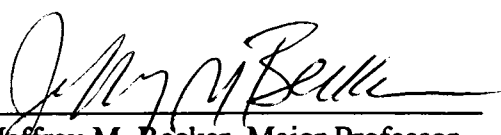
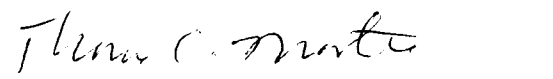


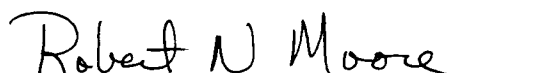
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STRUCTURE-FUNCTION STUDIES OF YEAST MATING PHEROMONES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Leann Lovin

December 1994

DEDICATION

This thesis is dedicated to my mother Diane Lovin. Her love, encouragement, and endless sacrifices have made this degree possible. My mother is an incredible lady, and I am very lucky to have her in my life. I would also like to thank my father, Juan Lovin, and my sister, Lisa Lovin McCurdy. Without their encouragement and constant support, I would not have had the will to complete my education. My grandfather, Cecil Roberts, has also played a major part in the completion of my degree. He encouraged me to pursue a Master's in Microbiology; even when he was in the hospital, he urged me to get back to the lab. To the rest of my family and friends, I give my sincerest thanks. I hope that their patience has finally been rewarded.

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ABSTRACT

Mating in *Saccharomyces cerevisiae* is initiated by extracellular peptides, known as mating pheromones. The mating factors are recognized by membrane-bound receptors which are similar to receptors in mammalian cells. The pheromone system of the *S. cerevisiae* can be used as a model to understand the mode of action of peptide hormones in more complex mammalian system. The purpose of this thesis is to provide information regarding the yeast mating pheromones. The first part of this project, described in Chapter 2, was to characterize α -factor analogs with an inserted p-Benzoylphenylalanine (Bpa) residue. This residue is photoactivatable by UV light, and it has been inserted because studies have shown that photoaffinity labeling can be used for the identification and localization of receptors. By performing biological assays, an analog with activity mimicking that of wild type α -factor (Norleucine¹²) was identified. This analog can be used in future studies in attempts to elucidate the binding site of the α -factor with its receptor. The second phase of this thesis project was to develop an assay which could be used to define the binding of **a**-factor to its receptor. As described in Chapter 3, an efficient binding assay could not be developed due to the hydrophobicity of the **a**-factor pheromone; however results achieved from these experiments could be a starting point for future attempts to develop a successful binding assay.

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CHAPTER 1

INTERCELLULAR COMMUNICATIONS BETWEEN THE TWO MATING TYPES OF *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, commonly referred to as baker's yeast, provides an excellent model for the study of cellular processes of eukaryotic cells. *S. cerevisiae* is a unicellular organism which is easily grown and manipulated; furthermore, there is an abundance of genetic and biochemical information known about this organism. Studies involving *S. cerevisiae* have revealed the mating process involves intercellular interactions (Sprague and Thorner 1992). The secretion and recognition of yeast mating pheromones can be used as a model for intercellular signaling pathways in higher eukaryotic organisms. Previous studies have shown that the mating pheromone receptors are members of the seven-transmembrane-segment family of GTP-protein (termed G-proteins) coupled receptors. Other members of this family include the rhodopsin and β -adrenergic receptors (Dohlman, et al 1991). Therefore, this organism can be used as a model for studies involving hormone-receptor interactions.

S. cerevisiae can exist either as haploid cells (a or α) or these haploids can undergo conjugation to form diploid (a/ α) cells. When diploid cells become starved for nutrients, they undergo sporulation which produces four haploid spores (Herskowitz 1988). Haploid cells mate efficiently with cells of the opposite mating type, but do not mate with cells of the same mating type. Diploid cells (a/ α) do not mate with either haploid cells or other diploid cells (Mortimer and Hawthorne 1969). This life cycle is summarized in figure 1.

The mode of sexual reproduction of the *S.cerevisiae* involves the secretion and recognition of mating pheromones. These mating pheromones are termed α -factor and

Figure 1. Life cycle of *Saccharomyces cerevisiae* . In the presence of pheromone from cells of opposite mating types, agglutination occurs, cells become fused, and a diploid zygote is produced. Under favorable conditions, the diploid cell will reproduce asexually by budding. When the cell is deprived of necessary nutrients, meiosis occurs within an ascus, and four haploid ascospores are formed. When the ascus is returned to a favorable environment, four haploid cells are released and mating can occur.

Source: Sprague, G.F. and J. Thorner. 1992. Pheromone Response and Signal Transduction during the Mating Process of *Saccharomyces cerevisiae* . In *The Molecular Biology of the Yeast Saccharomyces cerevisiae* , 2nd edition (eds. J.R. Broach, J.R. Pringle, and E.W. Jones.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

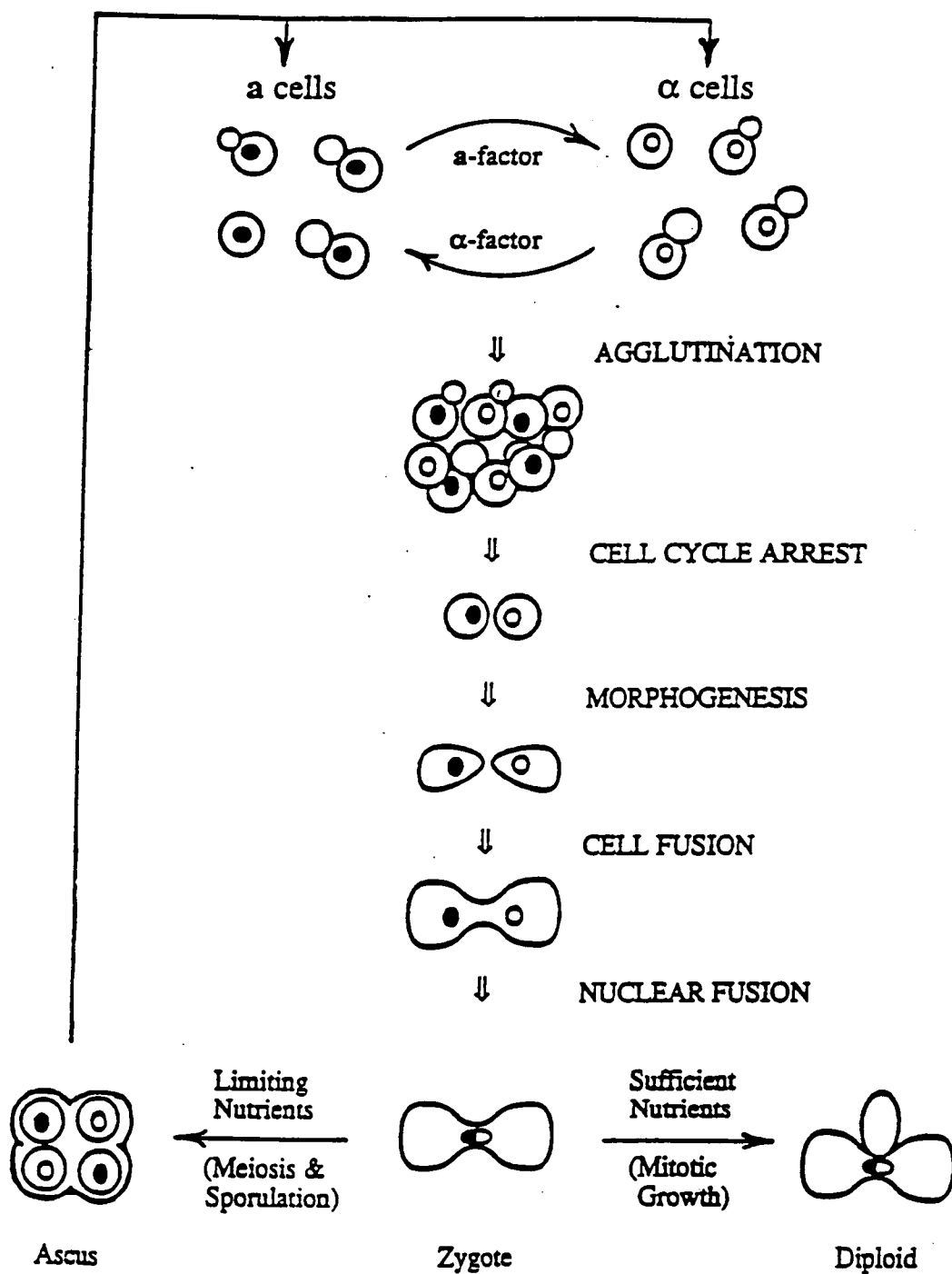


Figure 1 (continued).

a-factor. The α -cell produces and secretes α -factor which is recognized by the α -factor receptor on the **a**-cell. The **a**-cell produces and secretes **a**-factor which is recognized by the **a**-factor receptor on the α -cell (Herskowitz 1988, Sprague and Thorner 1992). The mating pathway is diagrammed in figure 2. When a pheromone acts upon the opposite mating type a number of events occur including: growth arrest at the G₁ phase of the cell cycle, the production of cell agglutinins, and morphological changes which results in the appearance of projections (Jackson, Konopka, and Hartwell 1991). The latter response has been termed "shmooing" (Thorner 1981).

Studies have shown that the mating type is controlled at the transcriptional level by information encoded at the *MAT* locus, a region on chromosome III (Oliver, et al. 1992). The **a**-cells contain a segment of unique sequence known as the *MAT_a* allele and α -cells contain a segment of unique sequence known as the *MAT α* allele. The **a**-cells express **a**-specific genes, such as the gene which encodes **a**-factor; α -cells express genes that are specific to the α -cells, such as the gene encoding α -factor.

Mating is initiated by extracellular peptides, mating pheromones, that are produced by each haploid cell type. α -cells secrete α -factor by the classical vesicle-based secretory pathway, a tridecapeptide (WHWLQLKPGQPMY) which acts on the α -factor receptor present on the **a**-cell (Fuller, Sterne, and Thorner 1988). This bioactive pheromone is translated from precursors encoded by either the *MF α 1* or *MF α 2* gene (this is diagrammed in figure 3) (Kurjan and Herskowitz 1982, Singh et al. 1983). Both of these primary translation products (termed prepro- α -factor) have an N-terminal hydrophobic signal sequence, a hydrophilic domain, and a C-terminal segment which contain repeats of the α -factor sequence which are separated by a pair of basic residues followed by either two or three dipeptides. The hydrophobic leader sequence enables the pheromone to be directed to the endoplasmic reticulum to be transported

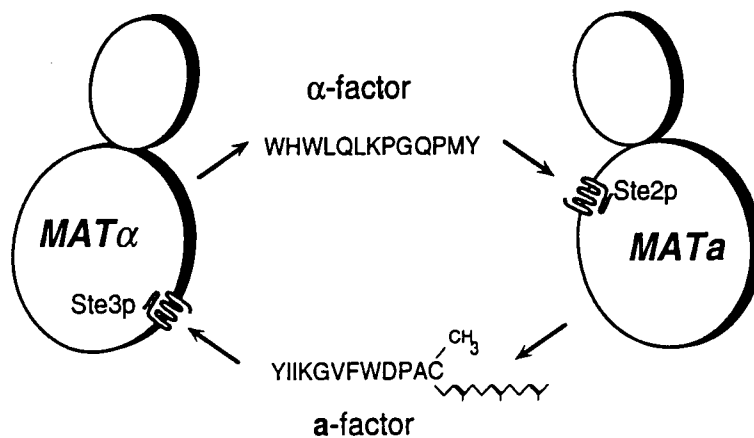


Figure 2. An overview of yeast mating. Two opposite *S. cerevisiae* mating types (*MATα* and *MATa*) secrete pheromones (α -factor and *a*-factor) which interact with their respective receptors (Ste2p = α -factor receptor, Ste3p = *a*-factor receptor) as a requirement for mating.

Figure 3. Scheme for the processing of the α -factor precursor. The first step is the hydrophobic leader sequence directs the primary translation product to the endoplasmic reticulum. Here, the signal sequence is removed by a signal peptidase. As passage through the Golgi occurs, the core oligosaccharides of the precursor are extended by the addition of outer chain oligosaccharides. In the late Golgi compartment, maturation occurs. The first step is the cleavage by *KEX2* endoprotease which liberates each of the α -factor repeats. This is followed by the removal of the C-terminal -KR-residues which had been exposed by *KEX2* enzyme. The removal of these repeats is accomplished by *KEX1* enzyme. N-terminal maturation is achieved by the dipeptidyl aminopeptidase encoded by the *STE13* gene. The mature α -factor is then secreted from the cell.

Source: Sprague, G.F. and J. Thorner. 1992. Pheromone Response and Signal Transduction during the Mating Process of *Saccharomyces cerevisiae* . In *The Molecular Biology of the Yeast Saccharomyces cerevisiae* , 2nd edition (eds. J.R. Broach, J.R. Pringle, and E.W. Jones.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

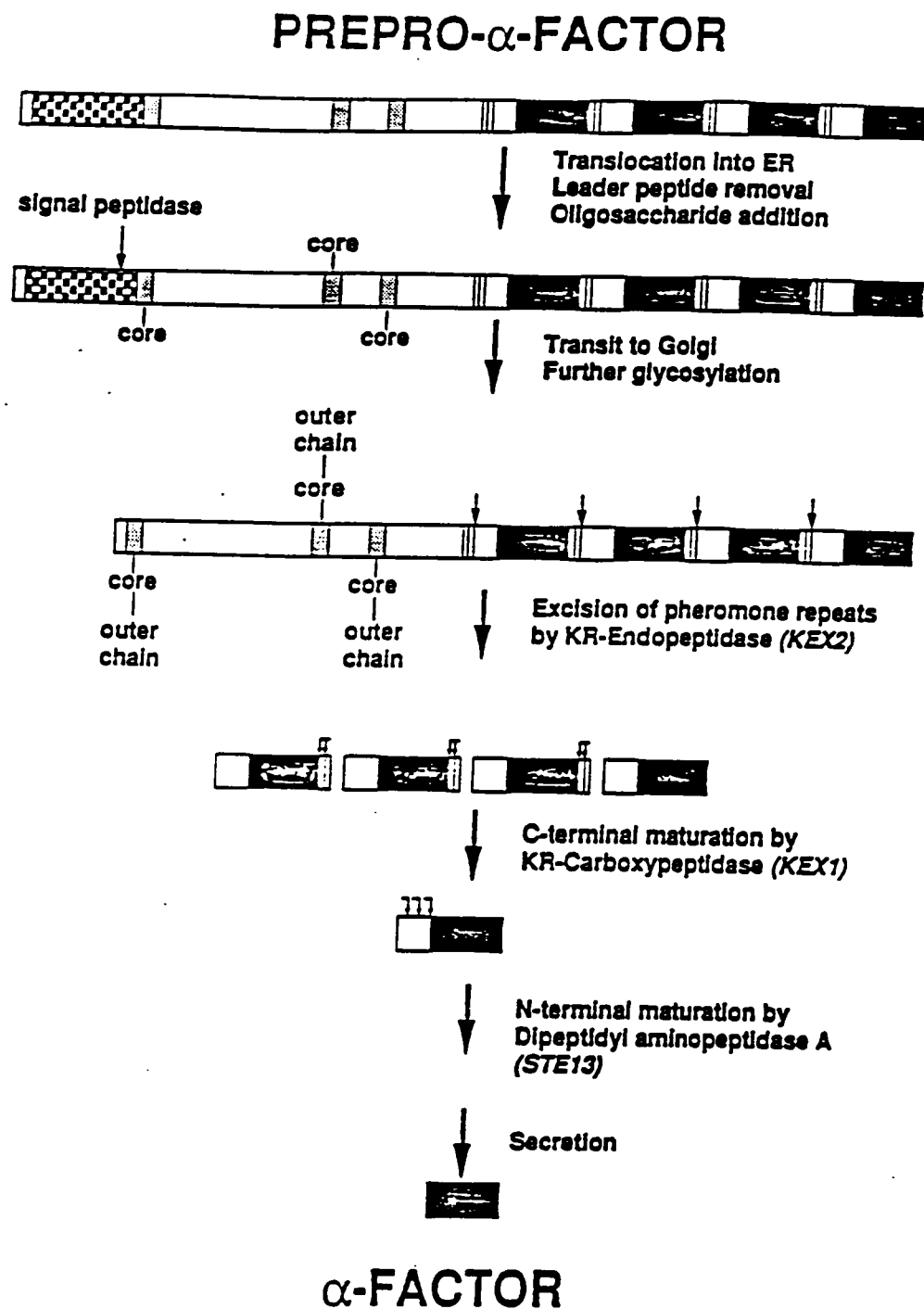


Figure 3 (continued).

from the cell (Waters, Evans, and Blobel 1988). The α -factor pheromone is secreted from the α -cell by the normal secretory pathway. The *MF α 1* gene product is processed to yield four mature α -factor peptides all of the sequence H₂N-WHWLQLKPGQPMY-CO₂H. The *MF α 2* gene product is processed to produce two units of α -factor; one copy is identical to that of *MF α 1*, and the other has the variant sequence H₂N-WHWLNLRPGQPMY-CO₂H (Sprague and Thorner 1992). To liberate individual units of the pheromone, the *KEX2* protease cleaves at lys-arg residues (Julius et al 1984). The *STE13* protease is responsible for the removal of x-ala dipeptides from the N-terminus (Brake et al 1984). Removal of the lys-arg dipeptide from the C-terminal end is accomplished by the *KEX1* protease (Dmochowska et al. 1987). Once processed, the mature α -factor will be secreted. This pathway is important because many human hormones are processed in approximately the same manner.

The **a**-cell secretes **a**-factor, a dodecapeptide of sequence YIIKGVFWDPAC(farnesyl)-OMe which acts on the **a**-factor receptor on the α -cell. This peptide is post-translationally modified by farnesylation and carboxymethylation as shown in figure 4 (Anderegg et al 1988). The biologically active **a**-factor is translated from two genes, *MFa1* (36 residues) and *MFa2* (38 residues). Both precursors have only one copy of the pheromone near the C-terminal end. Furthermore, both precursors lack a hydrophobic signal sequence. The *MFa1* and *MFa2* genes are specific for the **a**-cell, and both are induced by the α -factor pheromone (Dolan, Kirkman, and Fields 1989). The **a**-factor peptide is not secreted by the secretory pathway; the **a**-factor pheromone is secreted by a novel pathway (Sterne and Thorner 1986). Maturation of **a**-factor begins with a modification of the cysteine near the C-terminal end of the peptide; a protein prenyl transferase adds a farnesyl group at this site (Schafer et al. 1990). The CAAX box found at this C-terminal end is the target for the

Figure 4. The paradigm for **a**-factor maturation. Pro-**a**-factor is subjected to N-terminal proteolysis. Isoprenylation of the peptide then occurs at the cysteine of the CAAX (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid) box. The addition of the farnesyl group is accomplished by the farnesyl transferase RAM (which was termed for Ras and **a**-factor modification). Proteolysis of three C-terminal amino acids (AAX) follows. After carboxy methyl esterification, a mature **a**-factor is achieved. The mature **a**-factor is secreted through the plasma membrane of the **a**-cell via a mechanism involving the *STE6* gene product (the MDR homolog).

Source: Caldwell, G.A. "Functional consequences of structural alterations to the isoprenylated yeast mating pheromone **a**-factor." Doctor of Philosophy dissertation in Life Sciences, The University of Tennessee, Knoxville, December 1993.

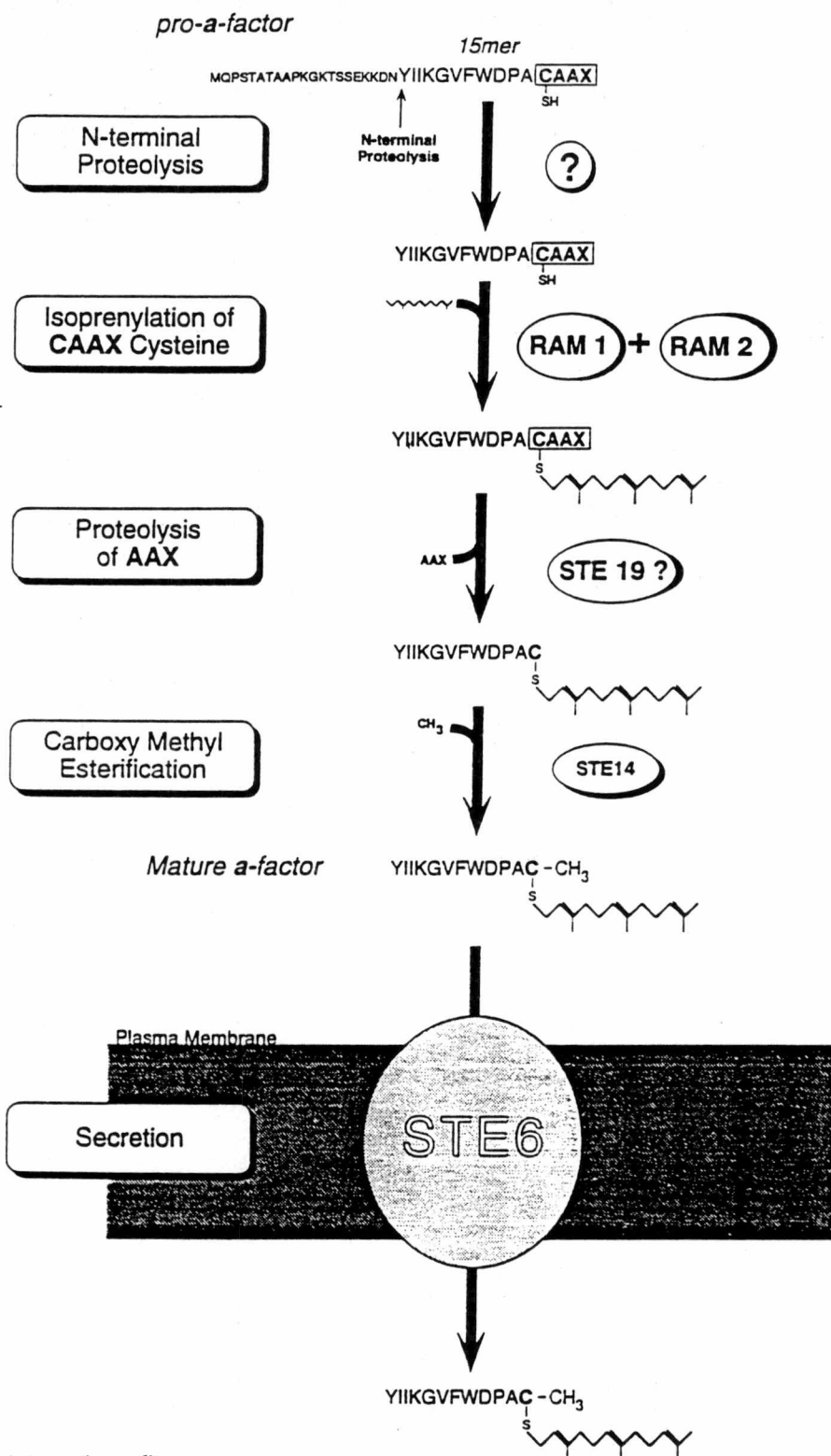


Figure 4 (continued).

prenylation event (Schafer and Rine 1992). An enzyme homologous to the one responsible for farnesylation in yeast is responsible for the farnesylation of mammalian oncogenic Ras proteins (Reiss et al. 1990, and Chen et al. 1991). The Ras oncoprotein been associated with 90% of human pancreatic and 50% of human colon cancer (Barbacid, 1987). The *STE6* gene product is required for **a**-factor export from the **a**-cell. This gene was discovered by experiments which complement an **a**-cell mutation, *ste6*, originally identified as sterile due to its inability to secrete **a**-factor (Wilson and Herskowitz 1987). The Ste6p is similar to the mammalian multi drug resistance (MDR) protein. The MDR protein plays a crucial part in providing drug resistance to transformed cells. Presently, the only known naturally occurring substrate for the MDR protein is the yeast **a**-factor (Raymond, Gros, Whiteway, and Thomas 1992).

In response to mating pheromones, the cells of opposite mating types undergo physiological changes (see figure 5). In order for the changes which lead to mating to occur, pheromones must bind to specific receptors. The *STE2* gene encodes the α -factor receptor (Ste2p) while the *STE3* gene encodes the **a**-factor receptor (Ste3p). The *STE2* gene is found only in **a**-cells, and the *STE3* gene is transcribed only in α -cells (Hartig et al. 1986 and Sprague, Jensen, and Herskowitz 1983). Both the **a**- and α -factor receptors are coupled to G-proteins. In the absence of the specific pheromone, the G-protein is inactive. When the pheromone binds, the receptor undergoes a conformational change which activates the G-protein. The immediate target of the pheromone action remains unknown. The responses of the cells to the mating pheromone include growth arrest in the G₁ phase of the cell cycle, increase in cell agglutinins, and a morphological change which allows the cell to elongate toward the opposite cell type (Kurjan 1992).

Figure 5. The signaling pathway. The α and α -factor receptors are coupled to heterotrimeric G-proteins. The activated G-protein signals a cascade of events. The *STE20* gene product is the most immediate known component of the pathway. The function of Ste20p may be the activation of the G $\beta\gamma$ complex so that it can interact with its effector. It is thought that phosphorylation by the Ste20p kinase activates *STE5* product. The physiological role of the Ste5p protein remains unknown. The action of Ste20p and Ste5p stimulates a series of protein kinases: Ste11p, Ste7p, Fus3p, and Kss1p. Fus3p is required for the pheromone induced cell cycle arrest. Ste12p is a transcription factor which is necessary for the pheromone response. The activation of Ste12p leads to the induction of genes whose products are critical in mating competence, cell cycle arrest, polarity and morphological changes, cell fusion, nuclear fusion, and adaptation and recovery.

Source: Sprague, G.F. and J. Thorner. 1992. Pheromone Response and Signal Transduction during the Mating Process of *Saccharomyces cerevisiae*. In *The Molecular Biology of the Yeast Saccharomyces cerevisiae*, 2nd edition (eds. J.R. Broach, J.R. Pringle, and E.W. Jones.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

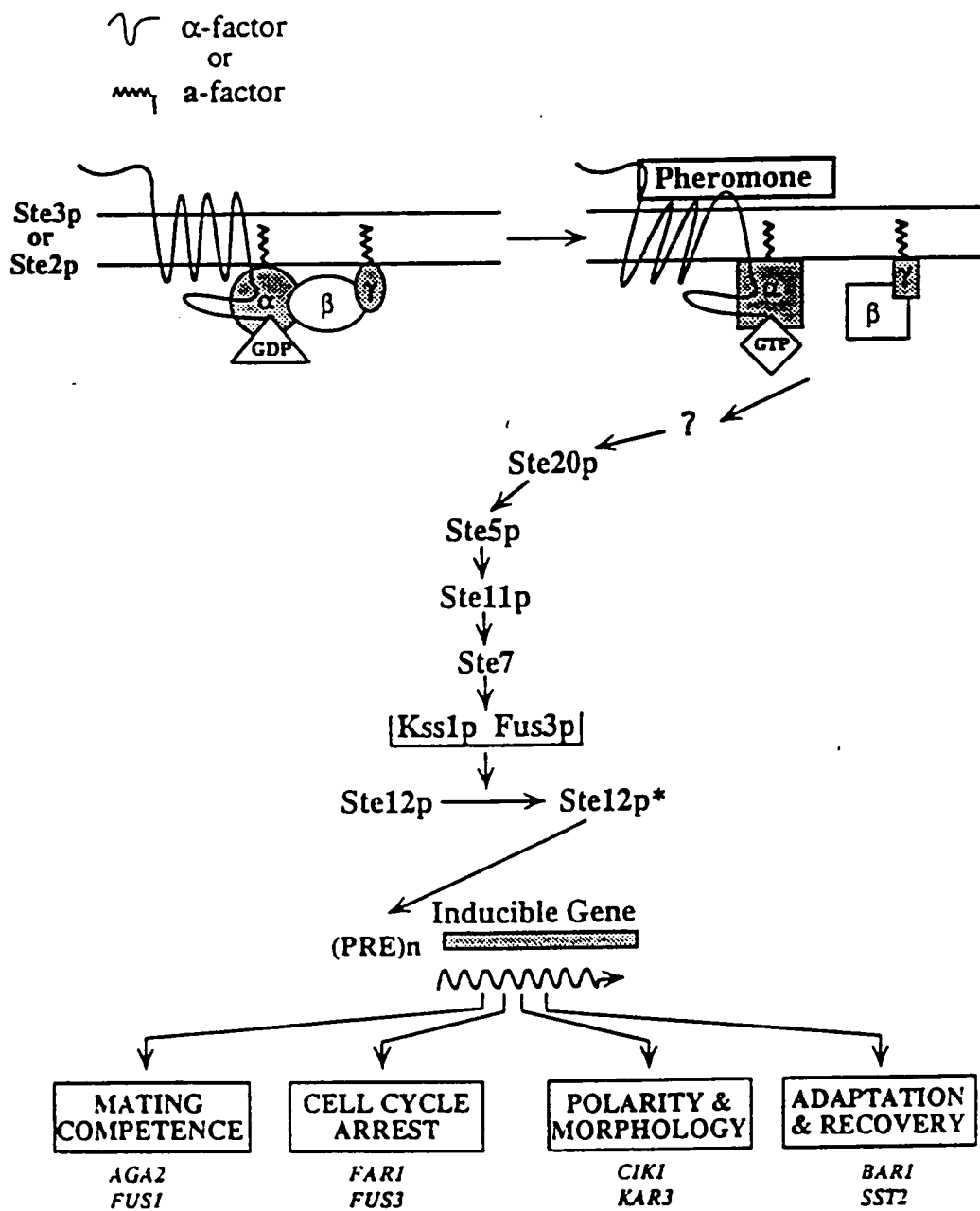


Figure 5 (continued).

The mating process in yeast is similar to hormone signaling used in multicellular eukaryotic systems. Studies involving intercellular interactions could provide vital information to the further understanding of intercellular communication in complex eukaryotic organisms. Since the unicellular *S.cerevisiae* is easily grown and manipulated, it provides an excellent model to study peptide hormones and their receptors.

The purpose of this thesis was two-fold. The first goal of this project was to successfully characterize α -factor analogs with an inserted photoactivatable residue, p-Benzoylphenylalanine (Bpa), by biological assays. A Bpa analog with activity mimicking that of wild type can be used to identify the binding site of α -factor with its receptor. The second objective of this project was to develop an assay which could measure the binding of α -factor to its receptor. This assay could provide information on the interaction between α -factor and its receptor.

CHAPTER 2

DETERMINING BIOLOGICAL ACTIVITY OF PHOTOACTIVATABLE α -FACTOR ANALOGS

Introduction

Mating between *S. cerevisiae* haploids of opposite mating types is initiated by diffusible pheromones. The two pheromones that control mating in these unicellular yeasts are α -factor and a-factor. These mating pheromones act upon the opposite mating types by binding to specific receptors. α -factor has been the focus of many studies involving the biological activity, conformation, and primary structure (Naider and Becker 1986, Eriotou-Bargiota, Xue, Naider and Becker 1992, Blumer, Reneke, and Thorner, 1988, Rath, Naider, and Becker 1988). However, which specific residues of the α -factor receptor interact with the bound pheromone remains unknown.

In an attempt to elucidate the binding site of the α -factor mating pheromone, photoaffinity labeling can be utilized. Previous research has shown that photoaffinity labeling has been widely successful for the identification and localization of macromolecular receptors (Williams and Shoelson 1993, Miller and Kaiser 1988, Kauer, Erickson-Viitanen, Wolfe, and DeGrado 1986). α -factor analogs which are photoaffinity labeled could be utilized to elucidate the binding site of the α -factor in its receptor. Analogs containing the photoactivatable amino acid p-benzoylphenylalanine (Bpa) have been created. Bpa was chosen as the label for these α -factor analogs because it has a high chemical stability which allows it to be used in conventional solid phase synthesis, it can be activated by low energy UV light, and there is a rapid reaction of the activated intermediate.

In order to create analogs believed to have a high biological activity, results from previous structure-function studies were considered. These studies of structure-activity relationships revealed that the histidine in position 2 and tyrosine in position 13 were extremely important for α -factor activity. Trp¹ could be removed from the pheromone or Trp³ could be substituted by certain hydrophobic residues with only a slight reduction in activity (Shenbagamurthi, et al 1983). Based on these results, α -factor analogs were synthesized in which Trp in position 1 or position 3 was replaced by p-benzoylphenylalanine . When the sulfur group of methionine is oxidized, the α -factor loses activity. To reduce the chance of oxidative degradation, Met¹² was replaced with the isosteric norleucine. Arginine can be used to replace lysine at position 7 with no loss in biological activity of α -factor (Eriotou-Bargiota, Xue, Naider, and Becker, 1992). This replacement was used in these studies to prevent interference by the ϵ -amino group of lysine during acylation of the N-terminal amino group with Bolton Hunter reagents.

In order for photoaffinity labeling of the α -factor receptor to be successful, the labeled α -factor analog must closely mimic the wild type α -factor. Characterization of the Bpa analogs can be accomplished by growth arrest and shmoos assays. By identifying analogs which are highly biologically active we will be able to use them as a powerful tool to aid in the elucidation of the α -factor binding site in the α -factor receptor.

Materials and Methods

Strains

The following *S. cerevisiae* strain was used in this study: RC629 *MATa sst 1-2* (supersensitive to α -factor by virtue of a mutation in the BAR1 protease [Allelic to *sst 1*] which hydrolyzes α -factor). This strain was provided by R. Chan (University of Cincinnati).

Synthesis and Resolution of D- or L-Bpa Peptides

(D/L)Bpa analogs were synthesized in the laboratory of Professor Fred Naider at the College of Staten Island. The photolabile amino acid was incorporated into the peptide by standard solid phase methodologies using DIPC as coupling agent directly or via the 1-hydroxybenzotriazole active ester of N-protected Bpa.

Separation of the D-Bpa peptide and the L-Bpa peptide was achieved by using reversed-phase semipreparative HPLC. All final products were >99% homogeneous in two solvent systems and gave the correct molecular ion in fast atom bombardment mass spectroscopy.

Synthesis of Iodinated Peptides Containing Bpa

Iodinated Bpa analogs were synthesized in the laboratory of Professor Fred Naider at the College of Staten Island and were provided for biological analyses.

Growth Arrest (Halo Assay)

YEPD plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) were overlaid with 4 ml of *S. cerevisiae* RC629 cells (2.5×10^5 cells per ml) in Noble agar. Filter disks (sterile blank disks from Difco), 7 mm in diameter, were placed on the overlay, and 10 μ l portions of peptide solutions at various concentrations were pipetted

onto the disks. The plates were incubated at 30°C for 24 hours and then observed for halos (areas of clearing, indicating growth inhibition caused by the peptide on the disk.) around the disks. The minimum value for growth arrest is 8 mm which represents the disk diameter (7 mm) and a small zone of inhibition. The data were expressed as the diameter of the halo (including the diameter of the disk). The diameters of the zone of inhibition were then plotted against the amount of peptide applied to the disk. The amounts of peptide required to produce a halo diameter of given size were determined by interpolation as described previously (Stefan and Blumer, 1994).

Shmoo assay

A *S. cerevisiae* RC629 culture was grown in YEPD at 30°C to log phase. Cells were diluted to 1×10^6 cells/ml in YEPD and added in 90 μ l aliquots to microtiter wells with α -factor analogs at various concentrations. After a four hour incubation at 30°C, a 10 μ l fraction was microscopically counted to determine the total number of cells, the number of cells shmooing, the number of budding cells, and the number of non-budding cells. The proportion of shmoos was calculated for each concentration of the peptide tested.

Results

Halo Assay

The Bpa analogs listed in table 1 were analyzed by halo assays. The activity of the analogs was measured using a strain that was not able to degrade α -factor due to a mutation in the α -factor protease. After an overnight incubation, the lawn was examined for the presence of a zone of inhibition known as a halo. Halos on the series of lawns for each analog were measured and the results can be seen in table 2. The

TABLE 1. Amino acid sequences of the Bpa analogs analyzed by biological assays.

Peptide	Amino acid sequence
Nle ¹²	W-H-W-L-Q-L-R-P-G-Q-P-Nle-Y
I	Bpa-H-W-L-Q-L-R-P-G-Q-P-Nle-Y
II	W-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
III	W-H-(D-Bpa)-L-Q-L-R-P-G-Q-P-Nle-Y
IV	H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
V	W-H-Bpa-L-Q-L-K-P-G-Q-P-Nle-Y
VI	(I ₂ -BH)-W-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
VII	(I-BH)-W-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
VIII	(I-BH)-W-H-(D-Bpa)-L-Q-L-R-P-G-Q-P-Nle-Y
IX	(I ₂ -BH)-W-H-(D-Bpa)-L-Q-L-R-P-G-Q-P-Nle-Y
X	α -(I ₂ -BH)-K-W-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
XI	ϵ -(I ₂ -BH)-K-W-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
XII	(I ₂ -BH)-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
XIII	ϵ -(I ₂ -BH)-K-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y
XIV	(I ₂ -Y)-H-Bpa-L-Q-L-R-P-G-Q-P-Nle-Y

BH represents Bolton hunter reagent.

TABLE 2. Growth arrest of *S. cerevisiae* RC629 by α -factor analogs

Peptide concentration (μ g/disk)	Zone of inhibition (mm)														
	Nle ¹²	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
10	27	20	20	11	0	24	0	11	0	0	15	12	0	19	18
5	23	19	19	9	0	21	0	9	0	0	13	11	0	14	16
1	19	15	16	0	0	19	0	0	0	0	12	10	0	12	14
0.5	18	14	15	0	0	16	0	0	0	0	10	9	0	10	10
0.2	16	8.5	9	0	0	12	0	0	0	0	0	0	0	0	9
0.1	15	0	0	0	0	9	0	0	0	0	0	0	0	0	0
0.05	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

diameters of the zones of inhibition were then plotted against the amount of analog on the disk. The data was analyzed by this manner, so it was possible to determine what concentration of analog was needed to produce a halo of 20 mm even if the analog was not able to produce a halo of that diameter. This allows for a more accurate comparison of the synthesized analogs. These graphs are shown on fig 6. (When there was no response to analog, no graph could be made.) From the data presented, several observations can be made. When tryptophan at position 1 was substituted with Bpa, the resulting peptide was only four-fold less active than the wild type Nle¹² α -factor. If tryptophan at position 3 was replaced with Bpa, the peptide was also active. However, if this substitution was made with D-Bpa, the resulting peptide was considerably less active than wild type α -factor. The Trp³ substitution using D-Bpa was one hundred-fold less active than wild type Nle¹² α -factor, while the Trp³ substitution with L-Bpa was only four-fold less active than wild type Nle¹². In the Bpa analog peptide IV, the tryptophan was deleted without substituting another amino acid. The resultant peptide exhibited no activity even when 10 μ g of analog were tested. When lysine was replaced by arginine, the analog did lose some activity. In peptide V, the lysine was not substituted, and this analog was only two times less active than the wild type α -factor. However, when the arginine was substituted for the lysine (peptide II) there was only a two-fold reduction of activity (as compared to peptide V). The addition of diiodinated Bolton Hunter reagent to the analog resulted in a great loss of activity (peptide VI). No growth arrest can be detected even with the addition of 10 μ g of analog. The moniodinated Bolton Hunter reagent was tolerated better than the diiodinated form; however, the resulting peptide was one hundred-fold less active than wild type α -factor (peptide VII). The analogs in which the Bolton Hunter reagent was present and D-Bpa was substituted in place of Trp³ were not active as shown by no growth arrest even

Figure 6. Measurement of pheromone response by growth arrest assay. The amount of analog applied to the disk on the yeast lawn ranged from 0.01 μg to 10 μg . The amount of analog required to produce a halo of 20 mm was determined by interpolation.

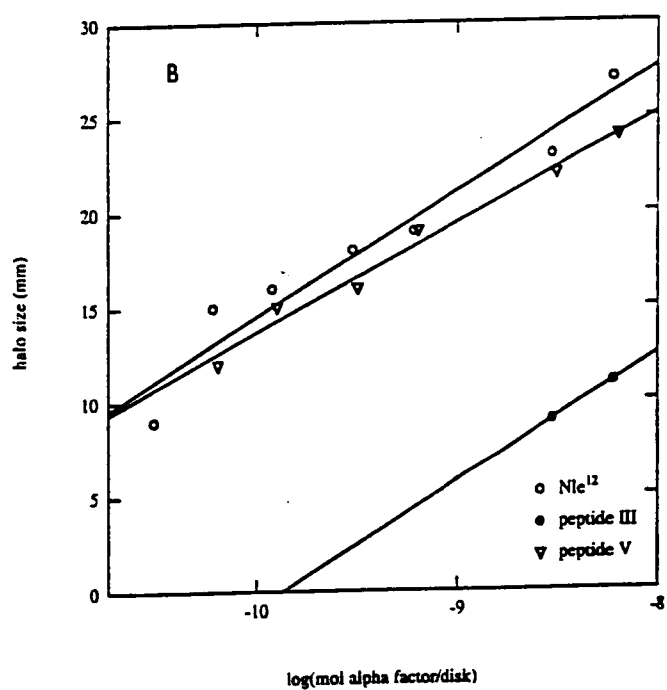
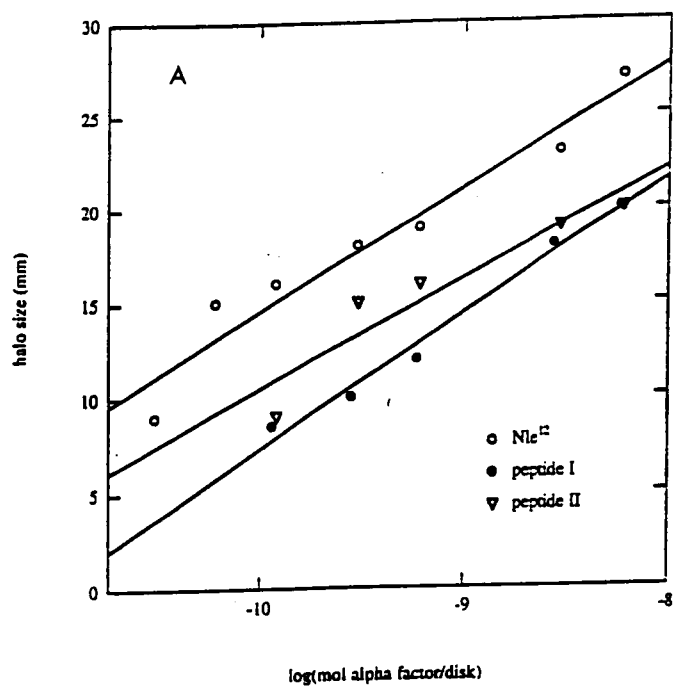


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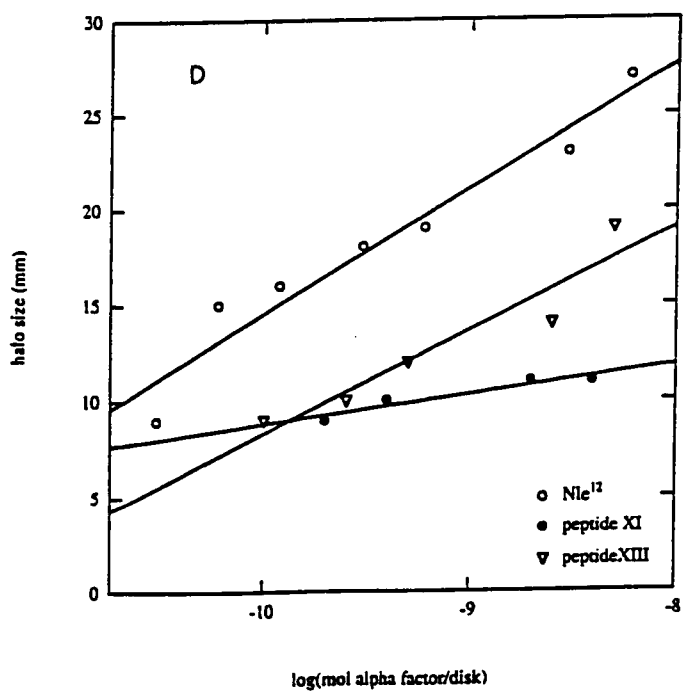
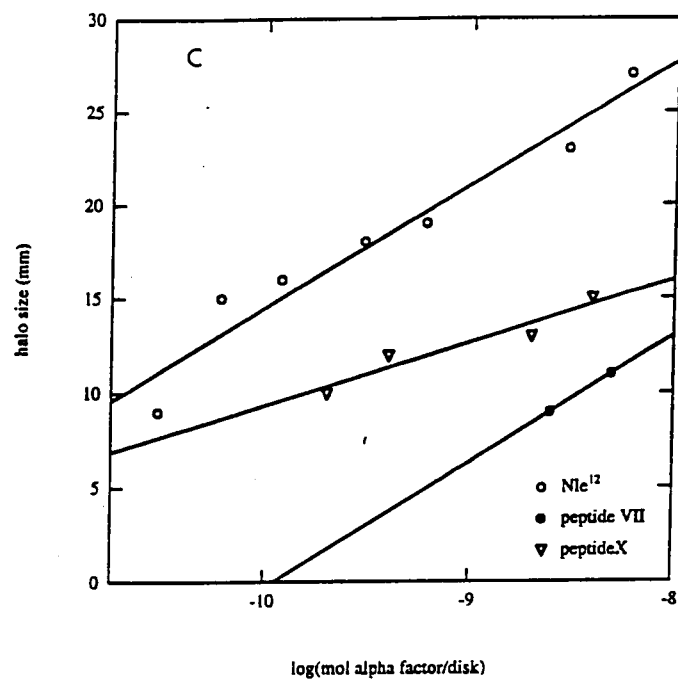


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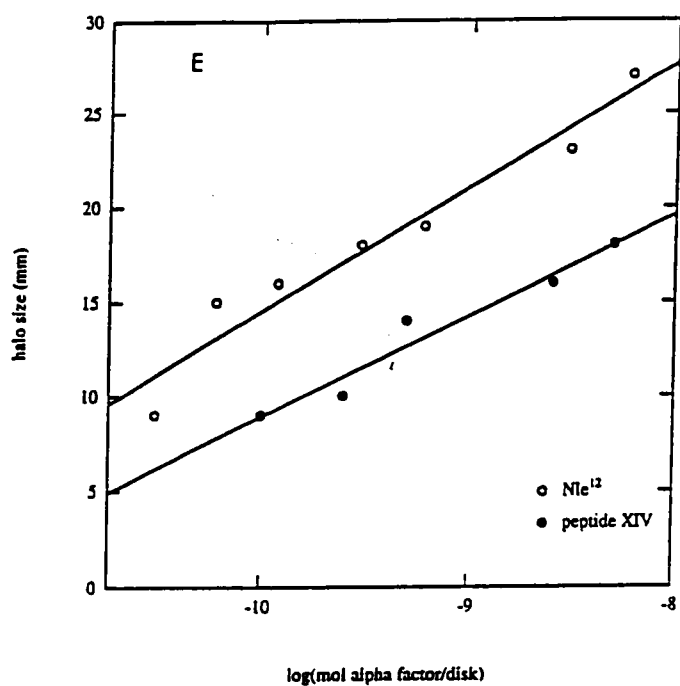


Figure 6 (continued).

when 10 μ g of analog were tested. When diiodinated Bolton Hunter reagent was used to replace the tryptophan at position 1, the resulting analog was inactive (peptide XII). However when spacers were used, either ϵ or α , the diiodinated Bolton Hunter reagent could be substituted for the tryptophan at position 1 with only a ten-fold effect on the activity of the analog (peptides X, XI, and XIII). The substitution of Trp¹ by an iodinated tyrosine resulted in an analog (peptide XIV) which was only four times less active than the activity of the wild type α -factor.

Shmoo Assay

Shmoo assays were also performed to test the biological activity of the Bpa analogs which are listed in table 1. After incubating RC629 with Bpa analog for 4 hours at 30°C, 10 μ l were removed and observed microscopically for the presence of budding and shmooing cells. The percentage of the total number of cells which were shmooing was calculated. After graphing the percentage of shmoos against the concentration of analog used (fig. 7), the amount of analog required to produce fifty percent shmooing was interpolated. These amounts are shown in table 3. When wild type α -factor was used, 0.056 μ g/ml was required to elicit fifty percent shmooing of the total number of cells. When Bpa was substituted for the tryptophan at position 1 of the analog (peptide I), the resulting peptide was active; in fact, it was only 1-fold less active than wild type α -factor. When Bpa was substituted for the tryptophan at position 3, the resulting analog, peptide II and peptide V, was 4 times less active than the Nle12 α -factor. Substitution using D-Bpa for the tryptophan at position 3, resulted in a peptide that was 15-fold less active than wild type. Loss of activity was also seen when the tryptophan at position 1 was deleted (peptide IV). When a Bolton Hunter reagent was attached to the amino terminal end of the analog, the results indicate that the activity of

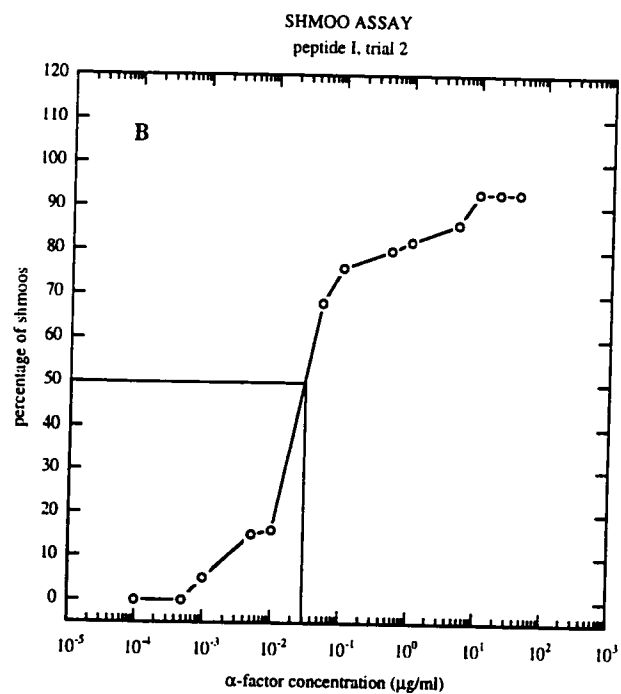
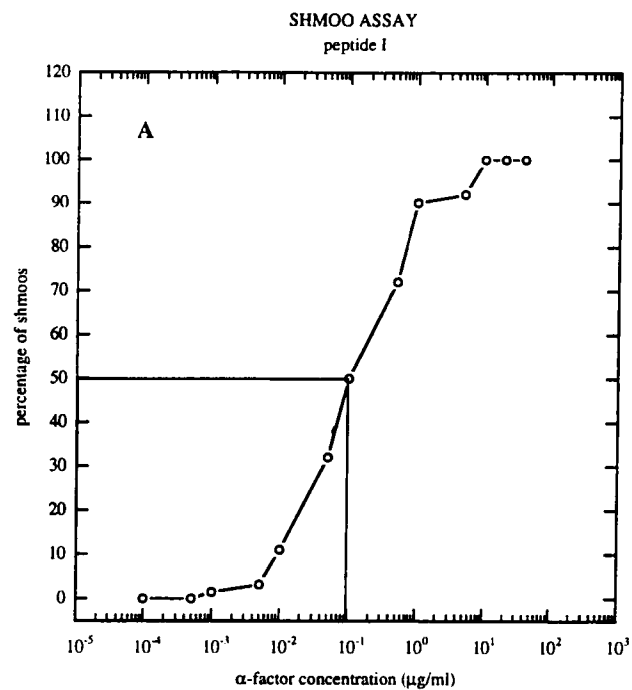


Figure 7. Endpoint shmoo assay.

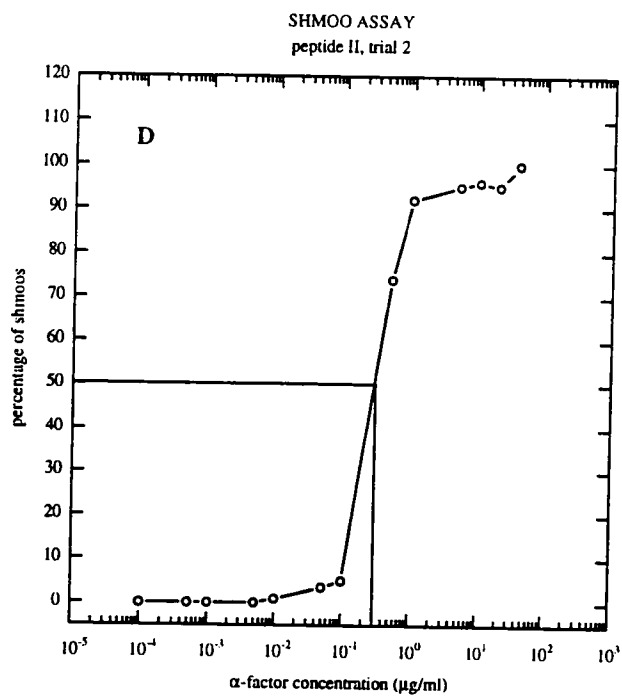
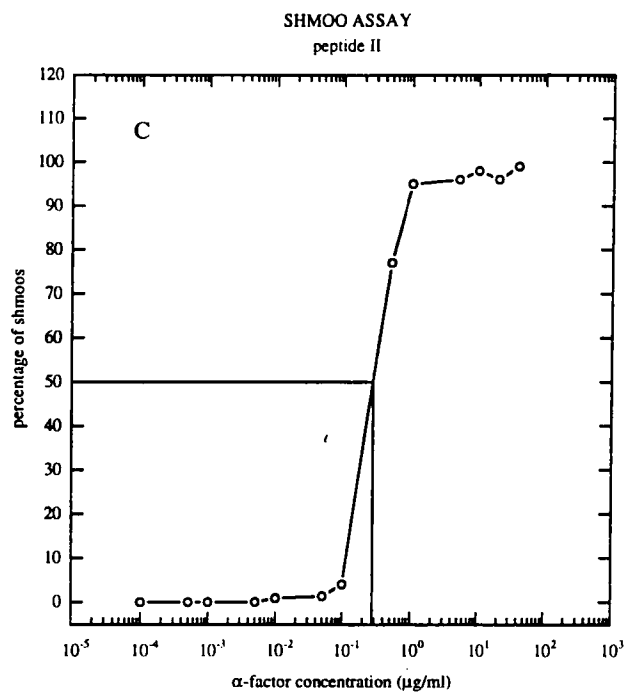


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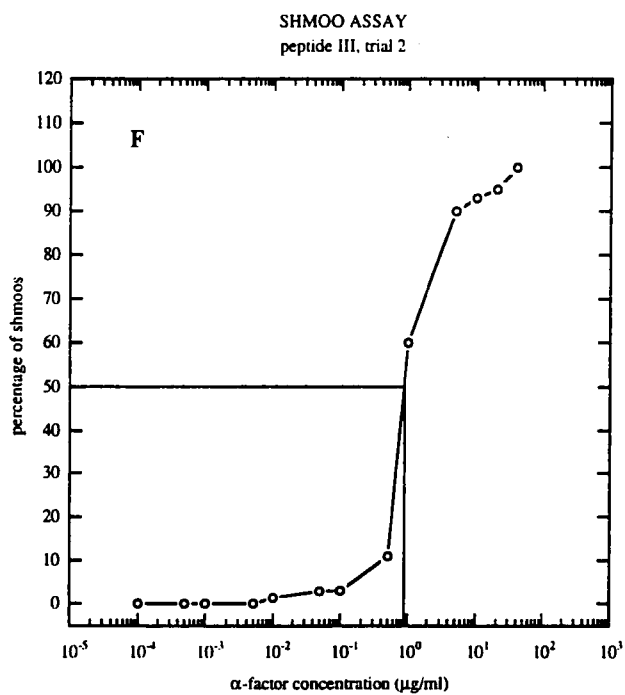
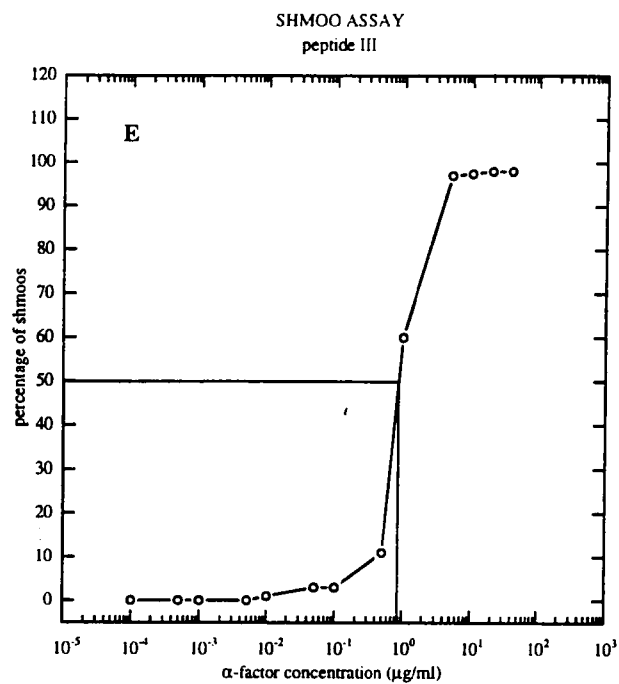


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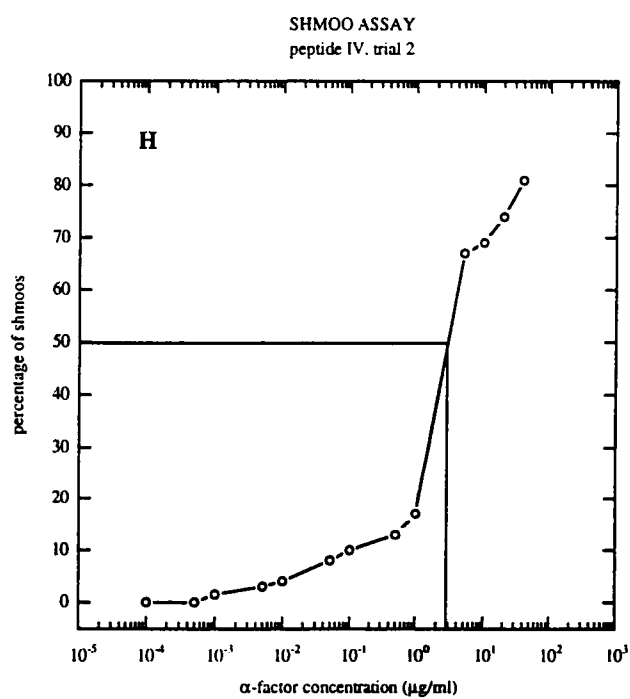
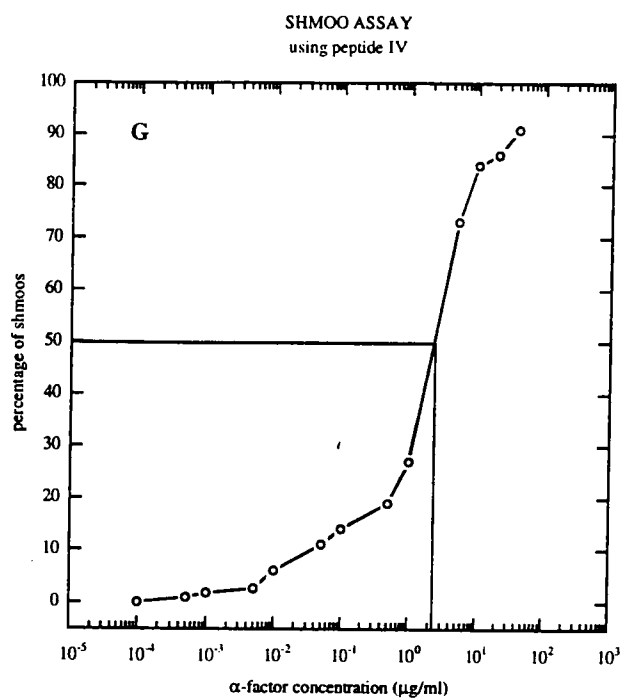


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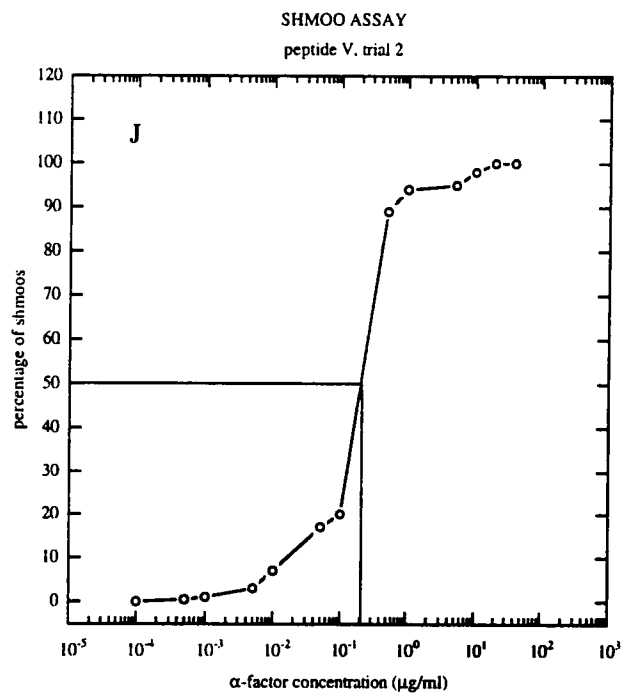
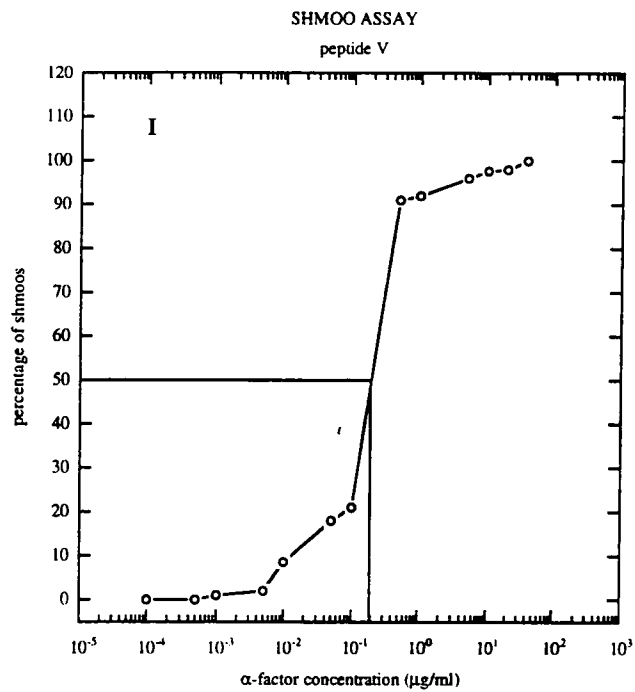


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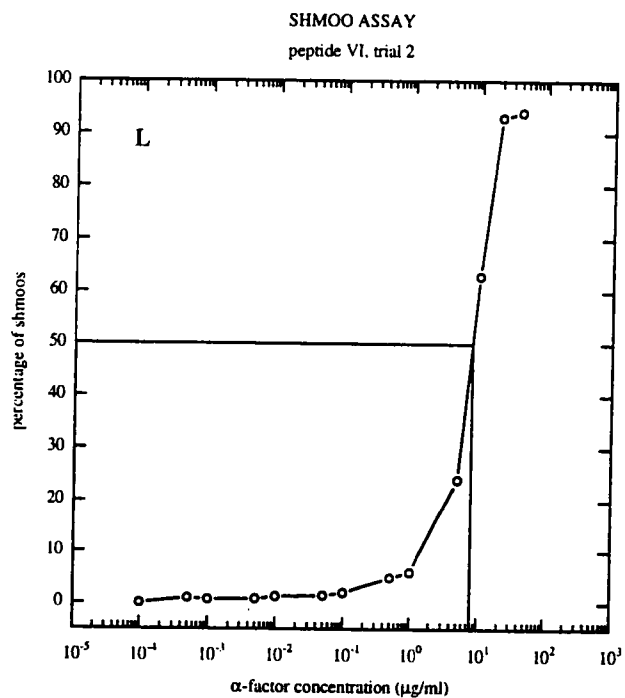
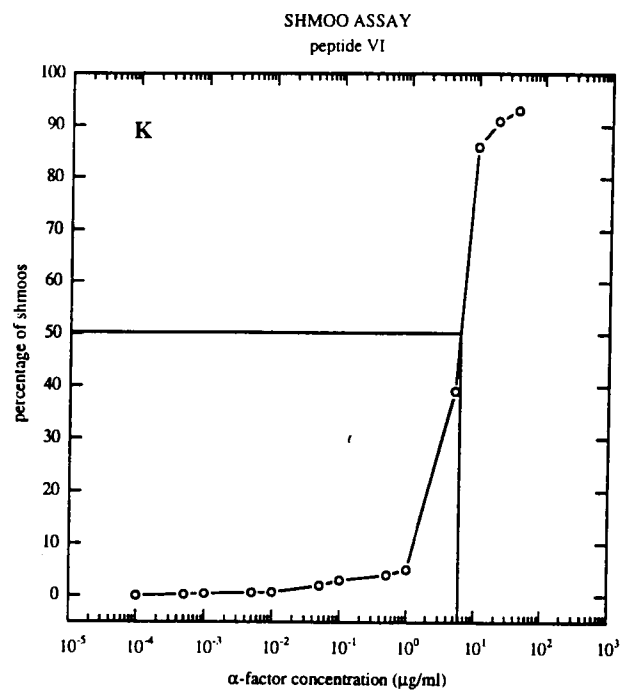


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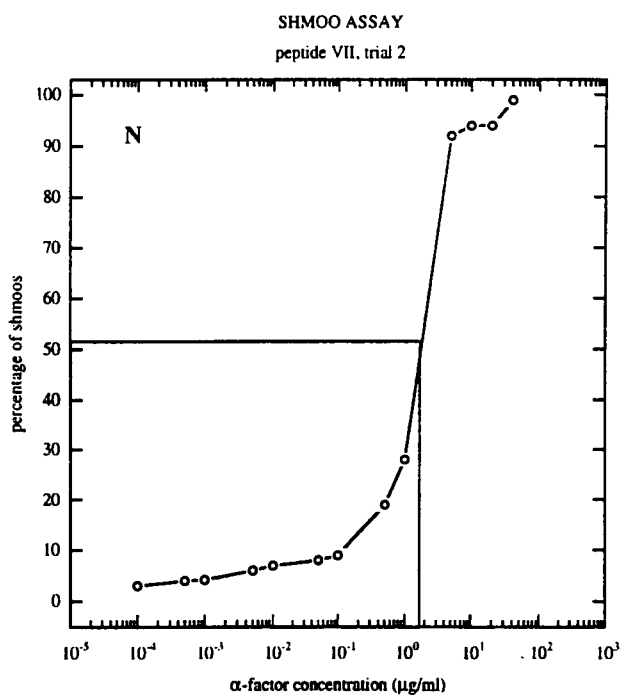
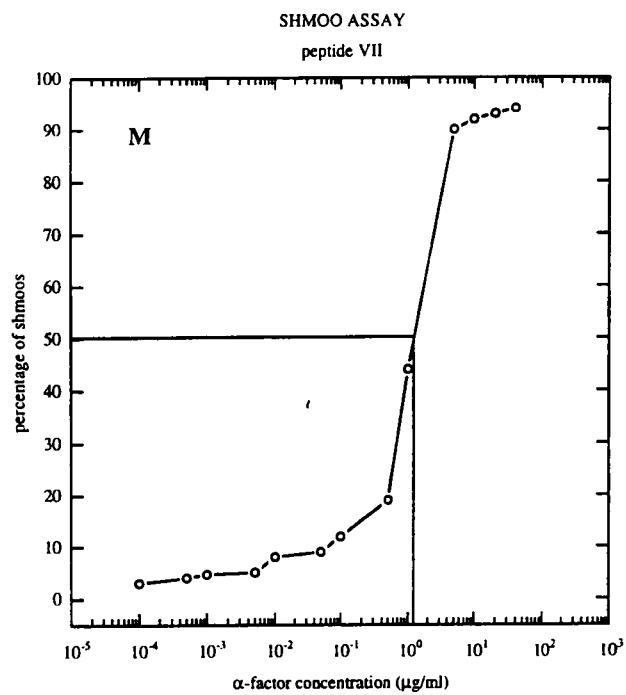


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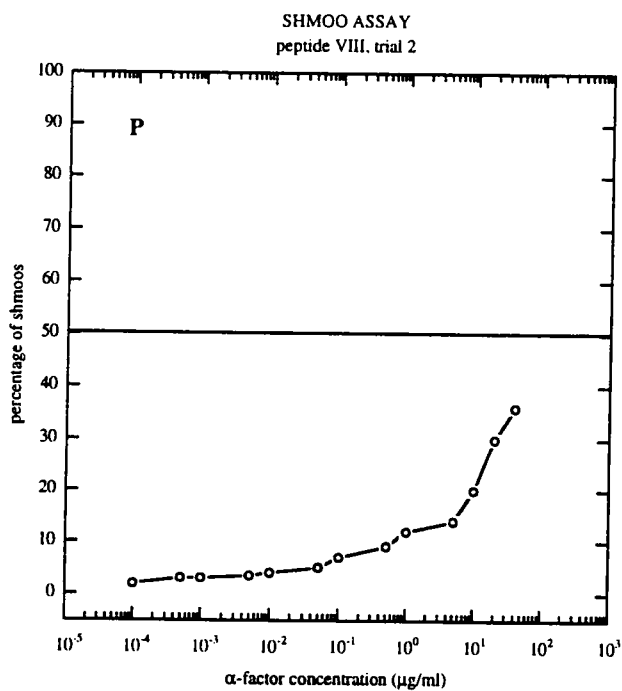
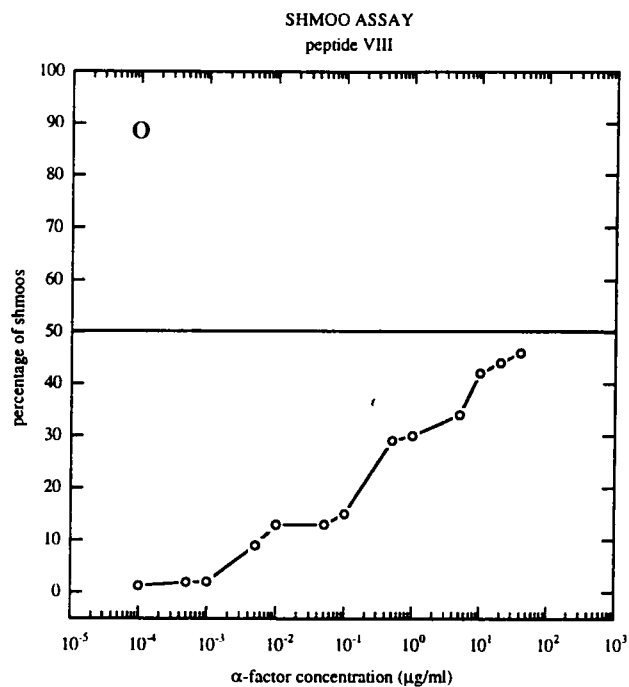


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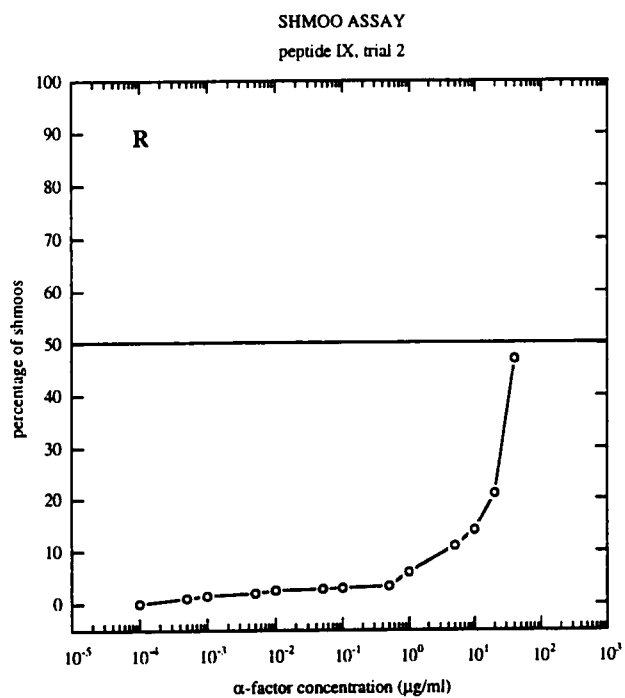
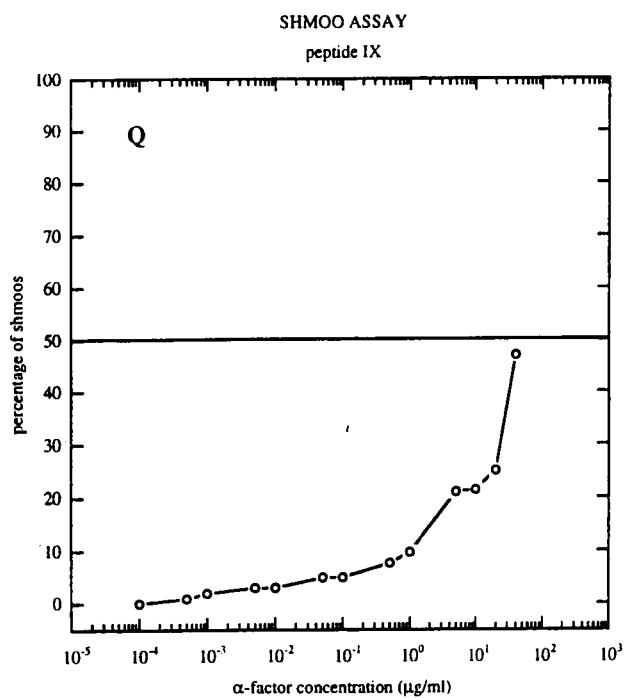


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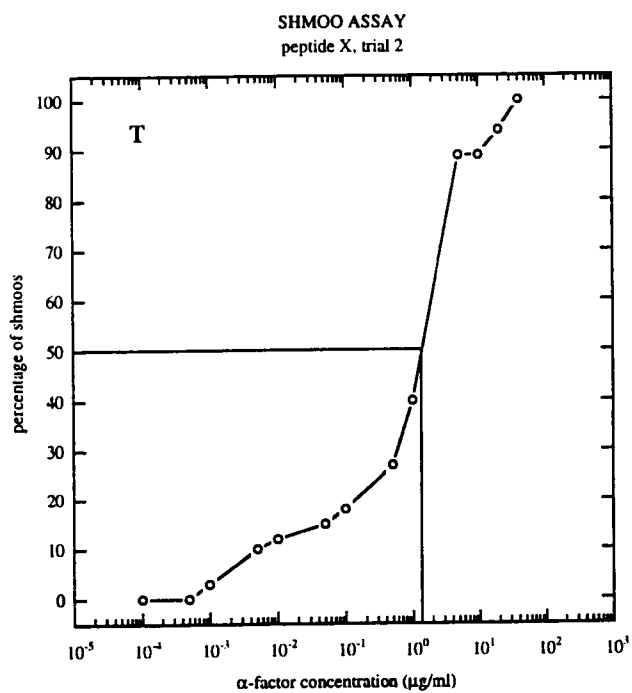
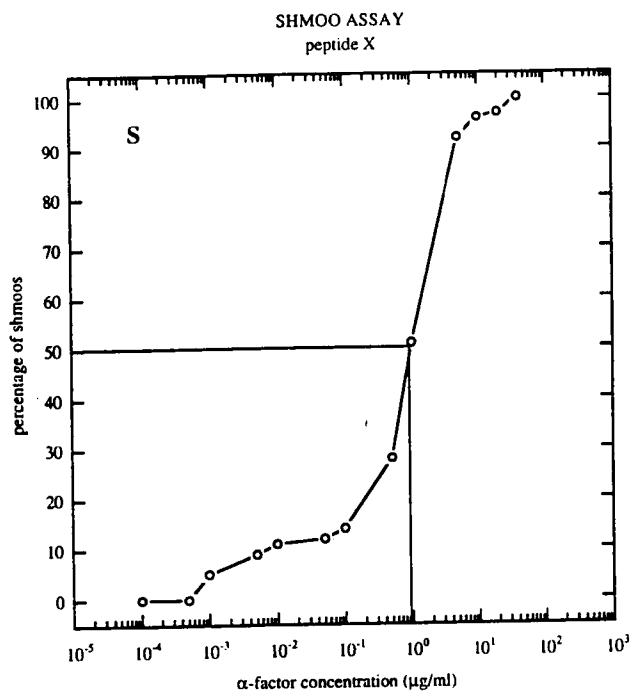


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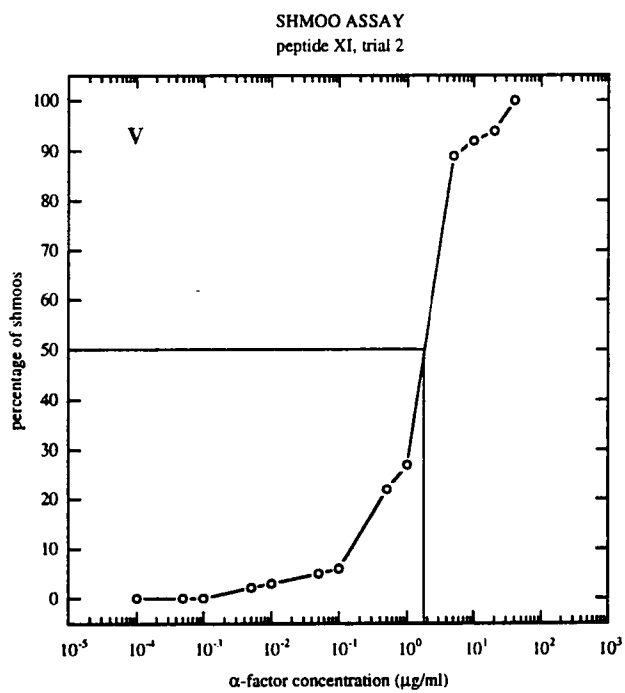
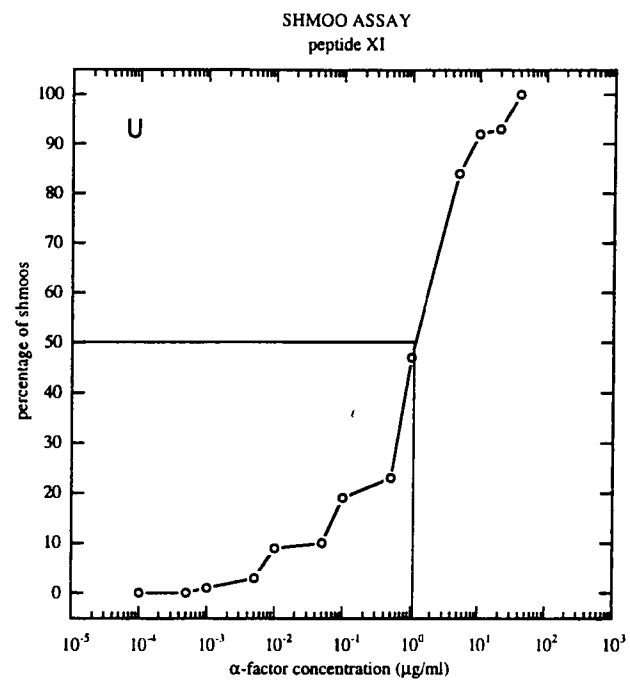


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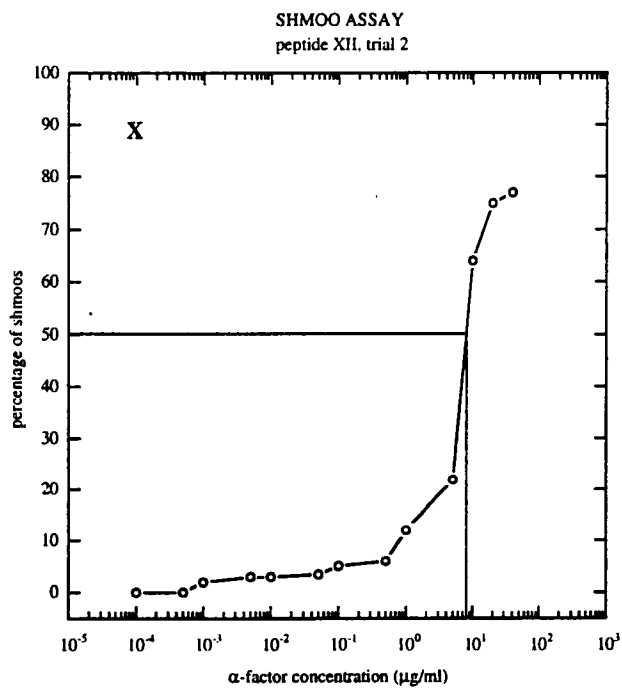
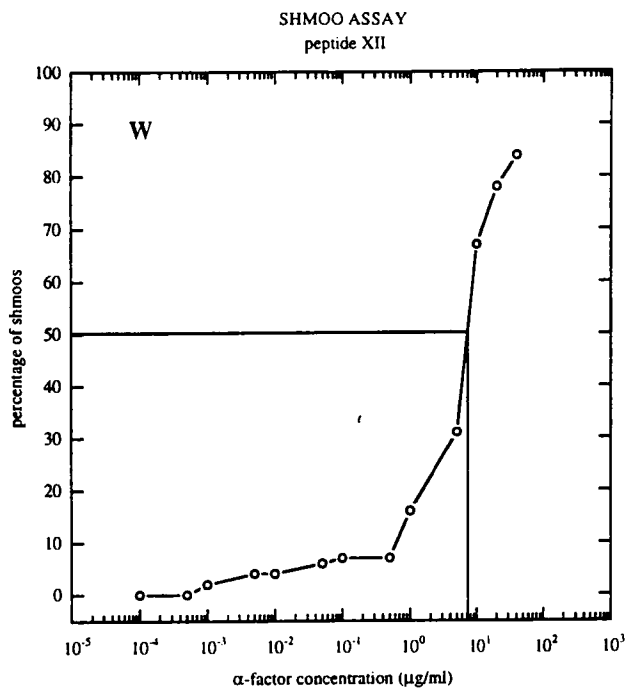


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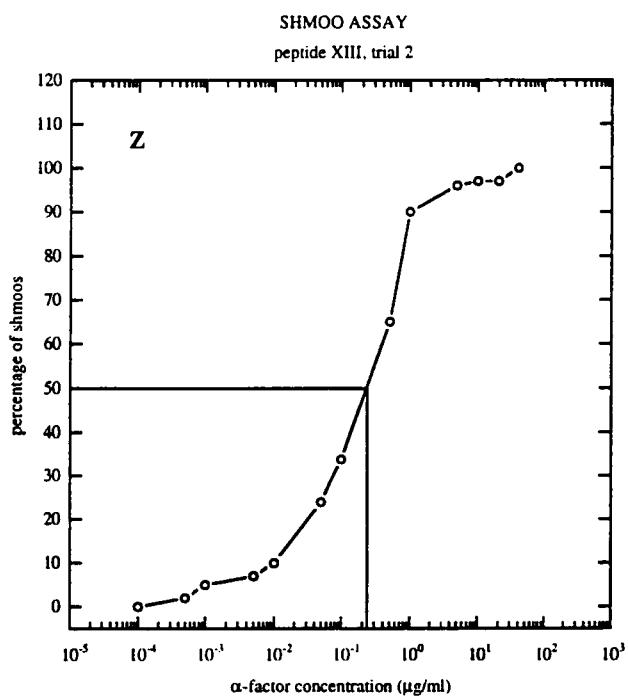
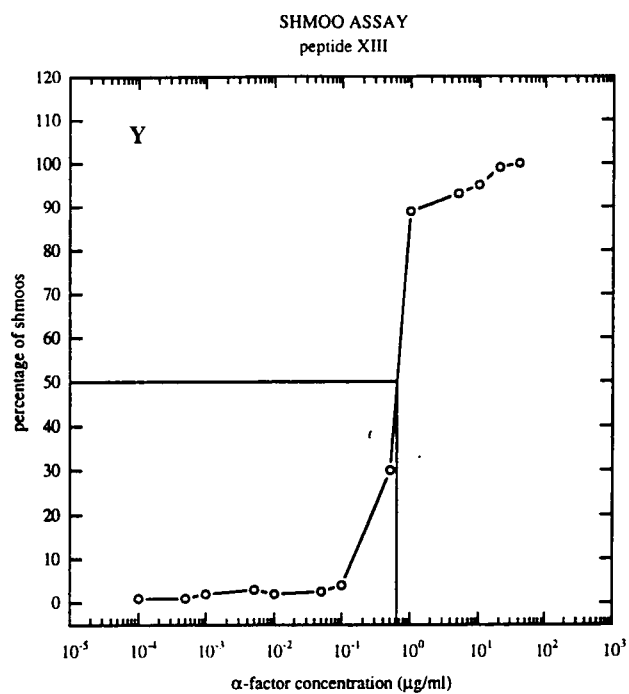


Figure 7 (continued).

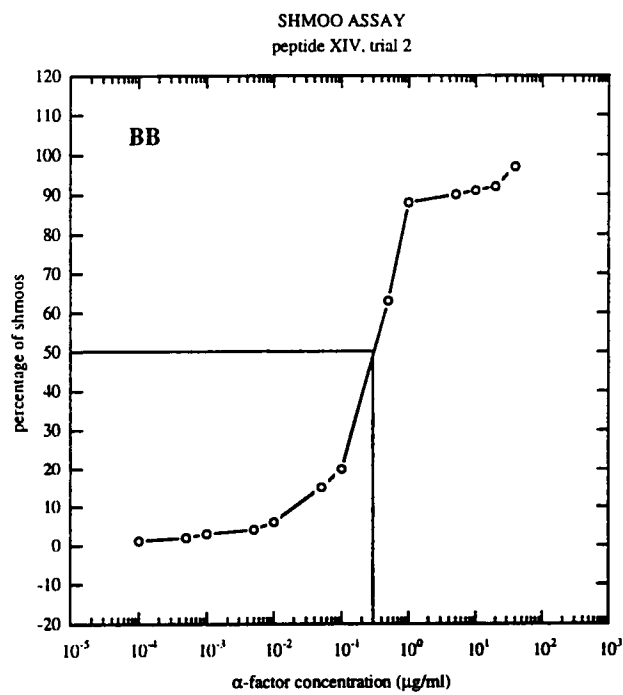
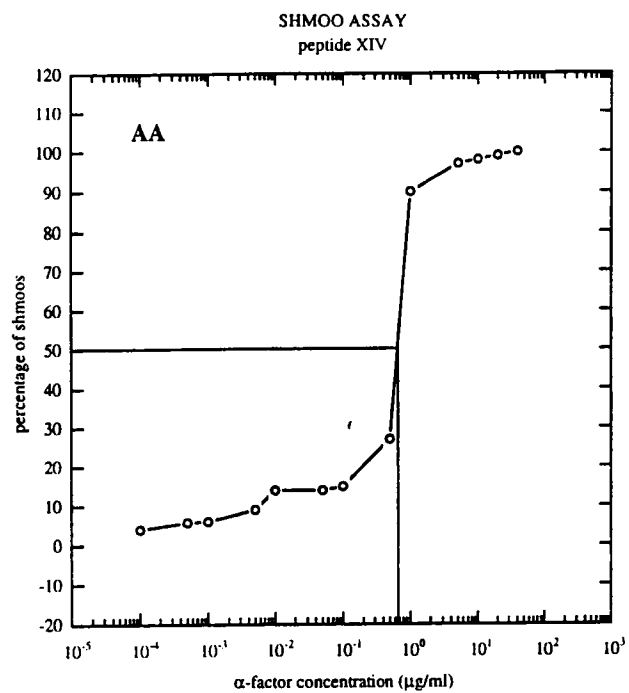


Figure 7 (continued).

TABLE 3. Biological activity of Bpa analogs measured by shmoo assay.

AMOUNT OF ANALOG REQUIRED FOR 50% SHMOOING (in $\mu\text{g/ml}$)		
ANALOG	TRIAL 1	TRIAL 2
Peptide I	0.09	0.03
Peptide II	0.19	0.2
Peptide III	0.85	0.85
Peptide IV	2.2	2.9
Peptide V	0.2	0.2
Peptide VI	6.0	8.0
Peptide VII	1.3	1.7
Peptide VIII	>40	>40
Peptide IX	>40	>40
Peptide X	1.0	1.5
Peptide XI	1.15	1.8
Peptide XII	7.0	8.0
Peptide XIII	0.6	0.22
Peptide XIV	0.6	0.3
Nle ¹²	0.056	0.056

the peptide does decrease. In the case of peptide VI, the addition of the diiodinated Bolton Hunter reagent decreases the activity of the analog by 125 times. When a monoiodinated Bolton Hunter reagent was added to the amino terminal end of the peptide, the resulting analog was 27-fold less active than the wild type pheromone (peptide VII). The same effect was seen when a monoiodinated Bolton Hunter reagent was added to the N-terminal end of the analog with D-Bpa in position 3. In this case, the peptide (peptide VIII) was over 700 times less active than the α -factor pheromone. The same effect was seen when the diiodinated Bolton Hunter reagent was added to the amino terminal end of the analog containing a D-Bpa residue in place of the tryptophan at position 3. The resulting activity of peptide IX, was over 700-fold less active than the wild type α -factor. In peptide XII, which does not have a tryptophan in position 1, the addition of a diiodinated Bolton Hunter reagent results in a decrease in the activity of the analog by 140-fold. The addition of the mono- or diiodinated Bolton Hunter reagent by a lysine spacer resulted in analogs with decreased activity. Peptides X, XI, and XIII, resulted in a 20, 30, and 8-fold reduction in activity. Peptide XIV, in which Trp¹ was replaced by a diiodo-Tyrosine exhibited a 8-fold reduction of activity when compared to wild type α -factor.

Discussion

The results derived from both the halo and shmoo assays indicate that when the tryptophan at position 1 was substituted with L-Bpa (peptide I), the resulting analog retains activity close to that of wild type α -factor. In assays performed on analogs in which the Trp³ was substituted with L-Bpa (peptide II), the peptide's activity also mimics that of the wild type α -factor. When this same substitution was made using D-

Bpa, activity of the peptide decreases (see results of peptide III). This result becomes more dramatic when either a mono- or diiodinated Bolton Hunter reagent was added to the peptide; the resulting analogs were considerably less active than the wild type α -factor. These findings suggest that substitution of tryptophan with the D-Bpa residue was not tolerated (as indicated by peptide VIII, and IX). When the tryptophan in position 1 is deleted (peptide IV), the resulting peptide exhibits no growth arrest, and the shmoo assays indicated a 50-fold reduction of activity. The addition of a mono- or diiodinated Bolton Hunter reagent resulted in analogs which were not as biologically active as wild type α -factor (as shown by peptides VI, VII, VIII, IX, and, XII). When the mono-or diiodinated Bolton Hunter reagents were added to the peptide with spacers, the resulting analogs (peptide X, XI, and XII) were not as biologically active as the wild type α -factor; however, these analogs do exhibit much more activity than the peptides in which Bolton Hunter reagent was added to the peptide without the use of spacers. Results from peptide IV indicated that an iodine group can be successfully added to the peptide using a tyrosine residue.

As stated earlier, the purpose of the characterization of the Bpa analogs was to identify an analog whose biological activity closely resembles that of wild type activity in order to elucidate the binding site of α -factor in its receptor. The results from the biological assays indicate that peptide XIV would be the best candidate for characterization of the α -factor binding site. This analog mimics the activity of α -factor, contains the photoactivatable amino acid; therefore, this analog could be activated by low energy UV light and crosslink with the receptor. The presence of the iodinated tyrosine is also important. When the tyrosine in position 1 of this analog is radiolabeled with ^{125}I , the peptide can be localized after it has crosslinked into the binding site of the α -factor receptor. Future experiments using the analogs characterized in this study may

reveal which amino acid residues in the α -factor receptor are involved in the binding of the α -factor pheromone.

CHAPTER 3

ATTEMPTS TO DEVELOP AN ASSAY TO MEASURE THE BINDING OF a-FACTOR TO ITS RECEPTOR

Introduction

Many studies involving biological activities of a-factor have been completed. Through this research, the importance of the prenylation of the mating pheromone for the secretion of a-factor has been established; however, the significance of the carboxyl methyl ester was not so clear (Powers, et al 1986). Recently, detailed structure-activity studies involving both the S-farnesylation and carboxy-terminal methyl esterification have provided insight to the importance of these modifications. In the absence of the farnesyl group, the resulting peptide has a 1000-fold reduction in biological activity. When the carboxy-terminal modification is not present, there is a 100-fold reduction in activity. Furthermore, when both modifications are absent, the resulting analog is virtually inactive. Further experiments revealed that the farnesyl group is not required for biological activity. S-alkyl modifications, other than farnesyl, resulted in biologically active analogs. However, the amount of biological activity of the pheromone increased as the length of the substituent prenyl moiety increased. (Marcus, Caldwell, Miller, Xue, Naider, and Becker 1991)

In order to understand the interaction between a-factor and its receptor, a binding assay must be achieved. This assay would provide a tool to describe the binding affinity of a-factor to its receptor and elucidate the number of receptors per cell. The development of a binding assay could give insight to how the a-factor interacts with its receptor by providing a method for describing the relationship between the

pheromone and its binding site in the **a**-factor receptor by the analysis of either mutant **a**-factor or α -cells with mutated **a**-factor receptors.

Materials and Methods

Strains

The following *S. cerevisiae* strains were used in this study: SY1187 (*MAT* α , *ste2-10::LEU2*) and SY1372 (*MAT* α , *ste2-10::LEU2*, *ste3* Δ). These two strains represent wild type α -factor cells which expresses the **a**-factor receptor (SY1187) and a mutant α -factor cell (SY1372) which does not have an **a**-factor receptor. The generation times of these cell strains were determined by a growth curve. These strains were received from George Sprague (University of Oregon).

Iodinated **a**-factor

The **a**-factor peptide was synthesized in the laboratory of Professor Fred Naider at the College of Staten Island and was generously provided for experimental purposes. All final products were >99% homogeneous in two solvent systems and gave the correct molecular ion in fast atom bombardment mass spectroscopy. The peptide was iodinated using Iodo-GenTM Iodination reagent (available from Pierce). The procedure used was as previously described with the following modification: during HPLC analysis, the flow rate was 1.5 ml/minute; gradient, 48% acetonitrile from 0 to 5 minute, 48 to 65% from 5 to 17 minute, and 65 to 48% from 17 to 22 minute (Marcus, et al. 1991).

Growth Arrest (Halo Assay)

YM-1 plates (0.5% yeast extract, 1% peptone, 1% succinic acid, 0.6% sodium hydroxide, 1% dextrose, 2% agar, 0.67% yeast nitrogen base without amino acids, 0.01%adenine sulfate, 0.01% uracil, pH 5.8) were overlaid with 4 ml of *S. cerevisiae*

SY1187 or SY1372 cells (2.5×10^5 cells per ml) in Noble agar. 10 μ l portions of ^{125}I a-factor at various concentrations were pipetted onto the lawn. The plates were incubated at 30°C for 24 hours and then observed for halos (areas of clearing, which indicates growth inhibition caused by the a-factor on the disk) around the disks. The appearance of a halo shows that the α -cell responded to the a-factor on the disk. This verifies the presence of an a-factor receptor in the α -cell.

Background binding using glass filters

The medium used in the assay to determine the amount of a-factor that would bind to the filters was YM-1 medium with inhibitors (YM-1+a/i). The following inhibitors were included: sodium azide and potassium fluoride (both are energy metabolism blockers which keep growth from interfering with binding); N α -p-tosyl-L-arginine methyl ester [TAME] and 1,10 phenanthroline (to inhibit a-factor proteases). BSA [fatty acid free from JRH Biosciences] was used to solubilize the hydrophobic a-factor. All reactions were performed using siliconized microfuge tubes (Midwest Scientific) since a-factor sticks to both glass and plastic. ^{125}I a-factor was added to 200 μ l of YM-1+a/i medium in siliconized microfuge tubes in a final concentration of $6.25 \times 10^{-9}\text{M}$ (duplicates of each were prepared). The solution was then vortexed. 100 μ l of each sample were removed and added to a filter on a vacuum apparatus (from YEDA Scientific Instruments). This apparatus consisted of a plexiglass base with ten wells which could be covered with filters and held in place and sealed by a clamping device; a vacuum could be applied to the apparatus. One sample was filtered on a GF/C filter, the duplicate sample of the same concentration was filtered on a 934-AH filter (both filters are glass filters and are available from Whatman). The filter was washed two times with 1 ml of 1% Tween80, 1% Ethanol wash. After filtration was complete, the filters were removed from the apparatus and put into a 12x75 mm glass tube for

counting. The remaining 100 μ l of the sample were placed in a 12x75 mm glass test tube for counting. Counts per minute were determined by a gamma counter.

Binding assay using cell suspensions filtered onto glass filters

S. cerevisiae SY1187 (wild type alpha) and SY1372 (alpha cells with a deletion of the α -factor receptor) were grown in YM-1 overnight at 34°C with moderate shaking. Approximately 200 ml of culture is required per binding curve. When the cell density reached 1×10^7 cells/ml (count determined by hemocytometer), cells were harvested by centrifugation (6K RPM/10min/JA-10 rotor prewarmed to ~34°C). Cells were washed twice with 100 ml of prewarmed YM-1+a/i. Cells were resuspended in YM-1+a/i at a concentration of 1×10^9 cells/ml and placed at 34°C. ^{125}I α -factor was added to 200 μ l of cell suspension in siliconized microfuge tubes in a final concentration of $6.25 \times 10^{-9}\text{M}$ (duplicates of each were prepared). The reaction mixture was incubated in a 34°C water bath for 6 minutes. 180 μ l of each sample were removed and added onto a prewet (using the wash solution) 934-AH filter on the vacuum apparatus. The remaining 20 μ l were placed in a 12x75 mm test tube for counting. After filtration of the reaction mixture was completed, the first 2 ml wash was applied to the filter. To ensure an even vacuum was applied to the whole filter resulting in the removal of all YM-1+a/i from the suspension, the vacuum of the filter apparatus was then broken and reapplied, and the remaining two washes were completed. After washing was complete, the filters were removed from the apparatus and were placed into 12x75 mm glass tubes for counting. Counts per minute were then determined by gamma counter.

Binding assay using oil centrifugation

SY1187 and SY1372 cells were grown in YM-1 medium overnight at 34°C. Cells were harvested by centrifugation (6K RPM/10min/JA-10 rotor prewarmed to ~34°C). Cells were washed twice with 100 ml of prewarmed YM-1+a/i and resuspended at 1×10^9 cells/ml in YM-1+a/i. 150 μ l of an inert oil mixture (dibutyl phthalate/dinonyl phthalate at 55:45) were added to a siliconized microfuge tube (Fasoli and Kerridge 1990). 100 μ l of the cell suspension were added over the oil. The microfuge tubes were placed in a bench top centrifuge. Labeled a-factor (to give a final concentration of 6.25×10^{-9} M) was added to YM-1+a/i, and then 100 μ l of this mixture were added to the microfuge tube. The tubes were centrifuged for 30 seconds at top speed. After centrifugation, the supernatant fluid was aspirated to remove unbound radioactive a-factor. YM-1+a/i (1 ml) was added on top of the oil layer twice to wash the reaction mixture. After the final wash, the oil layer was removed. The bottom portion of the test tube containing the cell pellet was removed using a microfuge tube cutter (Bel-art products) over 12x75 mm glass test tubes and then counted by gamma counter.

Results

Background binding assay using glass filters

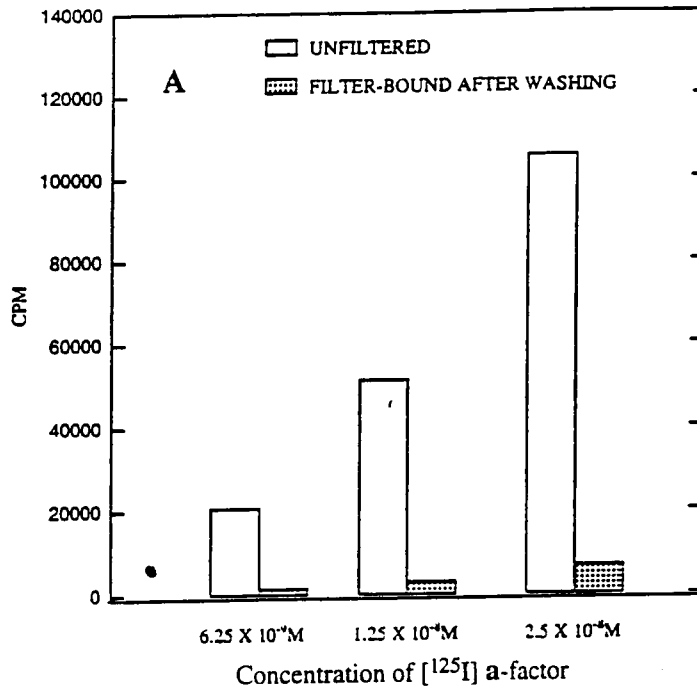
The development of an a-factor binding assay first required the development of a reproducible background binding assay which indicated low background binding of the ^{125} I a-factor to the filters. Research in our laboratory has shown that the binding of α -factor could be measured using a vacuum filtration apparatus; therefore, the use of the vacuum filtration apparatus was first used in attempt to develop an a-factor binding

assay. Using the procedure described in the *Materials and Methods* section, background binding of ^{125}I **a**-factor bound to the glass filters was determined. These results, shown in figure 8, indicate that when comparing the cpm's of ^{125}I **a**-factor bound to the filter (the shaded bars) versus the cpm's of the unfiltered reaction mixture (unshaded bars), the 934-AH filters retained less ^{125}I **a**-factor than the GF/C filters.

Since the amount of ^{125}I **a**-factor remaining on the filters indicated there was nonspecific binding of the **a**-factor to the filter, the conditions of the wash were changed. The filters were soaked overnight in a solution of 1% BSA in an attempt to reduce the background binding; however, after the amount of ^{125}I **a**-factor remaining on the filters was determined, it was evident that soaking the filters in BSA had not reduced the amount of ^{125}I **a**-factor remaining on the filters (these results are not shown).

Possibly, the 1% Tween, 1% Ethanol wash was not strong enough to sufficiently wash unbound ^{125}I **a**-factor off of the filters. Therefore, a background binding assay in which the amounts of Tween80 were varied was performed. The washes used were 1% Tween/1% Ethanol, 2% Tween, 5% Tween, and 10% Tween, and the wash solution was used to prewet the filter. The amount of reaction mixture applied to the filter was increased from 100 μl to 180 μl since it was easier to manipulate a larger volume. In case either the number of washes or the volume of wash used was not sufficient to wash the ^{125}I **a**-factor from the filter, the number of washes was increased to three, and the amount of the wash was increased to 2 ml. To ensure more even washing, the vacuum of the filter apparatus was broken after the completion of the first wash and reapplied for subsequent washes. For this background binding assay, two types of glass filters (GF/C and 934-AH) were used, and the iodinated **a**-factor was added to the medium at a final concentration of $6.25 \times 10^{-9}\text{M}$. The results shown in figure 9 indicated that the washes with the highest percentage of Tween were more

934-AH filter associated [^{125} I] a-factor after washing with
1% Ethanol 1% Tween



GF/C filter associated [^{125} I] a-factor after washing with
1% Ethanol 1% Tween

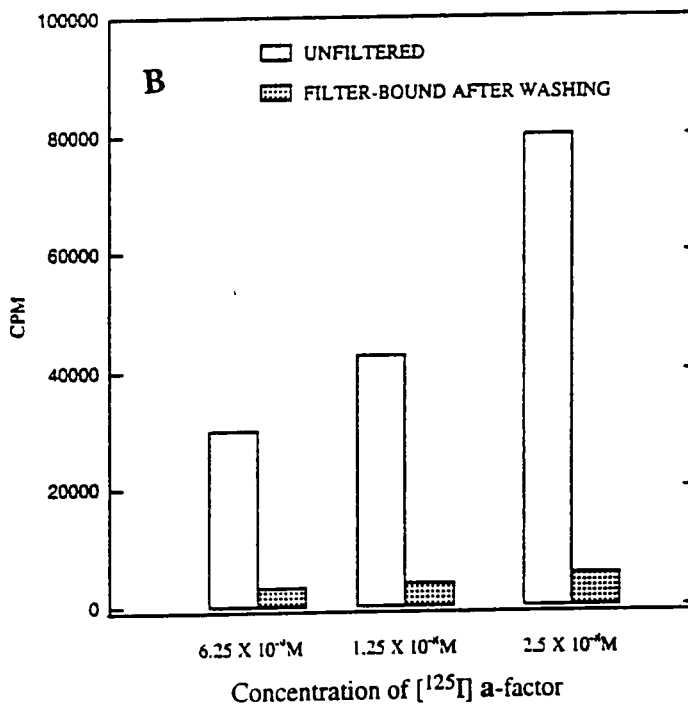
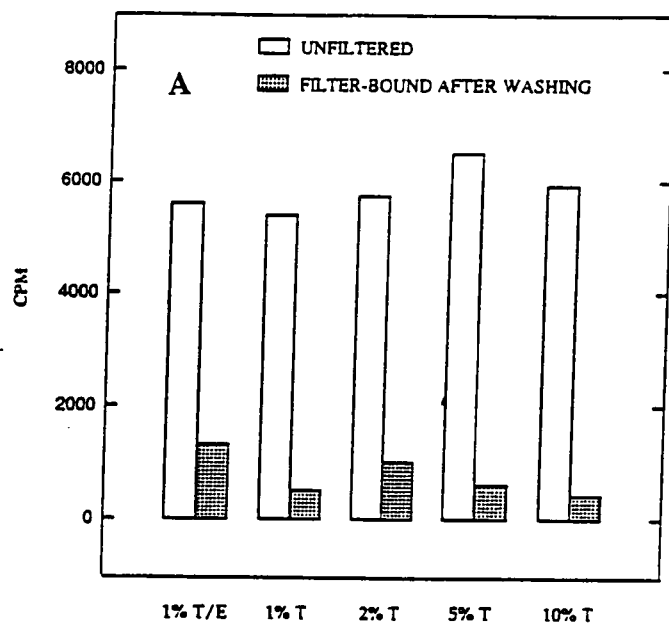
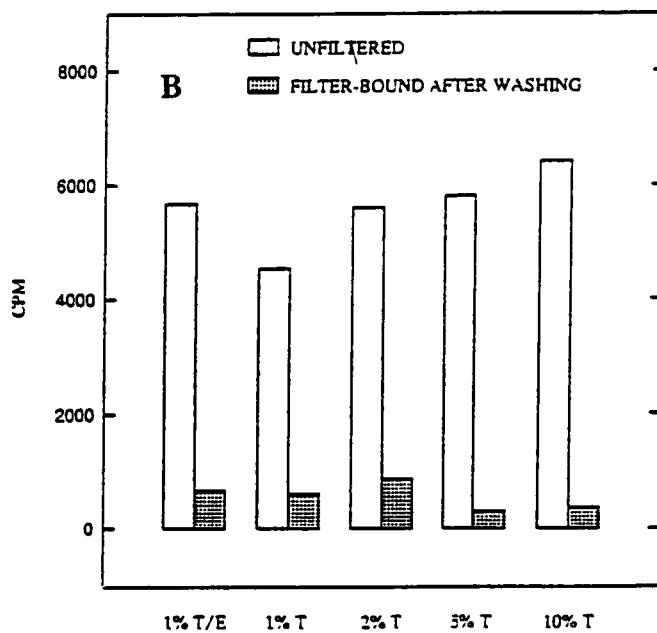


Figure 8. Background binding assay using 1% Ethanol, 1% Tween washes. Panel A: binding to 934-AH filters. Panel B: binding to GF/C filters.

934-AH filter associated [^{125} I] a-factor
after various washes



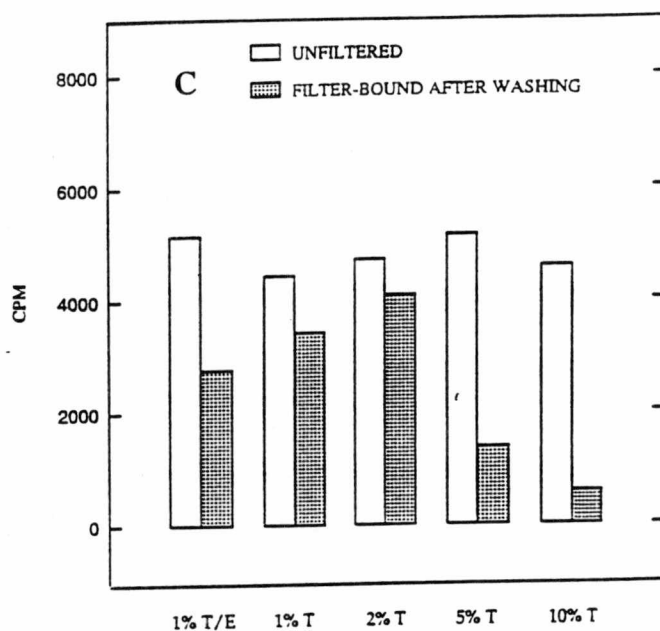
934-AH filter associated [^{125} I] a-factor
after various washes



T=TWEEN
E=ETHANOL

Figure 9. Background binding assay using various washes. Panel A: experiment 1 using 934-AH filters. Panel B: experiment 2 using 934-AH filters. Panel C: experiment 1 using GF/C filters. Panel D: experiment 2 using GF/C filters.

GF/C filter associated [^{125} I] a-factor
after various washes



GF/C filter associated [^{125} I] a-factor
after various washes

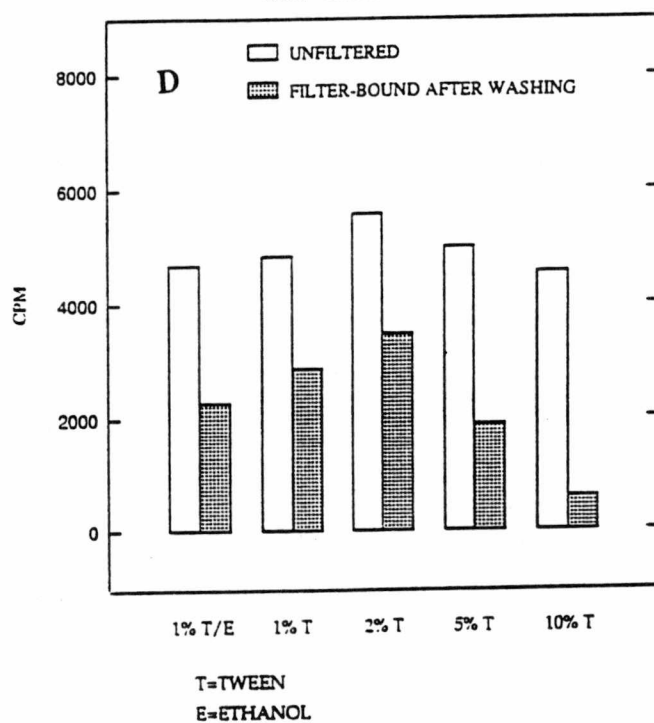


Figure 9 (continued).

successful in washing the a-factor off the filter. Again, the 934-AH filter retained less a-factor on the filter after washing was completed as compared to the GF/C filters. Since there was less a-factor bound to the 934-AH filters, these filters were used in further experiments. This background binding assay was reproducible (results not shown).

Characterization of generation times of SY1372 and SY1187

In *Candida albicans*, it has been shown that raising the incubation temperature decreases the hydrophobicity of the organism (Hazen, 1991). Since the hydrophobic nature of the a-factor peptide has been an obstacle in projects involving the binding of the pheromone, raising the incubation temperature was incorporated into the binding assay using α -cells.

Obtaining growth at a specific density has been shown to be critical for other binding assays (such as that for α -factor). Therefore, growth curves to characterize the generation times of both strains at a higher temperature (34°C) were performed. The growth curves of the two strains are shown in figure 10. From the data, generation times were calculated as 1.425 hours for SY1372 and 1.65 hours for SY1187.

Binding assay using cell suspension filtered onto glass filters

Since the background binding of the ^{125}I a-factor to the filters had been reduced, a binding assay using both SY1187 and SY1372 alpha cells was attempted by the method described in the *Materials and Methods* section. After analyzing the counts per minute, the results (shown in figure 11) revealed that there was as much ^{125}I a-factor bound to the cells without the a-factor receptor (as indicated by the shaded bars) as was bound to the α -cells with the receptor (represented by the white bars).

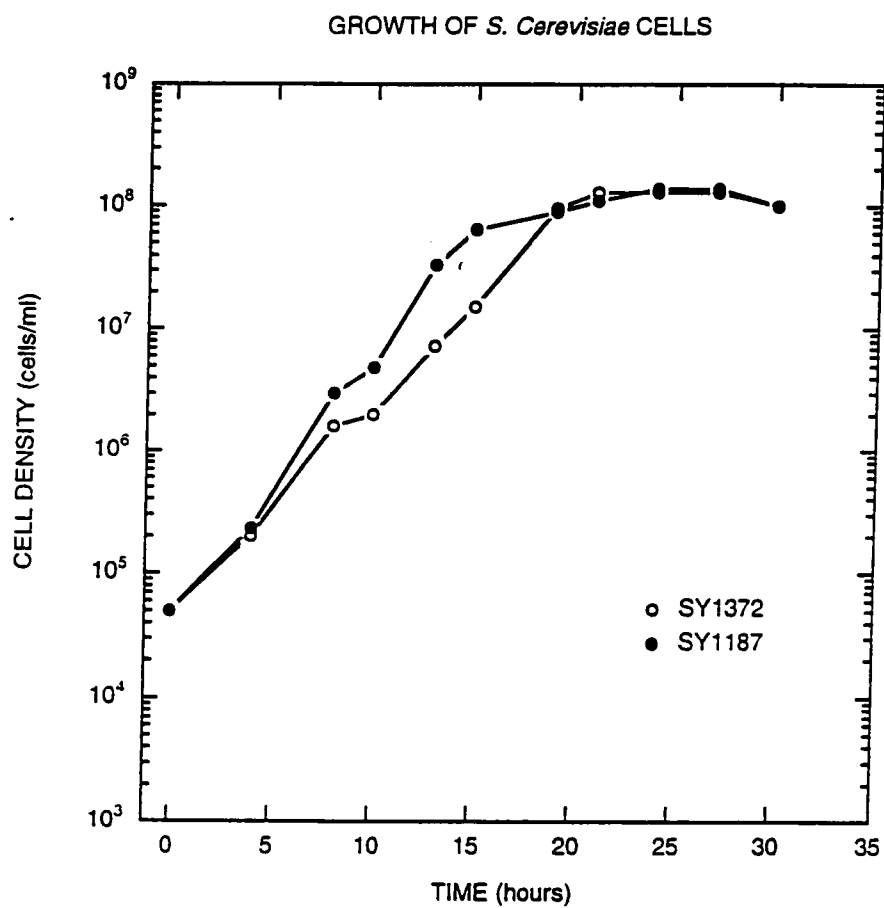
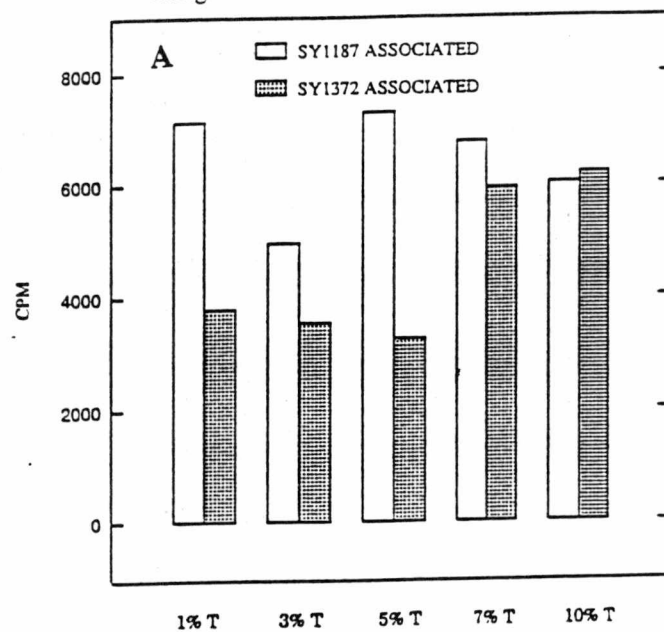


Figure 10. Growth curve of *S. cerevisiae* SY1372 and SY1187.

934-AH filter associated [^{125} I] a-factor
after various washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after various washes
using *S. cerevisiae* SY1187 and SY1372

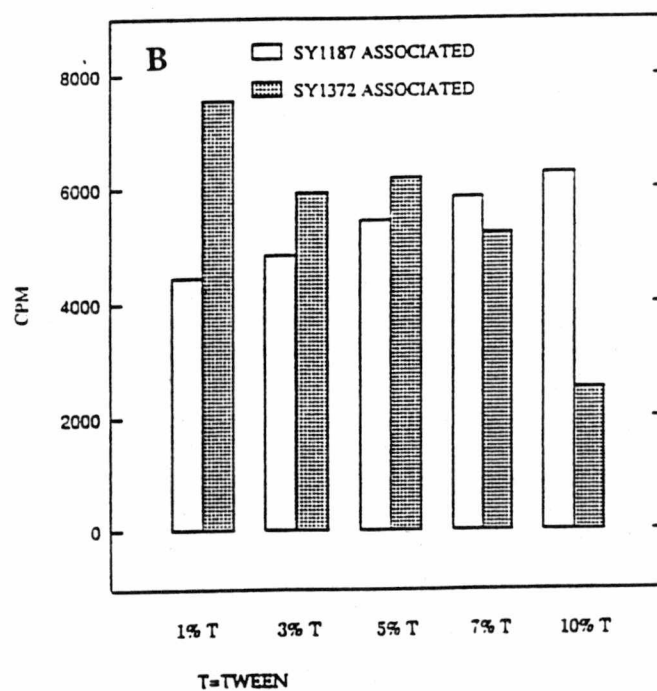
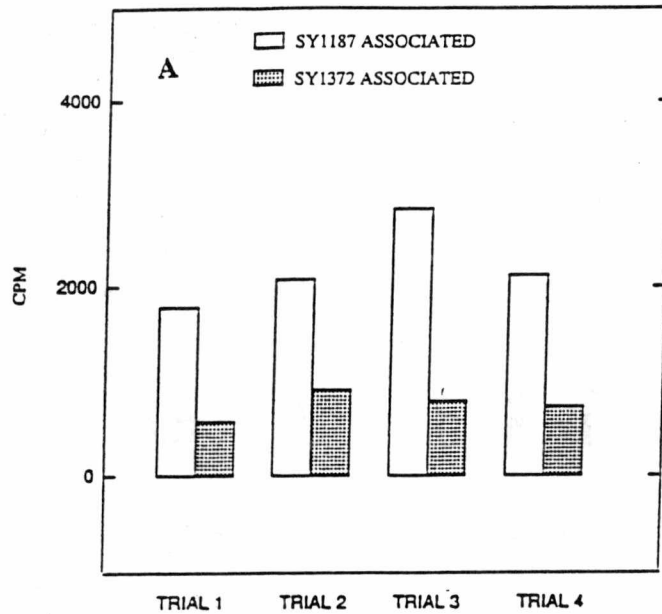


Figure 11. Binding assay using various Tween washes. Panel A: experiment 1. Panel B: experiment 2.

Since nonspecific binding continued to be a problem, a binding assay was performed using another detergent in the wash. Various percentages (1%, 2%, 3%, 5%, 7%, and 10%) of SDS washes were used instead of Tween washes. The use of these SDS washes were very promising in successfully washing ^{125}I **a**-factor from the filters (results not shown); however, when the 3%, 5%, and 7% SDS washes were used in a binding assay, specific binding to the α -cells expressing the **a**-factor receptor could not be achieved (results not shown). Next, the same experimental procedure was repeated except that the BSA (fatty acid free) in the YM-1+a/i medium was increased ten-fold. It was thought that the increase of BSA would help reduce the nonspecific binding of **a**-factor. The results from this assay using both 3% and 5% SDS washes revealed that there was a difference in the amount of ^{125}I **a**-factor binding to the two different strains (fig. 12). The graphs indicate that there was more ^{125}I **a**-factor bound to the α -cells with an **a**-factor receptor (SY1187 cells are shown by the white bars) than to the α -cells without a receptor (SY1372 cells are shown by the shaded bars). Although there was a difference in the amount of binding of the ^{125}I **a**-factor to the cells with and without the **a**-factor receptor, a further reduction of the amount of nonspecific binding was desired.

Since the nonspecific binding of ^{125}I **a**-factor to α -cells not expressing the **a**-factor receptor needed to be reduced, more modifications were made to the protocol. Cells were grown, harvested, and resuspended as detailed in the *Materials and Methods* section. Changes were then made to the binding assay procedure. From each reaction tube, 180 μl were added to a microfuge tube containing 1 ml of 3% SDS wash and then the tube was vortexed in order to prolong exposure of the reaction mixture to the detergent wash. All of the sample from the microfuge tube (wash and the 180 μl of reaction mixture) was added to a pre-wet (with 3% SDS wash solution) filter. The volume of the wash solution (3% SDS) remained 2 ml, and three washes were again

934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after 5% SDS washes
using *S. cerevisiae* SY1187 and SY1372

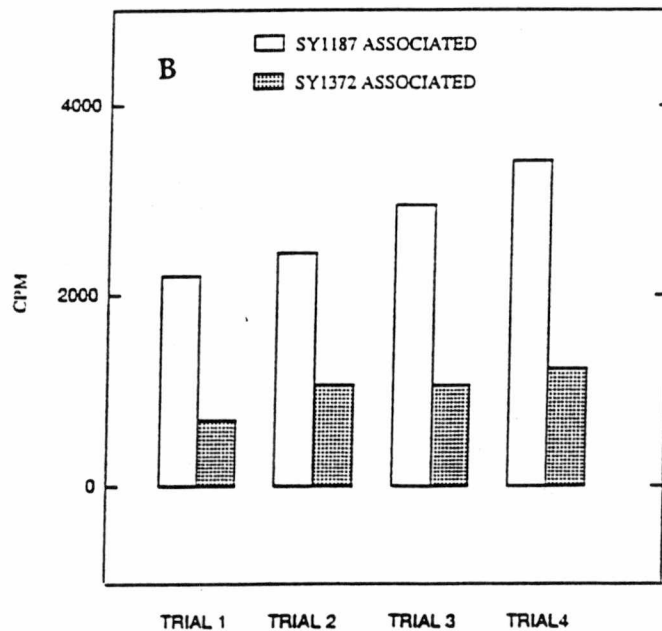
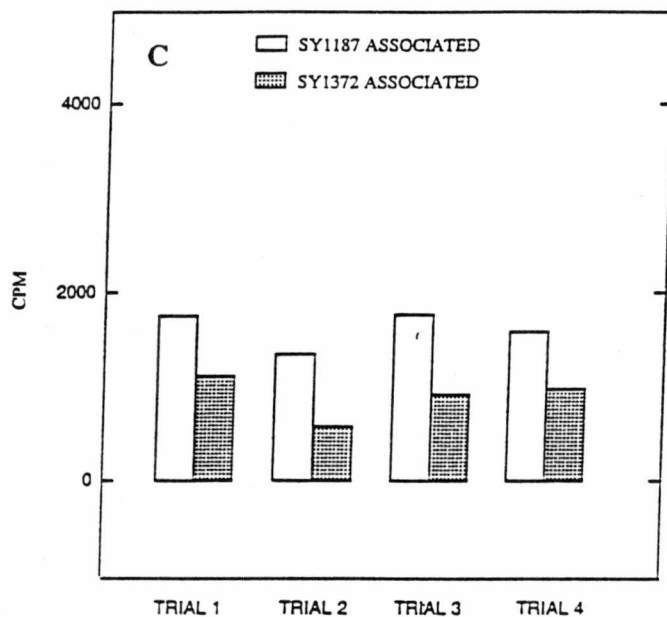


Figure 12. Binding assay using 3% and 5% SDS washes. Panels A through C: experiments 1 through 3, respectively, using 3% SDS. Panels D through F: experiments 1 through 3, respectively, using 5% SDS.

934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after 5% SDS washes
using *S. cerevisiae* SY1187 and SY1372

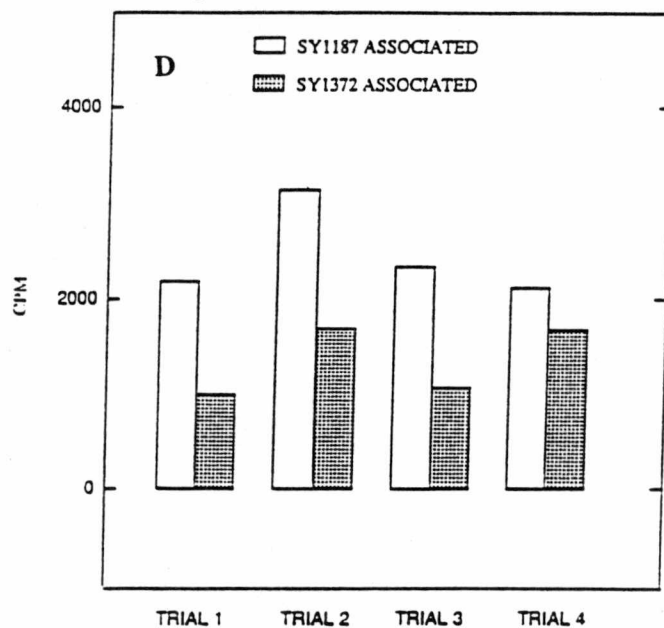
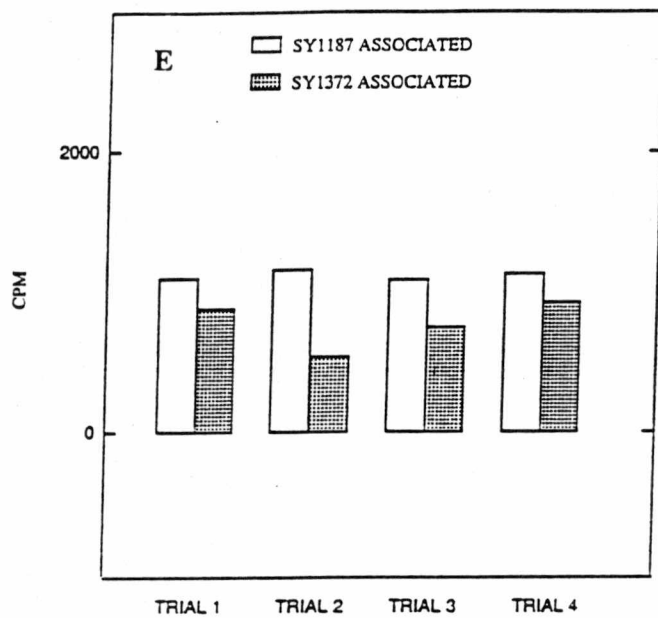


Figure 12 (continued).

934-AH filter associated [^{125} I] a-factor
after 5% SDS washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372

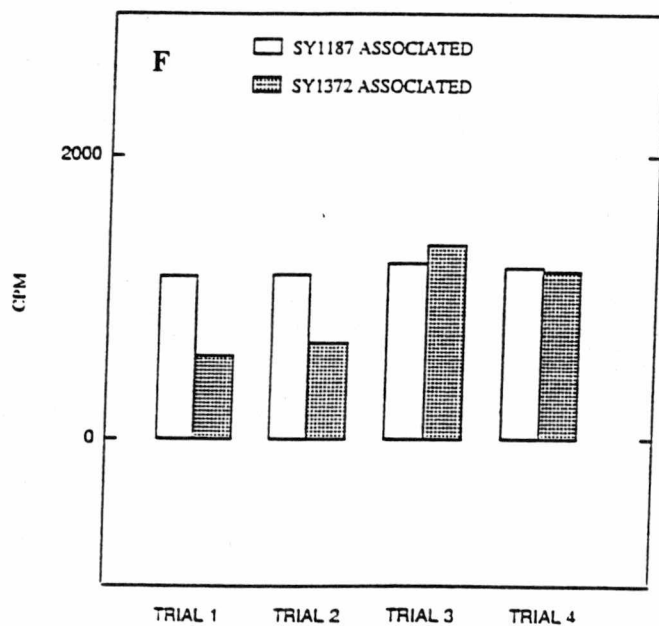
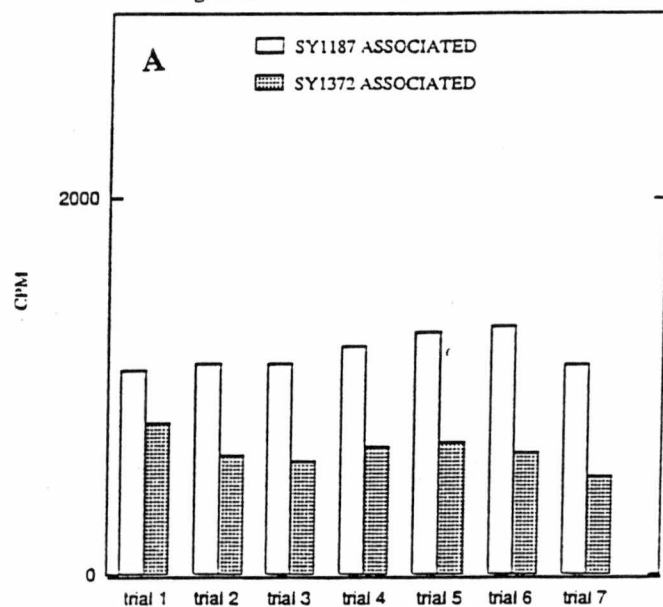


Figure 12 (continued).

performed. The vacuum was broken and then reapplied, and the remaining two washes (2 ml of 3% SDS) were performed. The original reaction tube (which contained 20 μ l of the original sample) was centrifuged in a bench top centrifuge briefly, the bottom of the microfuge tube was cut over a 12x75 mm glass test tube to ensure that an accurate count of the ^{125}I **a**-factor contained in the reaction mixture could be obtained. The test tube containing the portion of the original reaction mixture and the glass test tube containing the filter were counted by gamma counter. These changes in the protocol produced results showing more binding to the α -cells with the **a**-factor receptor than to the α -cells without the **a**-factor receptor (as shown in figure 13). From the results obtained from this assay, both a binding curve and a Scatchard plot were created for each strain. The resulting graphs indicate as the amount of ^{125}I **a**-factor increased, so did the binding (results not shown). Because there was never a plateau effect (where even as the ^{125}I **a**-factor increased the cpm's did not), saturation of the receptor was not achieved.

The final concentration of ^{125}I **a**-factor was increased from $6.25 \times 10^{-9}\text{M}$ to $2.5 \times 10^{-8}\text{M}$ in an attempt to reach a saturation point in the binding; however, even with the increased concentration of iodinated **a**-factor, saturation was not achieved. These results are shown in the saturation binding graphs in figure 14. In order to further increase the concentration of **a**-factor, the final concentration of **a**-factor was increased by the addition of cold **a**-factor to the ^{125}I **a**-factor. By utilizing cold **a**-factor, the final concentration of **a**-factor was increased to $1.26 \times 10^{-7}\text{M}$, $2.52 \times 10^{-6}\text{M}$, and $1.26 \times 10^{-5}\text{M}$. After performing a binding assay using these increased concentrations, the results indicated that as the amount of ^{125}I **a**-factor increased, so did the counts per minute. Once again, there was not plateau effect that would be achieved had the binding been saturated (results not shown). The results also show that specific binding had not been

934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372

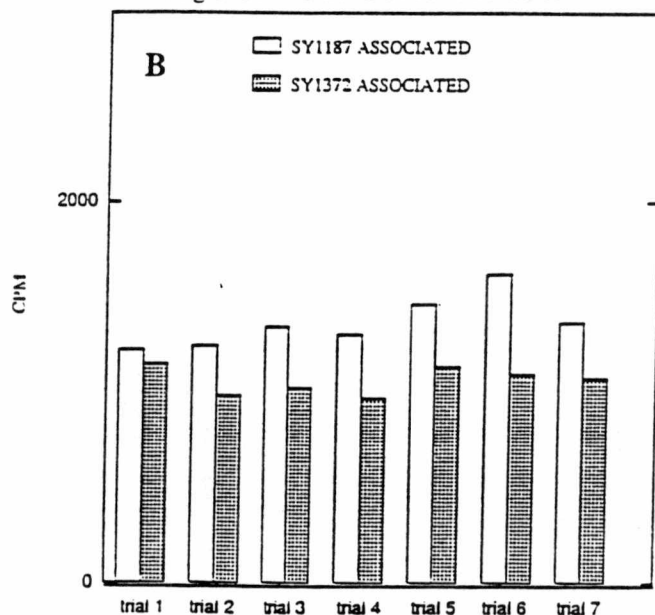
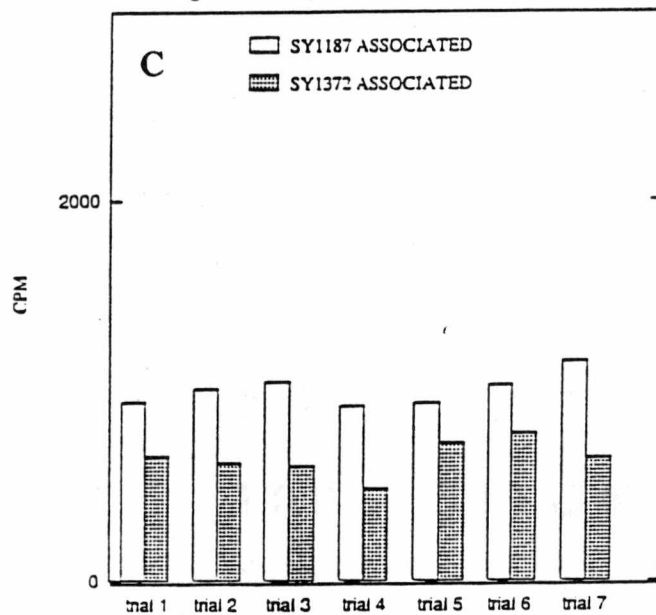


Figure 13. Binding assay using 3% SDS washes. Panels A through D: experiments 1 through 4, respectively.

934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372



934-AH filter associated [^{125} I] a-factor
after 3% SDS washes
using *S. cerevisiae* SY1187 and SY1372

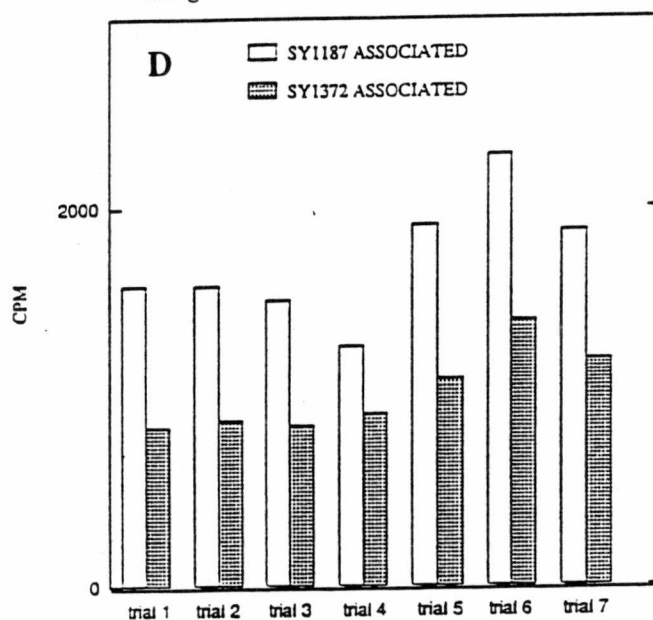


Figure 13 (continued).

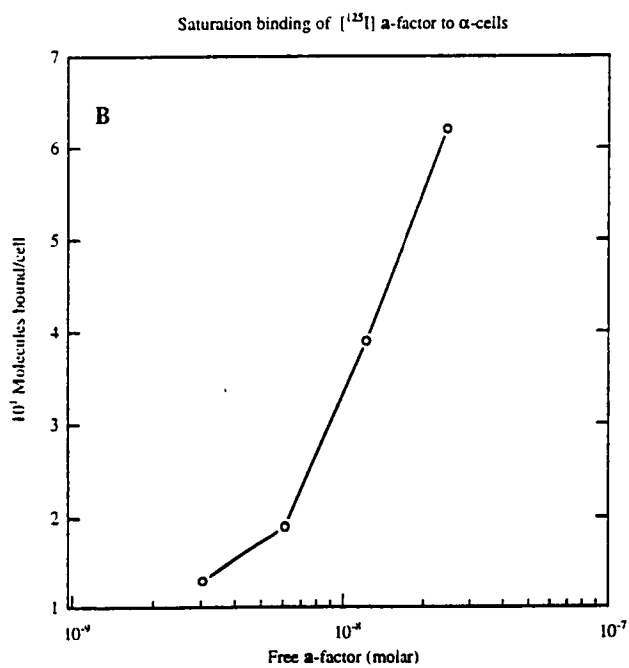
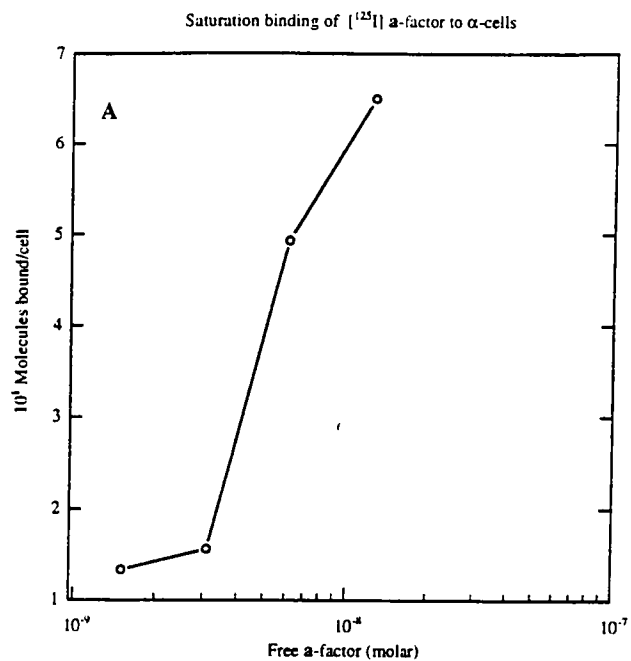


Figure 14. Saturation binding of α -factor to α -cells. Panel A: experiment 1. Panel B: experiment 2.

occurring; in fact, there was little difference in binding between the cells with and without the **a**-factor receptor.

Binding assay using oil centrifugation

Previously, our laboratory had attempted to develop an **a**-factor binding assay using oil centrifugation. Due to the unsuccessful results, this type of assay had been abandoned. Other researchers were able to achieve a binding assay using oil centrifugation, so their methods were incorporated to the **a**-factor binding assay (Fasoli and Kerridge 1990). A background binding assay in which no cells were present was performed using the protocol described in the *Materials and Methods* section. When the assay was performed using the α -cells, the procedure was unsuccessful. After the first wash, there was no longer a separation of the layers; and after analyzing the counts per minute, no difference could be seen between cells with and without the **a**-factor receptor. Therefore, changes were made to the procedure. For example, water was used instead of YM-1+a/i to extract the unbound ^{125}I **a**-factor from the reaction; however, no separation of the oil and aqueous layer could be seen after the first wash. Due to the erratic counts and the difficulty in using the oil centrifugation, this method was not further pursued.

Discussion

From the beginning of the development of an **a**-factor binding assay, the hydrophobicity of the **a**-factor pheromone has complicated the experiments. The hydrophobicity of the pheromone presented the problem of nonspecific binding. Many attempts were made to decrease the hydrophobic conditions of the assay and thus reduce the amount of nonspecific binding. Methods reported to help reduce the amount of

nonspecific binding (such as increasing the incubation temperature to 34°C) were tried with unsuccessful results. Perhaps the reason there was approximately the same binding of **a**-factor on cells with and without the **a**-factor receptor could be that the **a**-factor binds first to the cell wall of the α -cell, and only after this initial binding is the **a**-factor sequestered to the **a**-factor receptor. Since there was little difference in the amount of **a**-factor binding to the cells with and without the **a**-factor receptor, this theory is supported.

Some suggestions to decrease the hydrophobicity of **a**-factor could include the removal of the farnesyl group of the pheromone. This would solve the problem of hydrophobicity; however, this option is not realistic because it would not provide accurate results since the farnesyl group is important for the biological activity of the pheromone. This alteration in the pheromone would not provide an accurate description of the binding ability of the wild type pheromone. Another possibility would be to use isolated **a**-factor receptors instead of whole α -cells during the binding assay. Isolating the receptor has not yet been accomplished, but using the proteins alone should reduce the nonspecific binding significantly. This method would allow one to observe the binding abilities of **a**-factor and mutated analogs. The development of an **a**-factor binding assay has proven to be difficult.

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VITA

Leann Lovin was born in Huntsville, Alabama on December 10, 1969. She attended elementary and middle schools in Knoxville, Tennessee. In May of 1987, she graduated from Karns High School. Leann entered the University of Tennessee in September of the same year, and she earned a Bachelor of Science degree in Biology with high honors in May 1991.

In the fall of 1991, Leann entered graduate school in the Microbiology department. During her time in graduate school, she was a teaching assistant. Her thesis research was performed in the laboratory of Dr. Jeffrey M. Becker. In December of 1994, she received a Master of Science degree in Microbiology.