SOLID PHASE PEPTIDE SYNTHESIS OF α-FACTOR, A YEAST MATING PHEROMONE

Elena Ciejek, Jeremy Thorner, and Michael Geier

Department of Bacteriology and Immunology
University of California, Berkeley, California 94720

Received August 10,1977

SUMMARY

Based on analysis of highly purified preparations of natural α-factor and on the sequence recently reported by others, oligopeptides of the following structures were chemically synthesized by the solid phase method of Merrifield:

N-Trp-His-Trp-Leu-GlN-Leu-Lys-Pro-Gly-GlN-Pro-Met-Tyr-C
N-His-Trp-Leu-GlN-Leu-Lys-Pro-Gly-GlN-Pro-Met-Tyr-C

Both synthetic species arrested a cells in G1, inhibited their DNA synthesis, caused them to elongate markedly, and induced an increase in their adhesivity toward α cells. Neither synthetic material caused any of these effects in α cells or in a/α diploids.

The mating response is a developmental alternative open to the two haploid cell types, a and α, of the yeast Saccharomyces cerevisiae. Cells of mating-type α excrete into the medium a diffusible pheromone-like substance, called α-factor (1). The presence of this substance reversibly arrests the normal cell division cycle of a cells (2) and initiates other biochemical and morphological changes in a cells (3,4) preparatory to conjugation. One of the a cell responses is a pronounced elongation, an effect termed "schmooing" (5), which provides the basis of bioassays for detection of α-factor (6). Duntze and coworkers have purified α-factor in small amounts sufficient to permit its chemical characterization and recent work (7,8) indicates that the purified material is a family of four closely related oligopeptides with the basic sequence: N-(Trp)-His-Trp-Leu-GlN-Leu-Lys-Pro-Gly-GlN-Pro-Met(or MetSO)-Tyr-C. In marked contrast, Yanagishima and collaborators purified a peptide with similar biological activity (9), but with quite a different amino acid composition. These investigators found (10) their material lacked lysine, methionine, and tryptophan, and contained stoichiometric amounts of isoleucine, valine, and phenylalanine in addition to the other 6 amino acids, although this did not seem to be borne out in their subsequent work (11). In this report, we

ISSN 0006-291X

describe a new and rapid purification procedure that gives essentially homogeneous α-factor in good yield. Our findings are in essential agreement with the results of Duntze and not with those of Yanagishima. To further resolve this discrepancy, to confirm the assigned sequence, and to prepare sufficient quantities of α-factor for investigating its mode of action at the molecular level, we attempted the chemical construction of both the do- and tridecapeptides. We report here the successful chemical synthesis of biologically active material.

EXPERIMENTAL PROCEDURE

Materials- Reagent chemicals were obtained from the following sources: BOC[+]-derivatives of L-tryptophan, L-leucine, L-proline, glycine, and L-methionine, BOC, N^{im}-tosyl-L-histidine, BOC, N^{ε}-carbobenzoxy-L-lysine, BOC, O-o-bromocarbobenzoxy-L-tyrosine, and BOC-L-glutamine-p-nitrophenyl ester from Peninsula Laboratories; N,N-dicyclohexylcarbodiimide from the Institute for Protein Research, Osaka; chloromethylated polystyrene-2% divinylbenzene (0.98 meq chloride/g), fluorescamine, dansyl chloride, and ninhydrin from Pierce; pyridine, dichloromethane, methanol, acetone, dioxane, N,N-dimethylformamide, and chloroform from Mallinckrodt; methanesulfonic acid, triethylamine, and tryptamine from Eastman; anisole, N,N-diisopropylethylamine, and trifluoroacetic acid from Aldrich; Alumina W200 Basic from ICN; Bio-Rex 70 (10.2 meq/g) from Bio-rad; Whatman P-11 (3.8 meq/g) from Reeve-Angel; Sephadex LH-20, LH-60, and Octyl-Sepharose (∿40 μmoles ligand/ml) from Pharmacia; α-melanocyte-stimulating hormone from Bachem; and media constitutents from Difco. A wrist-action shaker for manual solid phase peptide synthesis was purchased from Schwarz-Mann. Reaction vessels were made by Berkeley Glass. All organic solvents were redistilled at least once before use and were stored under nitrogen and with Linde molecular sieve as drying agent. All other chemicals were reagent grade. All column chromatographic media were precycled and equilibrated with the appropriate buffer or solvent as recommended by the manufacturers.

Biological Assay of α-Factor Activity- Activity of α-factor preparations was monitored in four different ways. Arrest of a cells in G1 was scored by measuring the increase in the percentage of unbudded cells, essentially as described by Hartwell (12). Inhibition of DNA synthesis in a cells was measured by following the incorporation of [^{14}C]adenine into acid-precipitable alkali-resistant material, by minor modifications of the method of Throm and Duntze (13). Induction in a cells of increased agglutinability toward α cells was quantitated by the procedure described elsewhere (Fehrenbacher, G., K. Perry, and J. Thorner, Exp. Cell Res., submitted). Morphological elongation of a cells was scored microscopically and was used routinely for following α-factor activity during purification. For this purpose, samples (1-200 μl) of fractions were dried at 37° in the wells of a standard plastic microtiter plate. Exponential phase cells of strain X2180-1A (a gal 2 mal SUC 2 CUP 1) were diluted to 2 x 10^6/ml with fresh SD medium (14) and portions (200 μl) of the cell suspension were added to the wells. The plate was covered with an adhesive plastic strip and gently agitated. The state of the cells in the wells was observed after 4 hours at 30°. Control cells, in the absence of α-factor, were always budded and not detectably elongated. One unit of α-factor activity was

[+]Abbreviations: BOC-, tert-butylbenzoxycarbonyl-; dansyl-, 1-dimethylaminonaphthalene-5-sulfonyl-.

defined as the amount of peptide sufficient to produce a pronounced morphological change in at least 20% of the input a cells.

Purification of Natural α-Factor- Cells of Saccharomyces cerevisiae X2180-1B (α gal 2 mal SUC 2 CUP 1) were used to inoculate SD medium at 10^6/ml and were grown at 30° for 48 hours with vigorous aeration either on a gyratory shaker or in a fermenter (New Brunswick Fermacell). Just prior to harvesting, ethylenediamine tetraacetic acid, phenylmethylsulfonyl fluoride, and 2-mercaptoethanol were added to the culture, each to a final concentration of 1 mM. The cultures were then immediately chilled to 4° and the cells removed by centrifugation in a refrigerated Sharples supercentrifuge. The cell-free culture fluid (pH ∿2.5) was percolated at 4° through a bed (∿2 ml/100 ml culture medium) of Bio-Rex 70 equilibrated with 0.1 N acetic acid. The column was washed with 2 bed volumes each of 0.1 N acetic acid and 50% ethanol. The α-factor activity was eluted with 0.01 N HCl in 80% ethanol. All subsequent steps were performed at room temperature. The eluate was brought to pH 4.0 with NH_4OH, adjusted to a conductivity equivalent to 25 mM Na-acetate (pH 4.0), and applied slowly to a bed (1 ml/2 mg protein) of Whatman P-11 phosphocellulose. The column was washed with 2 bed volumes of 25 mM Na-acetate (pH 4.0) and then eluted with a linear gradient produced by 3 bed volumes of 25 mM Na-acetate (pH 4.0) in the mixing chamber and 3 bed volumes of 1.0 M Na-acetate (pH 6.5) in the reservoir. The α-factor activity emerged with a distinct peak of protein at about 0.5 M salt. The phosphocellulose eluate was lyophilized, resuspended in a minimal volume of 90% methanol, the salt removed by centrifugation, and the sample subjected to filtration through a bed (1-2% of sample volume) of Sephadex LH-20. The most active fractions were pooled, concentrated under a nitrogen stream, and re-run on Sephadex LH-60 (Preparation B). Alternatively, the phosphocellulose eluate was applied directly to a bed (1 ml/0.25 mg protein) of Octyl-Sepharose. The column was washed with 2 bed volumes of 0.5 M Na-acetate (pH 5.0), with 2 bed volumes of H_2O, and then the α-factor activity was eluted with 90% methanol. The peak fractions were pooled, concentrated under a nitrogen stream, and then subjected to filtration through Sephadex LH-60 (Preparation A). This purification scheme has been used on batches of culture medium from 10-200 liters, and either variation of the method resulted in yields of 15-30%.

Solid Phase Synthesis of α-Factor- In general, the procedures recommended for manual solid phase peptide synthesis by Stewart and Young (15) were followed, with modifications as suggested by Erickson and Merrifield (16). Batches of resin (1-10 g) were esterified with BOC, O-o-bromocarbobenzoxy-L-tyrosine (200-300 μmoles/g) and the appropriate amino acids coupled to the N-terminus of the growing peptide chain with N,N-dicyclohexylcarboiimide in dichloromethane in the presence of 2.5-fold excess of each BOC-amino acid and for a reaction time of 2 hours at room temperature. The exception was BOC-L-glutamine-p-nitrophenyl ester which was coupled using a 4-fold excess of reagent in N,N-dimethyl formamide for 20 hours. De-blocking was with 25% trifluoroacetic acid in dichloromethane, except after the addition of BOC-L-tryptophan 0.2% tryptamine was also present. The completeness of deprotection steps and coupling reactions was qualitatively assessed at each step by the ninhydrin method. The course of the synthesis was followed, in some cases, by examining the growing peptide at chain lengths of four and six by amino acid analysis. On occasion, it was necessary to double couple BOC-L-methionine, BOC-L-glutamine , and BOC-L-histidine . In one synthesis, any free N-termini remaining after a coupling were eliminated by reaction with fluorescamine (17). The finished peptide was cleaved from the resin and the side chain protecting groups removed by treatment with anhydrous HF in the presence of anisole. The peptide material was extracted with ethyl acetate to remove spirolactones and dissolved in 1 N acetic acid. The resin was removed by filtration and the filtrate and washes were lyophilized. The complete biologically active peptide was purified away from the excess anisole and from error peptides that result from side reactions and incomplete couplings by the purification scheme presented above.

TABLE I

AMINO ACID COMPOSITION OF PURIFIED NATURAL α-FACTOR

Amino Acid	Preparation A		Preparation B	
	nmoles	molar ratio	nmoles	molar ratio
Trp	36	2.2	106	1.5
Lys	16	1.0	45	0.6
His	15	0.9	51	0.7
Arg	Undetectable	-	8	-
Asx	Trace*	-	Undetectable	-
Thr	Trace	-	4	-
Ser	Trace	-	6	-
Glx	35	2.1	144	2.1
Pro	33	2.0	123	1.8
Gly	18	1.1	75	1.1
Ala	Trace	-	6	-
Cys	Undetectable	-	Undetectable	-
Val	Trace	-	5	-
Met†	16	1.0	77	1.1
Ile	Undetectable	-	4	-
Leu	33	[2.0]	140	[2.0]
Tyr	15	0.9	64	0.9
Phe	Undetectable	-	5	-

* Trace < 1 nmole

† Met + MetSO

The yield of tridecapeptide was 1 % and the yields for the dodecapeptide in two separate syntheses were 1 % and 2 %, respectively. In all, about100mg of biologically active synthetic material were prepared.

Other Procedures- Protein concentration was measured by the method of Lowry et al (18), using α-melanocyte-stimulating hormone as standard. Amino acid analyses were performed on either a Beckman 121M or on a Beckman 120B amino acid analyzer. Hydrolysates were usually prepared using 4 N methanesulfonic acid in the presence of 0.2% tryptamine (19). Dansylation and hydrolysis were done by the technique of Gray (20), followed by chromatography on polyamide thin layer plates (21). Since dansyl-tryptophan is destroyed in this procedure, in some cases methanesulfonic acid-tryptamine was used to prepare hydrolysates; and, after neutralization and lyophilization, dansyl-amino acids were extracted with water-saturated ethyl acetate and then subjected to chromatography (Jones-Brown, Y. and J. Thorner, unpublished method).

RESULTS

Natural α-Factor- The purification procedure described here yielded material

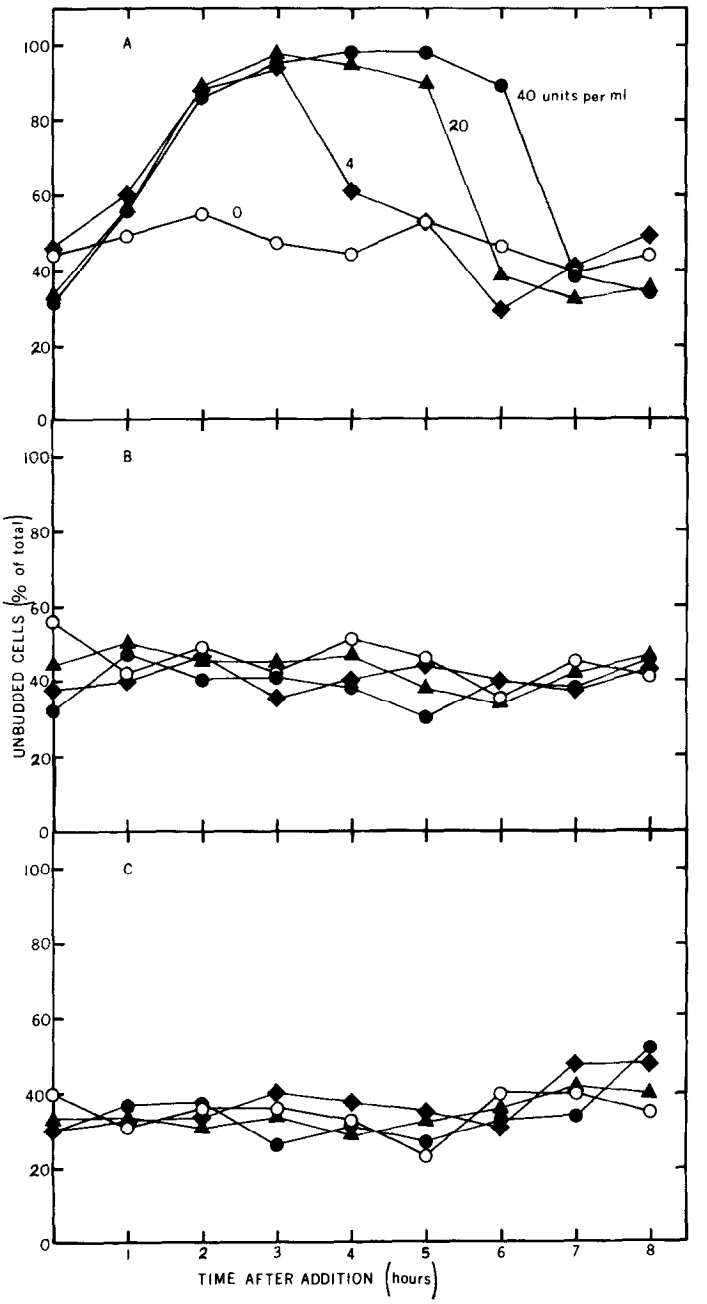

Figure 1. Accumulation of unbudded cells. Exponential phase cultures of X21-80-1A(a) (Panel A), X2180-1B (α)(Panel B), and a diploid P2180D (a/α) derived from mating the two haploids (Panel C) were diluted to 10^6/ml in SD medium and portions (1 ml) were placed in small screw-capped tubes in the absence (O) or in the presence of synthetic histidine-terminated, α-factor, at final concentrations of 4 (◆), 20 (▲), and 40 (●) units/ml. The tubes were placed on a rollerdrum at room temperature and samples were withdrawn at the indicated times for determination of the fraction of unbudded cells, as indicated in "Experimental Procedure". Results were similar for the synthetic tryptophan-terminated factor.

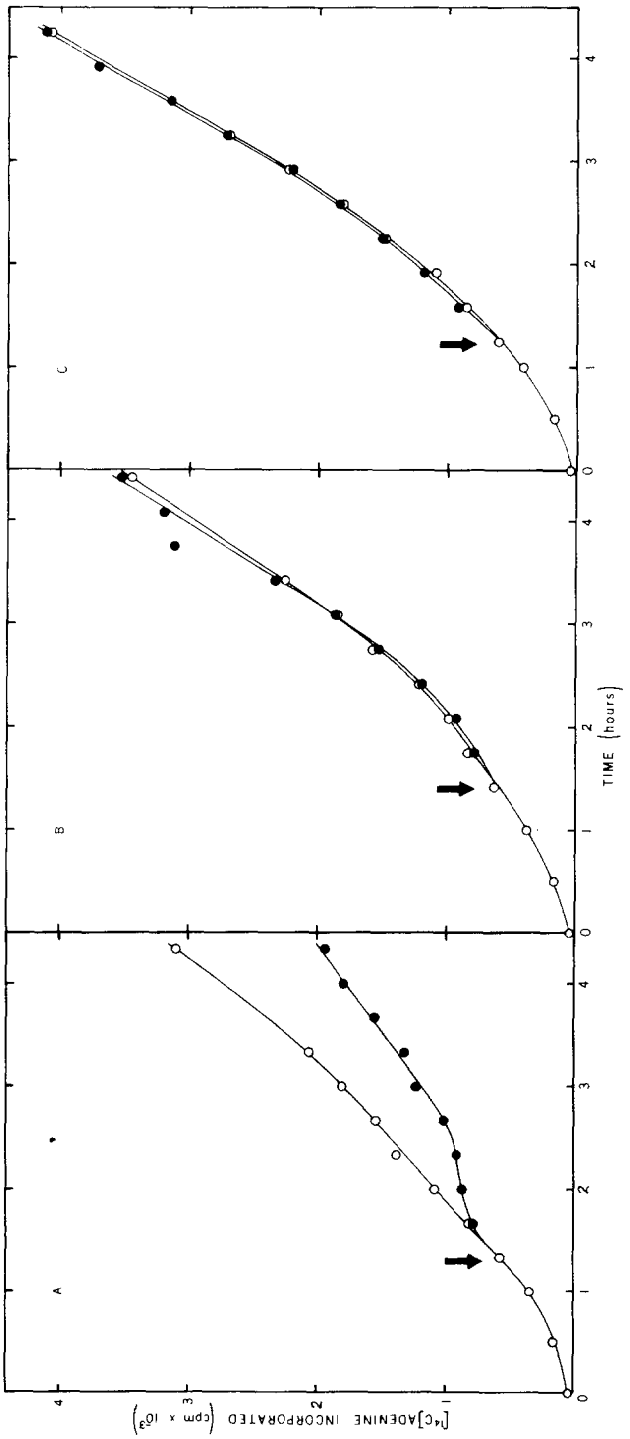

Figure 2. Inhibition of DNA synthesis. Exponential phase cultures of X2180-1A (a) (Panel A), X2180-1B (α) (Panel B), and P2180D (a/α) were diluted to 2 x 10^6/ml in fresh SD medium and incubated in a gyratory water bath at 30°. After one generation of growth, [^{14}C]adenine was added to each culture to a final concentration of 0.2 mM and 0.5 μCi/ml. After about one-half generation, a 5 ml portion of each culture was transferred to a screwcapped Erlenmeyer flask containing enough synthetic histidine-terminated α-factor to give a final concentration of 50 units/ml. At the indicated times samples (0.5 ml) were withdrawn from these cultures (●) and from the control cultures (○) and the amount of label incorporated into DNA determined, as stated in "Experimental Procedure". Results were similar for the synthetic tryptophan-terminated factor.

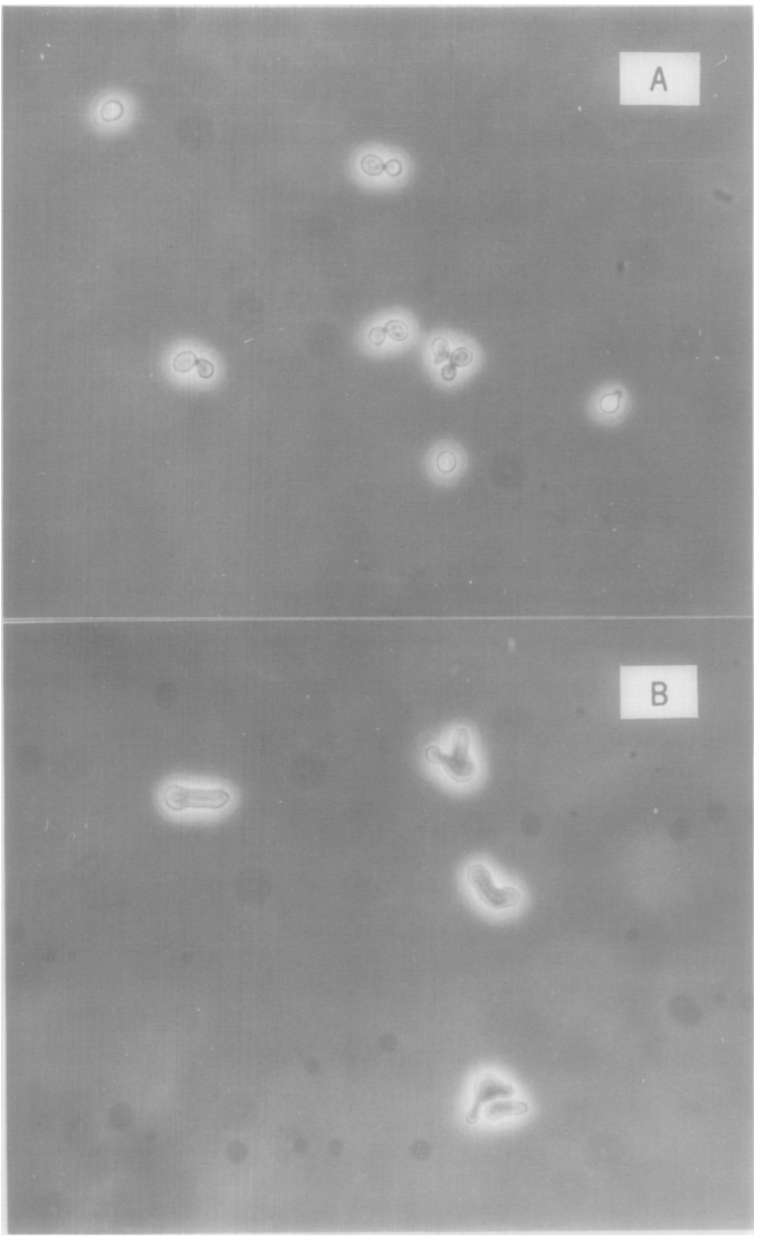

Figure 3. Morphological elongation. Exponential phase cells of X2180-1A (a) cultures were diluted to 2 x 10^6/ml in fresh SD medium and incubated at 30° in the wells of a microtiter dish for four hours in the absence (A) and in the presence (B) of synthetic histidine-terminated α-factor at a final concentration of 2 units/ml. Cells of X2180-1B (α) and P2180D (a/α) in either the presence or absence of the synthetic material were budded and displayed no morphological abnormalities. Similar results were found for the synthetic tryptophan-terminated α-factor.

TABLE II

STIMULATION OF AGGLUTINATION BY α-FACTOR

Concentration (units/ml)	Natural	α-Factor Added: Synthetic (N-trp)	Synthetic (N-his)
		(% adhesion)	
None	10	6	5
0.001	14	14	-
0.01	24	28	23
0.1	32	26	26
1.0	36	48	40
10.0	29	26	34

of the amino acid composition shown in Table I. About 20-40 μg were obtained per liter of original culture medium, with a specific activity of 10-20,000 units/mg. This material gave one major and one minor spot, as revealed by ninhydrin or fluorescamine, upon two-dimensional electrophoresis (in 12.5% acetic acid-1.2% pyridine, pH 3.5) and chromatography (in isoamyl alcohol: pyridine: water: ethanol: acetic acid::70:70:60:20:5) using microcrystalline cellulose thin layer plates. The two components differed slightly in mobility only in the chromatographic direction. On silica gel thin layer plates these two components were better resolved . Our material yielded only N-terminal histidine upon dansylation and HCl hydrolysis, but when methanesulfonic acid was used, dansyl-tryptophan was found to be the major N-terminal residue. These results are completely consistent with the findings of Duntze. He reported that his material was a mixture of tryptophan-terminated tridecapeptide and his-terminated dodecapeptide, as determined by Edman degradation. On the other hand, the major component in his preparations was the dodecapeptide.

Synthetic α-Factor- The chemically synthesized oligopeptides displayed the complete array of biological activities found for the natural peptides. The synthetic species arrested a cells in G1 (Fig. 1), transiently inhibited DNA synthesis in a cells (Fig. 2), induced a pronounced increase in the ability of

a cells to agglutinate to α cells (Table II), and produced a marked morphological change in a cells (Fig. 3). The specific activities of the several different preparations of synthetic material ranged from 1/5 - 1/10 that of the natural pheromone. Although subjected to rather extensive purification, thin layer chromatography of the purified synthetic material revealed that, in addition to the major spot which co-migrated with the natural peptide, there were present significant amounts of several other species. If these related peptides compete with normal α-factor for its "receptor", then it could account in part for the reduced specific activity. In addition, it is possible that certain amino acids in a significant portion of the synthetic peptides have suffered damage (e.g. racemization) under the conditions of synthesis.

DISCUSSION

We found that the purification schemes for natural α-factor developed by others were both irreproducible and unnecessarily laborious. Consequently, we devised the simple and gentle procedure presented here. Our method has been used successfully in at least one other laboratory (R. Schekman, personal communication). The somewhat low specific activity of the chemically prepared materials makes absolute confirmation of the assigned sequence still somewhat uncertain. Nevertheless, the effects of the completely synthetic oligopeptides unambiguously demonstrate that these pheromones are indeed the sole primary signal for eliciting the biological responses observed in a cells during the conjugation process. Furthermore, the availability of relatively large amounts of biologically active synthetic material is permitting us to prepare antiserum (for development of a radioimmune assay), functional radioactive derivatives (for localizing the site of pheromone action), and affinity adsorbants (for isolating α-factor-binding components).

ACKNOWLEDGEMENTS

This work was supported, in part, by NIH Grant GM21841 from the National Institute of General Medical Sciences. We are extremely grateful to Dr. Janis D. Young for her generous time and advice during the initial stages of this work.

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