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Purification of the inducible α-agglutinin of *S. cerevisiae* and molecular cloning of the gene

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The α-agglutinin responsible for mating type-specific agglutination of *S. cerevisiae* α-cells has been purified to homogeneity. The glycoprotein released from the cell surface under mild conditions has a relative molecular mass of 200 to 300 kDa as determined by SDS-gel electrophoresis. The protein moiety corresponds to 68.2 kDa. With an oligonucleotide corresponding to the N-terminal amino acid sequence, the α-agglutinin gene has been cloned and sequenced. From the DNA sequence, a protein of 631 amino acids with 12 potential *N*-glycosylation sites is predicted. The carboxy terminal one-third of the protein is not required for agglutination activity.

Agglutinin, α-; Glycoprotein; DNA sequence; *(Saccharomyces cerevisiae)*

1. INTRODUCTION

Haploid *a*- and α-cells of *S. cerevisiae* produce peptide pheromones, *a*- and α-factor, which, when experienced by the corresponding mating partner, cause a change of the cell surface and an increased agglutinability [1–4]. From *a*-cells, a glycoprotein of 22 kDa has been released from the cell surface which is present in 30-fold higher concentration after α-factor treatment [5]. This increase in amount corresponded well with the increase in biological activity [6]. From total extracts of α-cells, another glycoprotein has been enriched which possesses α-agglutinin activity [7].

In this paper, we describe the purification of a cell surface glycoprotein from α-cells, the amount of which increases 5–10-fold after treating the cells with *a*-factor, again paralleling the increase in biological activity of corresponding cell extracts. This protein was released from intact cells with mild zymolyase treatment and had only to be purified about 10-fold to be homogeneous. The N-terminal sequence of the protein was determined. With a corresponding oligonucleotide, the gene has been cloned and from the DNA sequence, the total amino acid sequence of α-agglutinin is predicted.

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2. MATERIALS AND METHODS

2.1. *Strains and media*

S. cerevisiae X2180-1A (Mat*a*) and X2180-1B (Matα) were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) at 30°C.

2.2. *Pretreatment of α-cells with a factor*

2.5 l of an overnight culture of X2180-1B (α-cells) with an OD_{578} of 4.0, were centrifuged and resuspended in 5 l *a*-cell medium. This preconditioned medium was obtained by removing X2180-1A cells of an overnight culture (OD_{578} = 2.0) by centrifugation and filtering (nitrocellulose, pore size 0.45 μm). The α-cells were growing for 90 min at 30°C in the *a*-medium.

2.3. *Preparation and purification of α-agglutinin*

Pretreated α-cells were harvested, washed 3 times with 100 mM Tris, pH 8.0, and 5 mM EDTA, resuspended in 60 ml of 20 mM potassium phosphate buffer, pH 7.0, resulting in 130–140 OD units/ml. 6 μg zymolyase 100 T and 20 μg cycloheximide were added per ml and the incubation was carried out for 90 min at 30°C. After centrifugation, the supernatant was run through an ion-exchange column (volume 12 ml;

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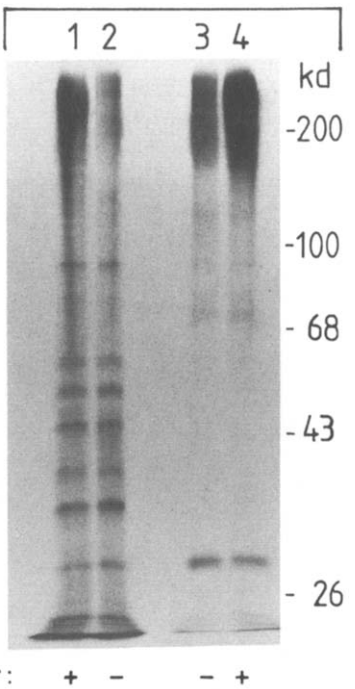

Fig.1. Fluorogram of ^{35}S-radiolabeled cell surface components of *S. cerevisiae* α-cells. 50 ml of α-cells (OD_{578} = 1.0) were incubated in a defined sulfate-free medium [15] until the culture reached an OD of 1.7. Then, 20 μCi of $^{35}SO_4^{2-}$/ml (spec. act., 100 mCi/mmol) were added and the cells further incubated in the presence or absence of *a*-factor (10 units/ml) for 90 min at 30°C. After harvesting the cells, their surface components were released and separated by SDS-gel electrophoresis as described in section 2.7. Crude extracts, lanes 1 and 2; active fractions after the first TSK column, lanes 3 and 4.

Tris-Acryl M-DEAE, Pharmacia) and the activity (inhibition of agglutination, see below) eluted with a KCl gradient (0–400 mM in 10 mM Tris-HCl 7.0). The active fractions were pooled, dialyzed against 2 mM Tris 7.0, and 5 mM EDTA and lyophilized. For HPLC gel filtration, the material was dissolved in 0.2–1.0 ml of 100 mM NH_4-acetate, pH 5.5 and separated on an LKB UltroPac TSK G 3000 SW column. The active fractions were pooled, dialyzed (as above) and concentrated in a Speed Vac.

2.4. *Agglutination assay (bioassay for α-agglutinin)*

Agglutination of pheromone-induced cells was measured by the procedure of Pierce and Ballou [8]. 50 μl of Mat*a*-cells (7.5×10^6 cells) induced with α-factor were added to 125 μl of 0.1 M sodium phosphate buffer, pH 6.0, in flat-bottom microtiter plates (15 × 17 mm wells); subsequently 25 μl of extract to be tested was added in increasing dilutions (2:1). After 1 h of gentle shaking, 50 μl of *a*-factor-pretreated α-cells (7.5×10^6 cells) were added. After 5–10 min of shaking, agglutination could easily be seen in non-inhibited controls. One assay unit is defined as the minimal amount of extract that clearly inhibited agglutination under these conditions. Further details are given in a previous publication [5].

2.5. *Digestion with EndoF*

250 mU endoglycosidase F (EndoF) from Boehringer Mannheim were incubated with 500 μg protein in 50 mM sodium phosphate buffer, pH 6.1, containing 35 mM EDTA in a total volume of 100–200 μl overnight at 37°C.

2.6. *Cloning of the gene*

The yeast genomic library, kindly provided by Dr C. Sengstag, contained yeast DNA fragments of about 10 kb that had been obtained by partial *Sau*3A digest of DNA from *S. cerevisiae* wild type S288C. The fragments were ligated into a centromere vector pCS 19 [9]. DNA sequencing was performed according to Sanger et al. [10], as detailed in [11]. RNA analysis was carried out as described previously [11].

2.7. *Other procedures*

SDS and native gel electrophoreses were carried out according to Laemmli [12] and Barbaric et al. [13], respectively. Protein was determined according to Bradford [14].

3. RESULTS AND DISCUSSION

When *a*-factor-treated α-cells were incubated with 6 μg zymolyase 100 T/ml for 90 min, the

Table 1

Purification of α-agglutinin

	Total protein (μg)	Total activity (units)	Spec. act. (units/μg)	Purification (-fold)	Yield (%)
Crude extract	5460	29120	5.3	–	100
DEAE-Pool	2000	28000	14	2.6	96
TSK 3000	730	25600	35	6.6	87
TSK 2000 after Endo F	130	9472	73	13.7	32

5 l α-cells with OD_{578} of 3.1 after induction in *a*-medium were treated with zymolyase, which resulted in the crude extract; for details and further purification see section 2

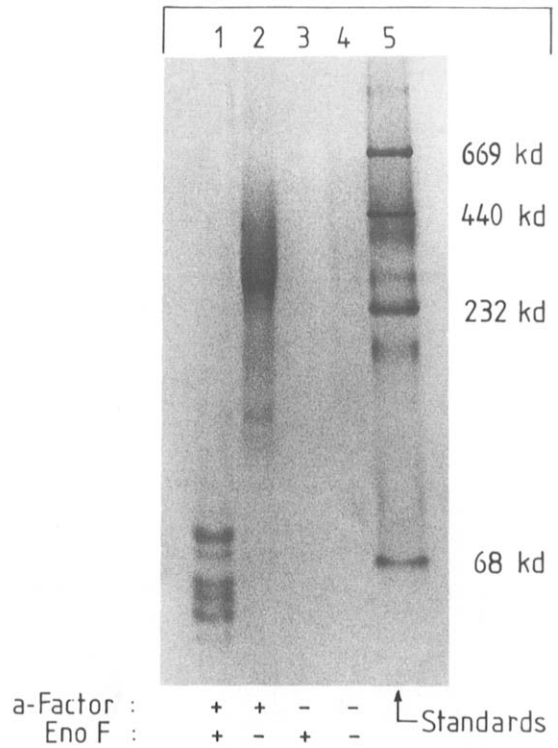

Fig.2. Coomassie stained non-denaturing gradient gel separating polypeptides by size (according to [13]). Lanes 3 and 4, α-agglutinin-active fractions after first TSK column; lanes 1 and 2, α-agglutinin-active fractions after second TSK column (see also table 1).

supernatant obtained after centrifugation strongly inhibited the agglutination of induced *a* with α-cells. Control α-cells released 5- to 10-fold less inhibitory activity. Cells treated in this way with zymolyase remained intact and 100% viable (data not shown). With α-cells previously radiolabeled with $^{35}SO_4^{2-}$, zymolyase released a number of cell surface components shown after SDS gel separation in the fluorogram of fig.1 (lanes 1 and 2). Clearly, a large molecular mass (>200 kDa) was the only one, showing an increase of 5- to 10-fold, due to *a*-factor pretreatment. After two column chromatographic purification steps (table 1), this large molecular mass material was enriched (fig.1, lanes 3 and 4). When the gel filtration column was repeated after Endo F digest of the active fraction (see below), an even higher specific activity was obtained (table 1).

Separation of the purified material (after the second column) in a native-gradient gel resulted in the picture of fig.2, lane 2; in this case the material showed a relative molecular mass of 250 to 400 kDa. The corresponding purified material of non-induced cells is hardly visible. After Endo F treatment, a fairly regular pattern of 5 to 6 bands was obtained (lane 1) with apparent relative molecular masses of 72, 68, 61, 57, 54, and 51 kDa (as determined by SDS-gel electrophoresis; data not shown). All these bands showed biological activity in the agglutination test. Since the activity obtained in each case correlated well with the in-

Table 2

N-terminal amino acid sequences of the various polypeptides of α-agglutinin obtained after Endo F treatment (fig.2, lane 1)

Polypeptide (rel. molec. mass, kDa)	N-terminal amino acid sequence
72	ININD
68	ININDITFSNLEITPLTANKQ
61	ININD
57	ININDITFSNLEIT
54	ININ
51	INI

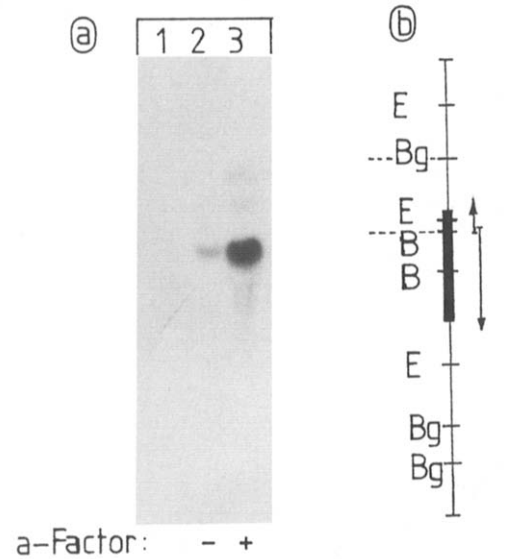

Fig.3. (a) Northern blot analysis. Total RNA of *a*-factor-treated (15 min) and control cells was isolated and hybridized (see [11]) with the *Bgl*II-*Bam*HI restriction fragment of clone 11 (see fig.3b, dashed lines). (b) Restriction endonuclease map of clone 11 carrying the whole α-agglutinin gene (black box). B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI. Arrows, sequencing strategy; for explanation, see fig.4.

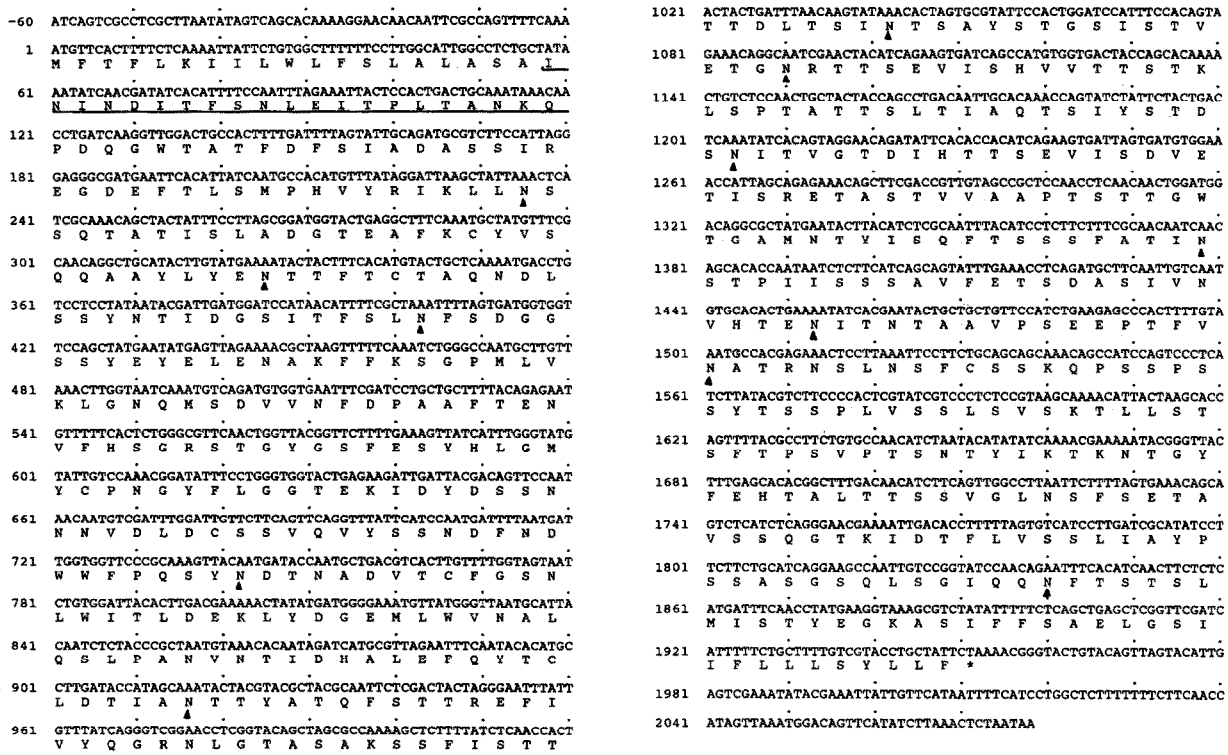

Fig.4. Nucleotide sequence and predicted amino acid sequence of the gene coding for α-agglutinin. The N-terminal sequence was detected in the *Bam*HI-*Bgl*II fragment; for the rest of the gene, 12 overlapping subclones were sequenced, according to the downward arrow of fig.3b. Underlined is the N-terminal amino acid sequence corresponding to that of table 2. Triangles indicate potential *N* glycosylation sites.

tensity of the Coomassie stain, the specific activity is estimated to be the same for each individual band. The protein characterized by Lipke et al. [7] also remained active after removal of carbohydrates; the molecular masses of their proteins, however, differed considerably from those shown here.

When the bands of fig.2 after blotting onto Immobilon foil were cut out and sequenced by Dr R. Deutzmann, Regensburg, the N-terminal amino acid sequences of all six bands were found to be identical (table 2). The reason for the difference in molecular masses of the bands is not known; proteolytic loss of carboxy termini of increasing size seems most likely, however (see below).

The oligonucleotide 5'GTG/TATGTCGTTG/TATGTTG/TAT was synthesized corresponding to the N-terminus ININDIT. It gave a strong signal on a Northern blot with mRNA from induced α-cells and a much weaker one with mRNA from non-induced cells (data not shown).

When a yeast genomic library was screened with the oligonucleotide, 16 positive clones were obtained. A 1.5 kb fragment of clone 11 with a total insert of 9.5 kb also reacted with mRNA of *a*-factor-induced cells considerably stronger than with that of control cells (fig.3). That this clone was a good candidate for the correct gene or part of it, was furthermore strengthened by the fact that mRNA of *a*-cells did not give a signal (fig.3a, lane 1) and that the signal of α-cells corresponded to a mRNA size of approximately 2.3 kb.

Subcloning clone 11 and sequencing according to Sanger et al. [10] resulted in the sequence given in fig.4. An open reading frame of 1950 bp corresponds to a protein of 650 amino acids containing the N-terminal 21 amino acids of table 2. Thus, the leader peptide is 19 amino acids long. The relative molecular mass of 68239 kDa of the mature protein agrees well with the 72 kDa determined for the largest polypeptide after Endo F treatment. Since 12 *N*-acetyl glucosamine residues

(= 2450 kDa) are likely to be attached to the polypeptide, according to 12 potential *N*-glycosylation sites in the sequence (fig.4), the protein moiety of the band most likely corresponds to 69500. The additional five bands on the gel (fig.2), therefore, seem to be proteolytic carboxy terminal deletions of increasing length. Since even the smallest polypeptide (51 kDa) is biologically active in preventing agglutination, the carboxy terminal one-third of the molecule seems not to be involved in the agglutination reaction.

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REFERENCES

[1] Betz, R., Duntze, W. and Manney, T.R. (1978) FEMS Microbiol. Lett. 4, 107–110.
[2] Fehrenbacher, G., Perry, K. and Thorner, J. (1978) J. Bacteriol. 134, 893–901.
[3] Terrance, K. and Lipke, P.N. (1981) J. Bacteriol. 148, 889–896.
[4] Yanagishima, N. (1984) in: Encyclopedia of Plant Physiology, New Series. Cellular Interactions, vol.17 (Linskens, H.F. and Heslop-Harrison, J. eds) pp.402–432, Springer, Berlin.
[5] Orlean, P., Ammer, H., Watzele, M. and Tanner, W. (1986) Proc. Natl. Acad. Sci. USA 83, 6263–6266.
[6] Watzele, M., Klis, F. and Tanner, W. (1988) EMBO J. 7, 1483–1488.
[7] Lipke, P.N., Terrance, K. and Wu, Y.-S. (1987) J. Bacteriol. 169, 483–488.
[8] Pierce, M. and Ballou, C.E. (1983) J. Biol. Chem. 258, 3576–3582.
[9] Sengstag, C. and Hinnen, A. (1987) Nucleic Acids Res. 15, 233–246.
[10] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[11] Watzele, G. and Tanner, W. (1989) J. Biol. Chem. 264, 8753–8758.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Barbaric, S., Kozulic, B., Ries, B. and Mildner, P. (1984) J. Biol. Chem. 259, 878–883.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Boller, T., Dürr, M. and Wiemken, A. (1975) Eur. J. Biochem. 54, 81–94.