Cell–cell recognition in yeast: Isolation of intact α-agglutinin from Saccharomyces kluveri

RICHARD D. LAISKY* AND CLINTON E. BALLOU†

Department of Biochemistry, University of California, Berkeley, CA 94720

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ABSTRACT The heat-labile sexual agglutinin from Saccharomyces kluveri strain 17 (α-agglutinin) has been isolated in an apparently intact form from wild-type and mnn1 (glycosylation-defective) cells. The wild-type agglutinin is polydisperse, due to variable degrees of glycosylation, and migrates on native gels with an apparent mass of >400 kDa, whereas under denaturing conditions it appears somewhat smaller. The mnn1 agglutinin is also heterogeneous but has a lower molecular mass due to the presence of shorter N- and O-linked polymannose chains. Both agglutinins are converted sequentially by proteolysis to active fragments of ~150 and 60 kDa, but the rate of proteolysis of the more highly glycosylated wild-type agglutinin is much slower than that of the mnn1 agglutinin. The 60-kDa fragment was isolated by HPLC and found to contain 46% carbohydrate as mannose, all of which was linked to serine and threonine. Thus, the N-linked oligosaccharides are restricted to that part of the agglutinin molecule that presumably anchors the agglutinin in the cell wall.

When haploid cells of opposite mating type of the yeast Saccharomyces kluveri are mixed, they agglutinate into clumps, an event that is followed by the fusion of paired cells to form a zygote (1). This process is mediated by species-specific agglutinins that are anchored in the yeast cell wall (2). In S. kluveri, as in other species, the a mating-type cell possesses an agglutinin that contains a heat-stable recognition site that is released in soluble form by reduction with diithiothreitol. In contrast, the a-agglutinin is a large mannoprotein that is unaffected by reducing agents but is readily denatured by heat.

Studies on the α-agglutinin from S. kluveri strain 17 revealed that an active component of 60 kDa was produced by Zymolyase digestion of the cells (1). Subsequently, by using a purified β-glucanase of reduced proteolytic activity, Weinstock and Ballou (3) obtained evidence for a larger active fragment of 134 kDa that appeared to be formed from a still larger agglutinin molecule of >200 kDa. It was found that a highly active protease in the Zymolyase preparation degrades the intact agglutinin sequentially to active fragments of 134 and 60 kDa.

In the present report, a protease-free β-glucanase (4) has been used to release the α-agglutinin from cells, and the solubilized agglutinin was purified by ion-exchange and gel chromatography. The agglutinins from wild type and a glycosylation-defective mutant of S. kluveri (designated mnn1) (5) have been compared by molecular size and for sensitivity to digestion by endo-N-acetylglucosaminidase H (endo H) and Oerskovia xanthineolectyta protease (4).

MATERIALS AND METHODS

Materials. S. kluveri strain 17 and the strain 17 mnn1 mutant, which is defective in synthesis of a1,3-mannosyl linkages (5), were from the laboratory collection. Cultures were grown on a rotary shaker at 250 rpm in a medium composed of 1% yeast extract, 2% (wt/vol) Bacto-peptone, and 2% (wt/vol) glucose at 30°C. Endo H from Streptomyces pilatus was prepared according to Tarantino et al. (6) and was provided by Lun Ballou (of this department), and a protease-free β-glucanase was obtained from the culture medium of Oerskovia xanthineolectyta (4).

Methods. Protein was determined by a modified Lowry (7) or the bicinchoninic acid procedure (8), whereas total carbohydrate was measured by the phenol/sulfuric acid method (9). Protease activity was determined using Azacoll (Calbiochem), and β-glucanase activity was detected by measuring yeast cell lysis in presence of 2-mercaptoethanol (4). Soluble α-agglutinin activity was estimated from the inhibition of agglutination between S. kluveri strain 16 and 17 cells (1). For proteolysis, 50 μg of agglutinin protein was treated with the Oerskovia protease (4.5 μg of protein) at 25°C or 37°C for the times indicated. The 60-kDa fragment was isolated by HPLC under isocratic conditions on a DuPont GF-250 gel filtration column operated with a Waters model 510 instrument and an LKB 2138 Uvicord S with a 206-nm filter. The column was equilibrated and eluted with 0.1 M sodium phosphate (pH 5.5) at 1.15 ml/min.

Agglutinin was prepared from a 12-liter culture of S. kluveri cells grown overnight at 30°C to late logarithmic phase in a 16-liter aerated fermenter. The cells were washed three times with wash buffer (1.2 M KCl/0.1 M Tris-HCl/1 mM EDTA/1 mM EGTA, pH 8.0). The final pellet was suspended in 300 ml of wash buffer/40 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride, and protease-free glucanase was added to a concentration of 20 lytic units/ml. The suspension was incubated for 6 hr at 30°C and then centrifuged at 2500 × g for 15 min at 4°C. The pellet was washed with 300 ml of wash buffer, and the combined supernatant fractions were centrifuged at 12,000 × g for 15 min at 4°C. The agglutinin in the extract was purified on a DEAE-Sephadex column by stepwise elution (1). The active fraction was concentrated to 68 ml on an Amicon PM10 stirred-cell filter and stored at 4°C with 0.002% chlorhexidine. Further purification was done in 3-ml portions on a Bio-Gel A-5m column (5 × 88 cm) equilibrated and eluted at 4°C with 0.15 M NaCl/20 mM sodium phosphate, pH 5.5/0.25% Triton X-100/0.02% sodium azide. The active fractions were combined, concentrated on an Amicon PM10 filter, and chromatographed on an Ultrogel AcA34 column (2.6 × 85 cm) equilibrated and eluted with the same buffer. The active fractions were combined and concentrated as described above.

NaDodSO4/polyacrylamide gel electrophoresis was done on a 4–15% gradient gel with a 5% stacking gel (10). Nondenaturing gel electrophoresis, with a 4–20% gradient of

Abbreviation: endo H, endo-N-acetylglucosaminidase H.
*Present address: Barnett Institute, Northeastern University, Boston, MA 02115.
†To whom reprint requests should be addressed.
acrylamide, was carried out with a continuous buffer system (0.09 M Tris/0.08 M boric acid/Na$_2$EDTA at 0.93 g/liter, pH 8.4) at 150 V for 15 hr at 4°C using an electrode buffer of the same composition. Gels were stained by the periodate/silver (11) or periodic acid/Schiff (12) technique. Immediately after electrophoresis, nondenaturing gels were washed for 1 hr at 25°C in 20 mM sodium phosphate, pH 5.5/0.15 M NaCl (buffer A) supplemented with 10% (vol/vol) methanol. The gel was then incubated for 6 hr in 50 ml of $^{125}$I-labeled 16-mnnl binding fragment (1-2 $\times$ 10$^5$ cpm/ml) in 5% (vol/vol) methanol in buffer A. Unbound radioactivity was removed by washing the gel once in a 100-ml portion of buffer A containing 10% (vol/vol) methanol, twice in buffer A containing 0.1% Triton X-100, and three times in buffer A alone. The gel was then wrapped in plastic and subjected to autoradiography. The 16-mnnl binding fragment (5 $\mu$g) was labeled with 1 mCi (1 Ci = 37 GBq) of $^{125}$I using the chloramine-T procedure (13). The reaction was carried out for 30 sec on ice, and the products were immediately separated on a 5-ml Sephadex G-25 column. The void-volume fraction contained the labeled product.

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**Fig. 1.** Gel filtration profile of $\alpha$-agglutinin preparations. The agglutinins were solubilized from S. kluyveri cells with protease-free $\beta$-glucanase and fractionated on a DEAE-Sephasel column, and the active fraction, eluted from the column, was chromatographed on a Bio-Gel A-5m column. (Upper) Wild-type agglutinin. (Lower) mnnl agglutinin.

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**Fig. 2.** Native gel electrophoresis of $\alpha$-agglutinins. Agglutinins from wild-type (lanes 1 and 2) and mnnl mutant (lanes 3 and 4) cells, eluted from the A-5m column, were divided into fractions A (lanes 1 and 3) and B (lanes 2 and 4) and electrophoresed on a 4-20% gradient gel under nondenaturing conditions overnight at 150 V and 4°C. The gel was washed and then incubated with $^{125}$I-labeled strain-16 mnnl fragment, after which it was washed free of unbound radioactivity and subjected to autoradiography.

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**Fig. 3.** Endo H digestion of agglutinins. Fraction B of wild-type (lanes 1 and 2) and mnnl (lanes 3 and 4) agglutinins were digested with endo H and subjected to NaDodSO$_4$/PAGE. Lanes 1 and 3 are the undigested controls, and lanes 2 and 4 are the digested samples.

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**Fig. 4.** Protease digestion of wild-type agglutinin. Fraction A (lanes 1-3) and fraction B (lanes 4-6) agglutinins were digested for 0 (lanes 1 and 4), 1 (lanes 2 and 5), or 4 (lanes 3 and 6) hr and then subjected to gel electrophoresis on a nondenaturing gel. Protein was stained by the periodate/silver procedure.
Endo H digestion of wild-type and \textit{mnnl} agglutinins (15 \textmu g of protein) was done in 50 \textmu l of 0.1 M sodium citrate, pH 5.5/2 mM phenylmethylsulfonyl fluoride/1 milliunit of endo H for 24 hr at 37\textdegree C. Samples were then boiled in reducing buffer for NaDodSO\textsubscript{4}/PAGE on a 4–15\% gradient gel, which was stained for carbohydrate using the periodic acid/Schiff reagent.

RESULTS

Isolation of the \textalpha-Agglutinin. The chromatographic profile of wild-type \textit{S. kluveyri} agglutinin on the Bio-Gel A-5m column is shown in Fig. 1 \textit{Upper}. The active material was eluted as a single broad peak, which was divided arbitrarily into two fractions, A and B, for analysis. The profile of the \textit{mnnl} agglutinin (Fig. 1 \textit{Lower}) revealed two well-separated peaks of activity. Differences between the A and B fractions of both agglutinins were apparent from the activity stained native polyacrylamide gels (Fig. 2). Whereas the two fractions of the wild-type agglutinin gave overlapping bands in the mass range of 300–600 kDa (lanes 1 and 2), the \textit{mnnl} agglutinin revealed distinct bands with some cross-contamination (lanes 3 and 4). The yield of wild-type agglutinin was \%5\% and that of the \textit{mnnl} agglutinin was \%0.4\%, both based on recovery of protein. The lower yield of the latter may be due to a greater sensitivity to proteolysis (see below).

Characterization of the Agglutinin. The agglutinin fractions were stable at room temperature for 24 hr but were usually stored at 4\textdegree C in presence of 0.002\% chlorhexidine to prevent microbial growth. Repeated freezing and thawing led to a loss of activity. The weight percents of carbohydrate in the wild-type agglutinin fractions A and B were 90 and 84, respectively, whereas the corresponding \textit{mnnl} agglutinin fractions contained 55 and 59\% carbohydrate. On NaDodSO\textsubscript{4}/PAGE, the wild-type agglutinin gave bands in the range of 300–400 kDa whereas the \textit{mnnl} agglutinin migrated as bands at 220–330 kDa.

Endo H Treatment. Digestion of the wild-type \textalpha-agglutinin (fraction B) with endo H increased the mobility and reduced the apparent size heterogeneity seen on NaDodSO\textsubscript{4}/PAGE (Fig. 3, lanes 1 and 2). Similar treatment of the \textit{mnnl} agglutinin (fraction B) had less of an effect (Fig. 3, lanes 3 and 4). The results confirm that some of the heterogeneity is due to differences in the amount of N-linked carbohydrate and that the \textit{mnnl} agglutinin contains less carbohydrate than the wild-type agglutinin. In neither instance was the activity, observed on nondenaturing gels with an activity stain, affected by the endo H digestion (data not shown).

Protease Treatment. Both fraction A and B wild-type agglutinins, when digested with \textit{Oerskovia} protease, yielded apparently identical fragments of \%150 and 60 kDa (Fig. 4). The kinetics of proteolysis of the wild-type and \textit{mnnl} agglutinins were quite different when analyzed by HPLC (Fig. 5). Undigested agglutinins of both types were eluted near the void volume of the column, but with time new peaks appeared with elution times of 7.0 and 7.7 min. For the \textit{mnnl} agglutinin, the peak at 7.7 min predominated after 20 min while the digestion of the wild-type agglutinin was far from complete after 1 hr. From the calibration curve for the HPLC column provided by the manufacturer, the intact

![Kinetic study of agglutinin proteolysis](image-url)

**Fig. 5.** Kinetic study of agglutinin proteolysis. Fraction B of \textit{mnnl} (A–F) and fraction B of wild-type (G–L) agglutinin were incubated with the \textit{Oerskovia} protease for various lengths of time and then analyzed by HPLC. Undigested controls (A and G), 5 min (B and H), 20 min (C and I), 40 min (D and J), 60 min (E and K), and 120 min (F and L) are shown. The intact agglutinins were eluted at positions near that of thyroglobulin (690 kDa), the peak at 7.0 min is near that of immunoglobulin G (150 kDa), and that at 7.7 min is near that of bovine serum albumin (68 kDa).
agglutinins migrate near the position for thyroglobulin (690 kDa), the peak at 7.0 min near that of immunoglobulin G (150 kDa), and the peak at 7.7 min near that of bovine serum albumin (68 kDa). The latter two values agree well with the apparent sizes of the fragments observed on gel electrophoresis.

The fragment from the protease digest of the mnnl agglutinin, which was eluted from the HPLC column at 7.7 min, was collected and analyzed by NaDodSO4/PAGE (Fig. 6). It migrated as a sharp band of ~55 kDa and contained 46% carbohydrate, but endo H treatment had no effect on the gel migration indicating that all of the carbohydrate must be O-linked. That this fragment appeared slightly smaller than the corresponding 60-kDa fragment from the wild-type agglutinin suggests that its O-linked oligosaccharides are smaller than those of the wild-type fragment.

**DISCUSSION**

Weinstock and Ballou (3) demonstrated that the 60-kDa α-agglutinin species described earlier (1) was a proteolytic product of a 134-kDa species that was, itself, formed by proteolysis of a glycoprotein of >200 kDa. In the present study, special care was used to avoid proteolysis in preparing the agglutinins from wild-type and mnnl cells of *S. kluuyveri* strain 17. The agglutinin from wild-type cells was heterogeneous in size and migrated on NaDodSO4 gels in the range of 330 to 400 kDa, which appears to be due to differences in the size of N-linked oligosaccharide chains because endo H treatment reduced the heterogeneity. Electrophoresis on nondenaturing gels gave an apparent mass of ~600 kDa, which suggests that the agglutinin might aggregate to a dimer under these conditions.

The mnnl agglutinin, which contains about two-thirds the carbohydrate of the wild-type agglutinin, was obtained in two distinct forms of 220 and 325 kDa by separation on a Bio-Gel A-5m column. The effect of endo H digestion on the mnnl agglutinin was less pronounced, in agreement with the fact that it contains smaller N-linked oligosaccharide chains.

Treatment of fractions A and B of the wild-type agglutinin with a bacterial protease yielded fragments of 150 and 60 kDa, which correspond to the 134- and 60-kDa fragments reported earlier (3). Thus, the heterogeneity in the agglutinin must reside in the part of the molecule distal from the active binding site. Proteolysis of the mnnl agglutinin yielded similar fragments, but the rates of proteolysis of wild-type and mnnl agglutinin differed considerably, with the latter being converted to the 60-kDa fragment in 20 min, whereas the former was only partly proteolyzed under the same conditions.

The small fragment from proteolysis of the mnnl agglutinin was readily purified by HPLC on a gel filtration column. It contained 46% carbohydrate but digestion with endo H had no effect on its mobility, which suggests that all of the carbohydrate was linked to serine and threonine in this part of the agglutinin molecule that retained the recognition activity. From the amino acid composition of this fragment (1) and its carbohydrate content, we calculate that it would contain ~60 hydroxyamino acids and that if all were glycosylated each would have ~3 mannose units attached to it. In the wild-type agglutinin, these oligosaccharides would be somewhat longer (5).

From the apparent mass of fraction B of the mnnl agglutinin (220 kDa) and its carbohydrate content (59%), we calculate that the polypeptide portion must have a mass of 90 kDa. Similarly, we calculate that the 60-kDa fragment contains a polypeptide portion with a mass of 32 kDa or one-third that of the intact molecule. Assuming that the 150-kDa fragment has a similar composition, it would contain about two-thirds of the polypeptide portion of the agglutinin. Thus, we conclude that the agglutinin has three domains with about equal polypeptide masses, but that they differ in carbohydrate content because the O-linked oligosaccharides are concentrated in the active site portion whereas the N-linked polymannose chains are located in the part of the molecule that is embedded in the cell wall.

Our results demonstrate that the α-agglutinin of *S. kluuyveri* exists as a large mannanprotein with considerable size heterogeneity that is due mainly to the variable amount of N-linked carbohydrate. In the mnnl agglutinin, a reduction in the extent of glycosylation on both asparagine and hydroxyamino acids has rendered it more susceptible to proteolysis, which accounts for the low yield in its isolation due, probably, to the action of endogenous yeast proteases. It has been demonstrated (1) that the carbohydrate portion of the agglutinin is not required for its recognition activity, and the evidence presented here suggests that the primary function of the polymannose chains is to anchor the molecule in the cell wall and to protect it from proteolysis.

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