledgments

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