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# THE ISOLATION OF BIOLOGICALLY ACTIVE MATING PHEROMONE, **a**-FACTOR, FROM THE YEAST, Saccharomyces cerevisiae

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Haploid cell-types of baker's yeast, Saccharomyces cerevisiae, secrete pheromones which are essential for conjugation. Recently a putative structure for the elusive **a**-factor pheromone has been reported. In this report we present a procedure to obtain **a**-factor from batch cultures of cells using hydrophobic Amberlite XAD-2 resin in the growth medium with subsequent differential washings of the resin with organic solvents. We have determined the biological activity of the **a**-factor preparation by verifying that there is an increase in transcription of the **a**-factor receptor gene, STE3, by Northern analysis of STE3 mRNA before and after exposure of the appropriate cell type to **a**-factor. Furthermore, a β-galactosidase assay of the putative receptor gene fused to the lacZ gene, coding for β-galactosidase (STE3-lacZ), was done to quantify the biological activity of the **a**-factor. 

Haploid Saccharomyces cerevisiae cells of the **a** and α mating type both secrete a low constitutive level of specific cell-type oligopeptide pheromone, **a**-factor and α-factor respectively. These pheromones are essential mediators of sexual conjugation in the life cycle of this yeast (1). Cells responding to pheromones undergo several dramatic physiological changes which include arrest of the cell cycle in the G1 phase and differential regulation of certain genes such as the receptors for the pheromones. In contrast to the α-factor, whose amino acid sequence, structure to activity relationship, and secretion have been studied in great detail (2, 3, 4, 5, 6, 7, 8, 9, 10), very little is known about the **a**-factor.

The DNA sequences of two structural genes (MFA1 and MFA2) coding for putative **a**-factor precursors which contain 36 and 38 amino acid residues were determined (11) and their secretion was shown to be essential for mating (12). Synthetic oligopeptides and several derivatives based on the sequences information failed to produce growth arrest in cells but blocked the effects of biologically active **a**-factor (13). Further biochemical and genetic observations suggested that the **a**-factor peptide was acylated and that the carboxy terminal was esterified (13, 14). Furthermore, secretory mutants blocked in the pathway involving endoplasmic reticulum, golgi, and secretory vesicles still appear to secrete **a**-factor (15). Post-translational processing of the MFA1 and MFA2 gene products by a novel secretory pathway is suggested by these observations (15). Recently, the amino acid sequences of two **a**-factor peptides were reported (16) to be modified by the attachment of a farnesyl moiety to the carboxy terminal cysteine residue which was blocked by a methyl ester:

$$\text{Tyr-Ile-Ile-Lys-Gly-}\begin{matrix}\text{VAL}\\ \text{LEU}\end{matrix}\text{-Phe-Trp-Asp-Pro-Ala-(S-Far)Cys-OCH3}$$

0006-291X/90 $1.50

Because of the hydrophobic nature of **a**-factor and its associated farnesyl group and the assays used to assess its biological activity, **a**-factor has been quite difficult to isolate reproducibly. Here we present a modification of the Strazdis and MacKay procedure (17, 18) for the rapid isolation of **a**-factor and the quantification of the activity by the use of a STE3-lacZ fusion protein product (19).

## Materials and Methods

### Yeast strains and growth conditions

The strains of Saccharomyces cerevisiae used were DC5 (MAT**a** leu2-3 leu2-112 his3 gal2 can1) and DC6 (MATα leu2-3 leu2-112 his4 gal2 can1), YY69 (MATα leu2-3 leu2-112 his4 gal2 can1; carrying the plasmid pSL35). The media used were YEPD and SD-Leu as described by Sherman et al. (20). Culture of YY69 were grown on SD-Leu agar plates and an isolated colony was then grown overnight in 5 ml SD-Leu liquid media. This culture was transferred to 25 ml of SD-Leu liquid media and grown in a shaking water-bath at 30° C. The overnight culture was centrifuged and resuspended at about 1 x $10^7$ cells/ml in YEPD medium. This culture was grown for two hours in a shaking water bath and **a**-factor was added at this time. Aliquots were then taken at appropriate times to assay β-galactosidase activity produced in response to **a**-factor. Cultures of DC5 were grown on YEPD.

### Northern Blot Analysis

Yeast cells of the DC6 strain were grown overnight in YEPD media. The following day, the cells were sedimented by centrifugation at 5000 rpm for 5 minutes in a Sorvall GSA rotor and resuspended in fresh YEPD at a concentration of 4X$10^7$ cells/ml. After one generation time (2 hrs), a 200 ml sample of the culture was removed and total cellular RNA was extracted (20). Twenty-five µl of **a**-factor preparation (962 µg protein) per 10 ml of culture was added to the remaining cells. Subsequent RNA samples were extracted from cells removed from the culture at 15, 30, 60, 90 minutes. Twenty micrograms of total RNA ($OD_{260}$ nm) was prepared and electrophoresed through a 1% agarose/2.2 M formaldehyde gel (21). The fractionated RNA was transferred to a Zeta Probe membrane and the membranes were baked for two hours under vacuum (21). The filter was prehybridized for 3-5 hours at 42° C in 50% formamide, 5X SSPE, 0.1% SDS, 5X Denhardt solution, 200 µg/ml denatured Salmon sperm DNA, and 10% dextran sulfate. Radioactive probes were made from the STE3 insert by random priming (Promega) and hybridization was allowed to proceed overnight at 42° C in fresh buffer. The filters were washed twice in 6X SSC/0.1% SDS at room temperature for 15 minutes, followed by a wash in 2X SSC/0.1% SDS at room temperature for 15 minutes and finally in 2X SSC/0.1% SDS at 65° C for 30 minutes. Autoradiography was performed (22). All membranes were also probed with a randomly primed URA3 gene probe as a control.

### Assay of β-galactosidase activity

The β-galactosidase assay was essential as described by Hagen and Sprague (19) with minor modifications. The YY69 cells were grown overnight in SD-Leu at 30°C, they were centrifuged at 7000 rpm for 5 min, resuspended in YEPD medium at a concentration of about 1x$10^7$ cells/ml and grown at 30°C four two hours. In all assays for **a**-factor, control cells were incubated with an equal concentration of the solvent used to dissolve the pheromone. Samples of 1 ml were taken at appropriate times, centrifuged in an Eppendorf tubes and the cell pellet was resuspended in 150 µl of 20 mM Tris-HCl, 85 mM NaCl, 1mM $MgCl_2$ (pH 7.4), a sample of 20 µl was pipetted into 1 ml of 0.15 M NaCl, 3.7% formaldehyde (v/v) for cell density determinations at 650nm. The remainder of the sample was treated with 2 µl of toluene and 2 µl of 5% (w/v) Sarkosyl. The samples were mixed vigorously then the toluene was allowed to evaporate for one hour at room temperature. Enzyme activity was measured at room temperature by pipetting 95 µl of the treated cells into 1.0 ml of 0.1 M sodium phosphate, 2.2 mM o-nitrophenyl-β-D-galactopyranoside (pH 7.0) and incubated for the appropriate times. Termination of the reaction was accomplished by the addition of 0.5 ml of 1 M $Na_2CO_3$. The utilization of ONPG as substrate was determined at 405nm and the light

scattering correction was measured at 562nm. The units of β-galactosidase activity are expressed as

$$\frac{1000 \times [OD_{405} - (1.03 \times OD_{562})]}{t \times OD_{650} \times ((1.02/0.02)\times 0.095)}$$

where the reading at 405 nm is a measurement of o-nitrophenol and light scattered by cellular debris, the value obtained at 562 nm is to account for light scattering, and the 600 nm value is for cell density. The values 1.02/0.02 and 0.095 represent dilution factors for the measurements at 650 nm and 405 nm respectively, 1.03 is a correction factor for light scattering, t represents time in minutes, and the β-galactosidase units are multiplied by 1000 for convenience. Optical density measurements were done with the Bio-Tek Instruments Microplate reader EL307C or with a Beckmann DU65 spectrophotometer.

ONPG was purchased from Sigma all other chemicals were of highest purity available.

Bead preparation

Amberlite XAD-2 resin was obtained from Sigma and it was prepared essentially as described by Strazdis and MacKay (17). The polystyrene resin was washed with excess double distilled water, then with 6 volumes of 1,2-dichloropropane: 1-propanol (1:3) and then 6 volumes of 1-propanol, both washes were done at 40°C for 3 hrs. The resin was stored in propanol at room temperature until needed. An appropriate quantity of resin (100 ml of well stirred prewashed resin per litre of culture) was then washed with excess double distilled water, sterilized by autoclaving in water and added to the culture. The beads were never allowed to go dry.

## Results and Discussion

The initial step of the purification procedure consisted in growing DC5 cells in one litre cultures with Amberlite XAD-2 resin. Overnight cultures were separated from the beads by decanting most of the liquid followed by several copious washings with sterile water and

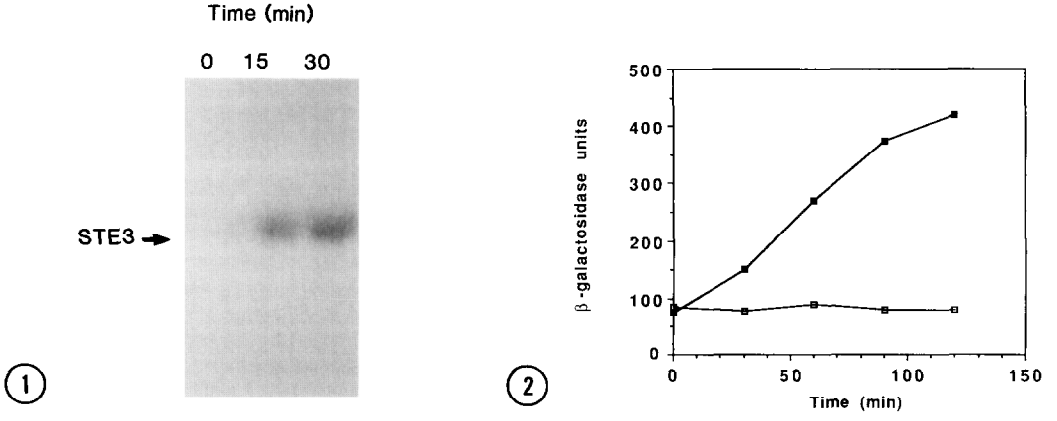


Figure 1. Induction of mRNA encoding the receptor, STE3. RNA extracted from MATα cells at various times after exposure to the **a**-factor preparation was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and blotted onto nylon membrane then probed with the randomly primed sequence of the gene coding for STE3.

Figure 2. Induction of β-galactosidase with the **a**-factor preparation. The induction of β-galactosidase was followed in MATα cells harbouring the STE3-lacZ fusion plasmid following **a**-factor addition. Cell cultures were incubated in the presence of a fixed concentration (926 ng protein/10 ml of medium) of **a**-factor preparation (■) or in the absence of a-factor but with an equal volume (25 µl/10 ml of medium) of DMSO (□).

removal of the liquid by suctioning. The beads were then immersed in 3 volumes of 40% methanol and incubated for 2 hrs at 40°C. This solvent was removed by suction and the beads were immersed in 3 volumes of 1-propanol for the final extraction. After an incubation of 2 hrs at 42°C, the propanol containing the **a**-factor was removed by suction and rotary evaporated to dryness at about 55-60°C. The brownish residue was dissolve in the minimal amount of dimethyl sulfoxide (DMSO) or methanol. This **a**-factor preparation was then stored at -20°C in aliquots or in bulk until tested.

It has been reported that the gene, STE3, encoding the putative receptor for the **a**-factor, is transcriptionally activated by the presence of **a**-factor (19). Therefore we tested the activation of transcription of the STE3 gene in DC6 cells by probing total RNA isolated from cells in the absence or presence of **a**-factor with the STE3 sequence. There is an increase over time of the STE3 mRNA in DC6 cells exposed to the **a**-factor preparation (Figure 1). This demonstrates that the **a**-factor does indeed active transcription of the STE3 mRNA in cells that respond to the pheromone. Therefore the **a**-factor preparation is biologically active and produces the expected result: transcriptional activation of the putative receptor gene, STE3.

In order to quantify the response we used cells of the YY69 culture grown overnight in SD-Leu media. The cells were counted, washed and resuspended to 1 x $10^7$ cells/ml in YEPD to assay the biological activity of **a**-factor using the response of the receptor STE3-lacZ fusion product. The β-galactosidase activity is seen to increase in the presence but not in the absence of **a**-factor (Figure 2) over a period of 120 min. These results obtained at the enzymatic level corroborate the previous set of results obtained at the transcriptional level. Therefore, the **a**-factor preparation is biologically active and elicits the predicted response at both the mRNA and protein levels. To further quantify the biological activity of this **a**-factor preparation, a series of concentrations were evaluated for their effects in eliciting β-galactosidase. It was observed that with increasing **a**-factor concentration there was an increase in enzymatic activity (data not shown).

Both the analysis of the mRNA and the β-galactosidase assays have been used to verify that the **a**-factor preparation does elicit the correct set of changes at the transcriptional and translation levels in cells which should respond to **a**-factor. Therefore, use of this rapid technique to isolate and concentrate **a**-factor from spent media should make it possible to increase yields and facilitate purification.

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