4. BACTERIAL EXPRESSION AND FUNCTIONAL ANALYSIS OF ACPR

4.1 INTRODUCTION

*E. coli* has been routinely used as a host to express proteins 1) which are difficult to isolate in large amounts, 2) those for which the cloned genes are available, 3) for expressing ORF of unknown function so that the protein product can be used to identify the gene and for its functional analysis. Expression in *E. coli* has been facilitated by the ability to introduce foreign gene sequences cloned into plasmid vectors. The cloned gene is then expressed by using the transcription and translation machinery of *E. coli*. Expression may be affected by the inability of the cloned sequences to provide proper signals for ribosome binding and translation initiation in *E. coli*. Therefore, strategies are used wherein the gene is fused either to the bacterial ribosome binding site to synthesize an authentic product or fused to the NH₂ terminal of a bacterial gene to synthesize a fusion protein. Once cloned into one such vector, the expression is driven by a constitutive or an induced promoter. The expression can be monitored by direct visualisation of the resolved proteins on the gel, immunodetection or functional assay. The functional identity of the cloned gene can be determined by 1) detection of a novel function imparted to the host, 2) complementation, 3) assay of the whole cell extract for activity and 4) assay of the purified or partially purified protein.

Expression in *E. coli* poses several disadvantages also. 1) High level expression of proteins in *E. coli* can result in the compartmentalisation of these products in inclusion bodies. The product is generally inactive and must be extracted with detergents.
or other solubilising agents. It may not be possible to regain the biological activity of the protein, 2) some proteins which are toxic are expressed poorly, 3) genes that contain introns are not spliced to result in the correct expressed protein, 4) proteins expressed in *E. coli* may not acquire biological activity due to improper folding and disulphide structure, 5) proteins to be secreted lack the NH$_2$ terminal specificity for targeting, 6) other post translation modifications like glycosylation and phosphorylation do not occur.

ACPR has homology to the NH$_2$-terminal DNA binding domain of STE12. It was of interest to see if this homology could translate into function. Also, since there exists no homology with the rest of STE12, we wanted to see if the difference could abolish DNA binding. With the nucleotide sequence of ACPR on hand, the obvious choice was to use Polymerase Chain Reaction (PCR) (Saiki et al., 1988) to synthesize the truncated ACPR and the whole gene with engineered sites to facilitate cloning into an expression vector of choice. Primers were designed to suit cloning into pMAL-c expression vector (Guan et al., 1987). The amplified products were sequenced to check the fidelity of Taq DNA polymerase during amplification.

Since this protein shared homology with the DNA binding domain of STE12, it was of interest to see if the homologous region of ACPR could bind DNA. The ability of a protein to bind DNA can be detected by any one of the following methods. a) nitrocellulose filter binding assay, b) gel mobility shift assay, c) exonuclease III assay and d) DNase I protection assay. Gel mobility shift assays as described by Fried and Crothers (1981) was used to detect
the ability of ACPR to bind PRE sequences. The advantages of gel mobility shift assays over the other methods are - 1) the method is more sensitive as it provides information about the number of complexes (factors) involved in binding, 2) the specificity of the complex formed can be confirmed by competition experiments and 3) the size of probe (target) can vary from short oligos upto 1-kb fragment, although large fragments would require further narrowing down of the binding site. We have used the upstream control regions of Ty 1, a retroposon from yeast (Errede et al., 1980), and STE2 UAS which have PRE elements as targets for gel mobility shift assays. The ability of the E. coli expressed truncated ACPR to bind these targets was determined.

The ability of secretory acid proteinase (SAP) to clot casein at acidic pH (5.3) in a milk agarose plate assay is used as an indicator of activity of the enzyme (Banerjee et al 1991). The acid proteinase activity of ACPR expressed in E. coli was determined on a milk agarose plate assay. Detection of acid proteinase activity in a heterologous system can pose problems due to 1) the background contributed by the host proteinases. Since in E. coli the levels of the acid proteinases (if any) are below the detectable limits of the assay, whatever activity is detected would be due the encoded gene only, 2) the stability of the toxic product in E. coli and 3) improper folding of the protein in E. coli may render the protein inactive. With the MBP-ACPR(1-699) fusion, proteinase activity is not detected. In the event of not being able to detect activity, the option is to find structural similarities between the two proteins. The presence of common peptides would indicate that the protein could fold similarly in the same context resulting in common
epitopes which can be detected by antibodies. The relatedness of ACPR and SAP was determined by peptide mapping and the related epitopes identified by immunodetection with SAP antibodies.

4.2 MATERIALS AND METHODS

4.2.1 Strains used

*E. coli* - DH5α, BL21(DE3)

*S. cerevisiae* - EG123 - MAT a *STE*⁺ (trp1, leu2, ura3, his4)

SF167-5a - MAT a ste12::LEU2(trp1,ura3,his4)

4.2.2 Media and solutions

LB medium containing 50μg/ml ampicillin and 0.2% glucose was used to grow bacterial cultures containing plasmids.

YPD - 1% yeast extract, 2% peptone and 2% dextrose.

YPD agar plates - YPD medium containing 2% agar to solidify the medium.

SD minimal medium - 0.67% yeast nitrogen base w/o amino acids (Difco), 2% dextrose. Wherever required, amino acids (SRL or Sigma) were added to 20μg/ml from stock solutions(2mg/ml).

SD minimal plates contained 2% agar to solidify SD minimal medium.

Omission plates were made by supplementing the SD minimal plates with the required amino acids.

For generating transformants, the transformation mix was plated on SD minimal plates containing the required amino acids and 1% YPD. Penicillin G (Sigma) was added to 100U/ml from a stock made in ethanol.

0.1M IPTG - 23.8mg of IPTG (BRL) was dissolved in 1ml of sterile water.

30% acrylamide - 30g of acrylamide and 0.8g of bis-acrylamide was
dissolved in 100ml of water and deionised using mixed bed resin AG 501-X8(D) (Biorad Labs.). The solution was filtered twice and used.

4.2.3 Polymerase Chain Reaction (PCR)

Design of oligonucleotides for use in PCR (Saiki et al., 1988):

The oligo corresponding to the ATG codon of ACPR with a 3 nucleotide 5' overhang was synthesized. The ATG codon is underlined

5' CCTATGTCAATTACTAAAACATAC 3'

The downstream oligo for amplifying ACPR(1-230) was generated with an Xba I site to facilitate cloning into the Xba I site of pMAL-c. The Xba I site created is underlined.

5' GCTCTAGAAGTAGAGCTTGTG 3'.

To amplify the whole ACPR gene the oligo used was

5' CTAATCTAATGATCTTGTCCA 3'

which corresponded to 120-bp downstream of the 3' end of the ACPR gene.

Amplification of ACPR(1-230) and ACPR(1-699) fragments from genomic DNA of C. albicans SC5314.

PCR reactions were performed in a 100µl reaction volume according to the specifications of the suppliers (Perkin Elmer, Cetus Corp.). 10ng of genomic DNA was used as template and 20pmoles of primers were used. After initial denaturation for 5 min at 94°C, 5 cycles were performed by annealing at 45°C for 2 min, extending at 72°C for 1 min., and denaturation at 94°C for 1 min. After the first five cycles, the cycling parameters were denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min. for 20 cycles. A final extension was done for 5 min at 72°C to complete the synthesis. PCR was performed in PTC-100-60 thermal cycler of MJ Research Inc. A 25µl reaction without DNA template was performed.
simultaneously as a control. 5μl of each of the PCR amplified mix was analysed on a gel along with DNA standards (lambda DNA cut with Hind III and pUC19 cut with Hinf I).

4.2.4 Cloning of the PCR products in pMAL-c expression vector

Processing the PCR products for cloning - The amplified fragments were filled in with Klenow polymerase to repair the ragged ends.

1) 10μl of the PCR product was diluted with equal volume of sterile water. 2.5μl of Klenow buffer was added.

2) 125μM of all dNTPs were added, mixed and supplemented with 5U of Klenow polymerase (NEB).

3) The reaction was carried out for 30min at room temperature. The samples were extracted with equal volume of TE saturated phenol:chloroform (1:1) and CHCl₃:IAA (24:1).

4) The DNA was precipitated by adding 1/10 volume of 3M NaOAc pH 5.2 and two volumes of ethanol.

5) The precipitate was washed with 70% ethanol and resuspended in sterile water.

pMAL-c plasmid was digested with Stu I for blunt end cloning of ACPR(1-699) and with Stu I - Xba I for directional cloning of ACPR(1-230). The Stu I-Xba I plasmid backbone was purified using Geneclean II kit (Bio 101).

Ligation was set up in a 25μl volume with 50ng of the vector. For blunt end ligation of ACPR (1-699), ligation buffer was supplemented with 25% PEG and 800U of T4 DNA ligase (NEB). The samples were incubated at 20°C for 16-18 hours for blunt end ligation and at 16°C for 16-18 hours for cohesive end ligation.

5μl of the ligation mix was used to transform DH5α by the protocol described in section 3.2.15. Recombinant clones (white colonies)
were analysed for the correct orientation of the inserts.

4.2.5 Induction of the recombinants for expression of the fusion proteins.

1) Preinoculum of each of the recombinants and a non-recombinant (carrying the vector only) were grown in 3ml of LB medium containing ampicillin and 0.2% glucose at 37°C for 16-18 hrs.

2) 1% of the preinoculum was transferred into 10ml of fresh LB medium containing ampicillin and glucose at 37°C until the \( A_{600} \) was 0.5 (2x10^8 cells/ml).

3) 1ml of the uninduced cells were spun down and the pellet resuspended in 50μl of 1X Laemmli gel loading buffer.

4) To the remaining culture, IPTG was added to 0.3mM and the cultures grown at 37°C for 2 hours. 0.5ml of the induced sample was spun down and the pellet resuspended in 50μl of gel loading buffer.

5) 15μl of the samples along with MBP standard (NEB), low and high molecular standards (Pharmacia) were heated in a boiling water bath for 5 min, spun down and resolved on a 10% discontinuous page.

4.2.6 Gel electrophoresis of proteins

The discontinuous buffer system described by Laemmli (1970) was used for electrophoresis in Mighty Small apparatus (Hoefer) at 150V constant voltage. Acrylamide was added from a 30% stock according to requirement to 0.375M Tris Cl (pH 8.8), 0.04% TEMED, 0.5% APS for preparing the resolving gel. Stacking gel had 4% acrylamide and 0.125M Tris Cl (pH 6.8), 0.04% TEMED, 0.5% APS. Reservoir buffer was 0.025M Tris base, 0.192M glycine and 0.1% SDS (pH 8.3). Samples were prepared in 0.0625M Tris-HCl (pH 6.8), 2% SDS, 5% BME, 10% glycerol and 0.002% bromophenol blue. After the run the gels were stained in 0.25% Coomassie Brilliant Blue(R) in 25% methanol and 10% acetic
acid.

4.2.7 Western blotting and immunodetection

1) After SDS page, the proteins were electroblotted onto nitrocellulose membrane(S&S) according to the procedure of Towbin et al.,(1979). Briefly, the gel was equilibrated in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). The transfer was carried out in a electrotransfer apparatus (Hoefer) at 150mA constant current for 3 hours at room temperature. The blots were stained in 0.5% Ponceau S (Salinovich and Montelaro, 1986) in 1% acetic acid for 5 min. The position of the standards were marked and the blot destained completely in water.

The blots were processed for immunodetection by the protocol described in Superscreen immunoscreening system, Amersham.

1) The blots were rinsed in TBS (10mM Tris Cl(pH7.5), 150mM NaCl) and blocking was done overnight in 5% non-fat dry milk (Amersham) in TBST containing 0.1% sodium azide.

2) Blots were rinsed thrice in TBST (TBS containing 0.05% Tween 20) for 10 min and incubated for 1hr at room temperature with primary antibody. Affinity purified proteinase serum was used at 1:200 dilution and MBP serum (NEB) was used at 1:100,000 dilution in TBST.

3) The blots were rinsed thrice in TBST and secondary antibody (rabbit IgG alkaline phosphatase conjugate (Biorad)) was added at 1:3000 dilution and incubated for 1 hr at room temperature.

4) The blots were washed thrice in TBST and developed using NBT/BCIP (Promega) in alkaline phosphatase buffer (0.1M Tris Cl pH9.5, 0.1M NaCl, 5mM MgCl₂). After the color developed the reaction was stopped by rinsing the blots in water.
4.2.8 Time course of induction of recombinant proteins.

1) 10ml of cultures were grown to 2X10^8 cells/ml (as described above) and induced by adding 0.3mM IPTG.

2) Samples were withdrawn from uninduced cultures and after induction for 30 min, 1hr, 2hr, 3hr and 4hrs as described above (section 4.2.5).

3) The cells were resuspended in gel loading buffer.

4) The samples and the markers were heated for 5 min and then resolved on a 10% SDS polyacrylamide gel in duplicate.

4.2.9 Determining the nature of the recombinant protein

The recombinant protein was induced by adding IPTG as described above (section 4.2.5).

1) The culture (20ml) was divided into two aliquots. The cells were spun at 4000g for 10 min. One of the pellet was resuspended in 1.25ml lysis buffer (10mM Sodium phosphate (pH 7.0), 30mM NaCl, 0.25% Tween 20, 10mM EDTA, 10mM EGTA,) - Sample A.

2) The other pellet was resuspended in 2.5ml of 30mM Tris Cl, 20% sucrose, pH 8.0 - Sample B

Sample A was processed as follows - The sample was lysed by rapid freeze-thaw cycles until the sample became viscous. Added 150μl of 5M NaCl, mixed and the sample was spun at 9000g for 20 min. The supernatant was stored as crude extract 1. The resulting pellet was resuspended in 1.25ml of lysis buffer. This insoluble suspension was stored as crude extract 2. 50μl of crude extract 1 was mixed with 50μl of amylose resin (NEB) in column buffer (10mM sodium phosphate, 0.5M NaCl, 1mM azide, 10mM βME, 1mM EGTA ) and incubated on ice for 15 min. It was spun briefly and the supernatant was discarded. The pellet was washed with 1ml of column buffer and spun
again. The pellet was resuspended in 50μl gel loading buffer.

Sample B was processed as follows - EDTA was added to 1mM and incubated 5-10 min with shaking at room temperature. The sample was spun at 8000g, 4°C, 10 min. The pellet was resuspended in 2.5ml of ice cold 5mM MgSO₄. The sample was stored for 10 min in ice water bath and centrifuged 8000g, 4°C, 10 min. This supernatant was collected as cold osmotic shock fluid.

Equivalent volumes of each of these samples was boiled for 5 min and the proteins resolved on 10% SDS page. The samples were visualised by staining with CBB and by immunodetection using SAP affinity purified serum for MBP-ACPR(1-699) and MBP serum for MBP-ACPR(1-230).

4.2.10 Purification of the fusion protein

The fusion protein was purified from the cytosolic extracts by amylose affinity chromatography (Protein fusion and purification system, NEB).

MBP-ACPR(1-230) fusion protein was purified from 100ml cultures from crude extract 1 (described above).

1) A 4ml amylose column was packed in econocolumn (Biorad). The column was washed with 3 column volumes of column buffer containing 0.25% Tween 20.

2) The crude extract was diluted 1:5 with column buffer Tween 20, clarified and loaded at a flow rate of 1ml/min.

3) The column was washed with 3 volumes of the same buffer. It was then washed with 5 volumes of the same buffer containing Tween 20. The protein was eluted with column buffer containing 10mM maltose. 1.5ml fractions were collected. The fractions containing the fusion protein was detected by checking the absorbance at 280nm in the UV
range. Fractions containing the protein were pooled and concentrated and dialysed in centricon 30 (Amicon). The protein concentration was determined by Coomassie blue dye binding method (Bradford, 1976) using protein assay reagent (Biorad).

4.2.11 Cloning into pET3c expression vector and analysis of the recombinants

pET3c (Studier et al., 1990) was digested with Nde I and the ends filled in using Klenow polymerase by standard recombinant DNA techniques (Sambrook et al., 1989). PCR amplified product of ACPR(1-230) was repaired at the ragged ends to create blunt ends and cloned into the Nde I site to use the ATG codon of ACPR as translation start. The 2.2-kb fragment of ACPR(1-699) was digested from MBP-ACPR(1-699) plasmid by Stu I-BamHI digestion and cloned into the end-filled Nde I and cohesive BamHI site of pET3c to facilitate directional cloning. The samples were ligated as described in section 4.2.4 and transformed into E. coli DH5α. The recombinants were screened and the orientation determined by restriction digests. The construct was then transformed into competent E. coli BL21 (DE3) strain.

4.2.12 Time course of induction of pET3c expressed proteins

20ml of LB medium containing 50μg/ml ampicillin and 0.2% glucose was inoculated with 1% inoculum from the recombinant clones. The cells were grown to A600 of 0.6. 1ml of the culture was spun down and resuspended in 50μl of sample buffer. IPTG was added to 0.4mM and 0.5ml samples recovered after 30 min, 1hr, 1.5hr, 2hr, 3hr, 4hr, 5hr, 6hr and 24hrs of induction. The cells were spun down and resuspended in 50μl of sample buffer. As controls, BL21(DE3) cells harboring only pET3c was used for induction. The samples were
resolved in duplicate on 10% SDS page. One of the gels was stained in CBB and destained as described previously (section 4.2.5). The other gel was electroblotted onto nitrocellulose and the overexpressed protein identified by immunodetection using affinity purified proteinase antibodies as described in section (4.2.7).

4.2.13 Determining the nature of the overexpressed proteins expressed in pET3c.
10ml of cultures expressing ACPR(1-230) and ACPR(1-699) were induced and the cells processed as described in section (4.2.9) to determine the nature of the protein.

4.2.14 Peptide mapping

Peptide mapping was done according to the procedure of Cleveland (1983).

Resolving the overexpressed proteins on SDS page:
ACPR(1-230) and ACPR(1-699) expressed in E. coli and 4μg of SAP were resolved on a 10% SDS page. The gel was stained for 30 min in 0.1% Coomassie blue(R) in methanol: water: acetic acid (5:5:1 by vol.) and then destained in 5% methanol(v/v), 10%(v/v) acetic acid for one hour. The gel was rinsed with water and the bands corresponding to ACPR(1-230), ACPR(1-699) and SAP were cut out with a razor blade and trimmed. The slices were equilibrated by soaking in 10ml of stacking gel buffer (0.125M Tris Cl pH6.8) containing 1% SDS for 30 min with gentle stirring. The gel slices can be stored frozen at -20°C.

Proteolysis and separation of polypeptides:
A second slab gel was made with a 2-cm long stack and a 15% resolving gel. The wells were filled with 0.125M Tris Cl, 0.1% SDS
and the gel slices were placed horizontally on the top of the well surface. The spaces around the gel were filled with the same buffer containing 20% glycerol. 10µl of the stacking gel buffer containing 0.1% SDS, 10% glycerol, 0.001% bromophenol blue and protease was added over the slices. Proteases used were Trypsin (Sigma) type III from bovine pancreas and Chymotrypsin (Sigma) of sequencing grade were used at the required concentrations from a 5mg/ml stock. Electrophoresis was carried out at 10mA until the tracking dye had traversed 2/3rds of the stack. The power was then shut off for one hour to permit proteolysis. Electrophoresis was continued at 25mA to resolve the peptides until the dye front reached the bottom of the gel. The peptides were then electroblotted onto a nitrocellulose membrane as described in section 4.2.7. The immunorelated peptides were detected using affinity purified proteinase antibodies (section 4.2.7).

4.2.15 Gel mobility shift assays

4.2.15.1 Preparation of E. coli extracts

Cultures of DH5α expressing MBP-ACPR(1-230) were induced for 2 hours at 37°C. The cells harboring only the plasmid were grown simultaneously as a control. The cells were spun down and resuspended in lysis buffer. The cells were lysed by rapid freeze thaw cycles as detailed in section 4.2.9. The lysates were spun down and the supernatant (crude extract) was used for DNA binding assays.

4.2.15.2 Transformation of S. cerevisiae

Yeast transformation was done by the lithium acetate method of Dunn (1984). Yeast strain EG123 (MATaSte+) was transformed with a YEpl3
plasmid carrying the cloned \textit{STE12} gene (pSY2) to produce a strain over expressing \textit{STE12} under its own promoter.

Preparation of yeast competent cells:

1) Preinoculum of the strain was made in 10ml YPD by inoculating from a fresh slant. The cells were grown overnight at 30°C with shaking.

2) 50ml of fresh YPD medium was inoculated with 1% preculture. The cells were grown until $A_{595} = 0.45 \times 10^7$ cells/ml. The cells were then chilled on ice for 30min.

3) The cells were then spun down and washed once in equal volume (50ml) of cold 10mM Tris Cl pH7.5, 1mM EDTA, 0.1M LiOAc. The cell suspension was agitated gently at 30°C for one hour.

4) The cells were chilled on ice. One third volume of chilled 60% glycerol was mixed and the cells were aliquot as 200μl samples, snap frozen in liquid nitrogen and stored at -70°C until use or stored at 4°C for 24 hours.

Transformation:

1) Competent cells were thawed on ice (if stored at -70°C) or used fresh. 200μl cells were used for each transformation.

2) 10μg of the transforming DNA along with 50μg of carrier DNA (sheared salmon sperm DNA) was added. The samples were mixed gently and incubated at 30°C for 30min.

3) 0.7ml of PEG solution (40% PEG in 10mM Tris Cl pH7.5, 1mM EDTA, 0.1M LiOAc) was added and incubated for 1hr at 30°C.

4) The cells were subject to heat shock in a 42°C water bath for 5 min.

5) The cells were collected by a brief spin and resuspended in 0.1ml of 10mM Tris Cl pH7.5, 1mM EDTA. The volume was measured.
6) 1/10 volume and the remaining 9/10 volume were plated on selective plates and incubated at 30°C until microcolonies appeared.

7) Samples used for transformation were - pSY2, YEpl3 and one transformation was performed without the plasmid DNA.

4.2.15.3 Preparation of yeast extracts

Yeast extracts were prepared according to the protocol of Company, et al., (1988). The cells were grown from a fresh slant into minimal medium containing the required amino acids to maintain the plasmids.

1) Cells were grown in 10ml of preculture at 30°C for 16-18hrs.

2) 1% of the preinoculum was transferred into fresh culture and the cells were grown until the A_{595} was 0.45.

3) The cells were chilled on ice and harvested.

4) The pellet was washed with 0.01 volume of extraction buffer (0.2M Tris Cl(pH8.0), 0.4M Ammonium sulphate, 10mM MgCl_{2}, 1mM EDTA, 10% glycerol, 1mM PMSF, 7mM BME). The pellet was resuspended in 0.001 volume of extraction buffer.

5) Cells were disrupted with a vortex mixer in the presence of an equal volume of glass beads (0.45mm diameter). The lysed cells were incubated for 30 min on ice. Cell debris was spun down with the glass beads by centrifugation. The supernatant was further centrifuged for one hour at 13000g.

6) Saturated ammonium sulphate (100%) in buffer (20mM Hepes (pH8.0), 5mM EDTA) was added to the supernatant fraction to give a final ammonium sulphate saturation of 40%. This mixture was incubated at 4°C with gentle agitation for 30min.

7) The protein precipitate was collected by centrifugation at 4°C.
for 10min. at 13000g.

8) The final protein pellet was suspended in 0.00025 volume of protein buffer (20mM Hepes(pH8.0), 5mM EDTA, 7mM BME, 1mM PMSF, 20% glycerol). The extracts were stored at -70°C. Protein concentration was determined by using protein assay reagent (Biorad Labs.).

4.2.15.4 Preparing the target DNA

Ty1 (carried on plasmid pNC188) and STE2 (carried on plasmid pGA1682) were used as targets for gel mobility shift assays.

Isolation of inserts - 5μg of pNC188 was digested in 30μl volume with Pst I and Xba I in the recommended buffer.

5μg of pGA1682 was digested in a 30μl volume with EcoR I and Xba I in the buffer provided.

The 100-bp fragments (in both cases) were resolved on 4% Nu Sieve GTG agarose (FMC) gel. The inserts were cut out from the gel and isolated using Geneclean II kit (Bio101 Inc.). The yield of the insert was determined by comparing the fluorescence of the insert with that of known amount of standards.

Labelling the target DNA - 100ng of Ty1 or STE2 DNA was labelled in a 50μl volume using Klenow polymerase to fill in the ends as described in Sambrook et al., 1989. A typical reaction contained - 100ng of DNA, 200μM of dNTPs excluding the one used as radiolabelled nucleotide, 10μCi of [α-32P] dATP or [α-32P] dCTP or both (3000Ci/mmole, BARC, India); 2 units of Klenow polymerase (Pharmacia) in 50μl volume containing Klenow buffer. The reaction was carried out at 30°C for 30 min. The samples were extracted with equal volume of phenol/chloroform and chloroform:IAA.

0.1 volume of 3M NaOAc pH5.2 was added and the DNA precipitated by adding 2 volumes of chilled ethanol at -70°C for 1hr. The DNA
precipitate was obtained by centrifugation at 13000g for 30 min. at 4°C. The pellet was rinsed in 70% ethanol and dried. The DNA was resuspended at 0.5ng/μl in TE pH8.0.

4.2.15.5 Protein - DNA binding reactions

Protein - DNA binding reactions were carried out in a 20μl volume containing 0.5ng of probe DNA and binding buffer (20mM Tris Cl pH8.0, 40mM NaCl, 4mM MgCl₂, 1mM DTT, 5% glycerol). Yeast extracts were added at 10μg for STE2 probe; 20μg for Ty1 probe. E. coli extract was added at 20μg for reactions containing Ty1 and STE2 probes. 2μg of salmon sperm DNA was included in STE2 assays only for the reactions containing yeast extracts. Each reaction was supplemented with 0.5μg of di-dC (Pharmacia). Binding reaction were incubated for 10-15min at room temperature. In competition assays, the specific competitor DNA (STE2 or Ty1) or non-specific competitor pUC19 DNA was added at 0-100 fold molar excess prior to addition of the protein extracts.

4.2.15.6 Resolving the protein - DNA complexes

The protein - DNA complexes were resolved on a 5% polyacrylamide gel(19:1 acrylamide:bis acrylamide) in 0.5X TBE (1X TBE is 89mM Tris, 89mM borate, 2.4 mM EDTA). The samples were run on a 1.5mm thick gel in Hoefer apparatus. Gels were run at 150V for 4-5hrs without pre-electrophoresis until the dye front just ran out.

4.2.15.7 Detection of Protein - DNA complexes by autoradiography

The gels were transferred onto a Whatman 3mm sheet and covered by a saran wrap on the top. The gels were dried under vacuum in a gel drier for 3 hours. The dried gel was exposed to Kodak X-OMAT AR film with an intensifying screen at -70°C.
4.3 RESULTS AND DISCUSSION

4.3.1 Bacterial expression of ACPR

A 0.7-kb fragment was amplified by PCR from SC5314 genomic DNA which corresponds to the first 230 amino acids of ACPR. At codon 230 an Xba I site was created to facilitate directional cloning into pMAL-c vector. The entire ACPR coding region and an additional 120-bp downstream of the stop codon was amplified as a 2.2-kb fragment (Fig.16). The pMAL-c vector was chosen to express these proteins due to the following reasons - 1) The protein is expressed as a fusion with maltose binding protein (MBP) which enables the proteins to be purified in a single step by amylose affinity column. 2) The vector has the lacZ gene which permits color selection of the recombinants, 3) The fusion protein after purification can be cleaved with factor Xa whose site is just 5' to the polylinker site. 4) The recombinants are produced under the control of tac promoter; and the lac repressor produced keeps the expression level low in the absence of induction.

Cultures of E. coli harboring ACPR(1-230) and ACPR(1-699) and the vector alone were induced for two hours to allow the expression of the fusion proteins. The fusion proteins could be seen in the induced lane which correlated with the size of product expected from the cloned fragment. With ACPR(1-699) however the level of expression was low. The synthesis of fusion protein was monitored over a period of time by withdrawing samples at 30min., 1hr, 2hr, 3hr and 4hrs of induction and resolving on SDS polyacrylamide gel (Fig.17). The expressed proteins were confirmed on an immunoblot probed with proteinase antibodies and MBP antibodies (Maina et
Fig. 16. Polymerase Chain Reaction of ACPR(1-230) and ACPR(1-699) - The nucleotide sequence corresponding to ACPR(1-230) and ACPR(1-699) were amplified from 10ng of C. albicans genomic DNA. The 0.7kb and 2.2kb fragments were resolved on 1% agarose gel with DNA molecular size standards (Hind III digest of lambda DNA and Hinf I digest of pUC19 DNA).
Fig. 17. Expression of MBP-ACPR(1-230) and MBP-ACPR(1-699) fusion proteins in E. coli. Cultures of DH5α expressing MBP (maltose binding protein) alone, (A); MBP-ACPR(1-230), (B); and MBP-ACPR(1-699), (C); were analysed on 10% SDS polyacrylamide gel for the expression of fusion proteins at the indicated time points (in minutes). The arrows indicate the position of the fusion proteins in the lanes marked A, B and C. L and H indicate low and high molecular weight standards. M, MBP; U, uninduced samples.
al., 1988) (Fig.18). MBP antibodies cross reacted with several
E. coli proteins at the suggested 1:10,000 dilution and was
therefore used at 1:100,000 dilution. A small fraction of the
fusion protein appeared to be cleaved at the site of fusion as has
been reported by the manufacturers. It is of importance to assess
the behaviour of the expressed protein before lysates can be used
for purification and functional analysis. For both the kinds of
fusion, 30% of the expressed protein was found in the soluble
cytosolic fraction; about 70% of the protein was however found in
the insoluble fraction. The proteins did not have any tendency to
be exported to the periplasmic space (Fig.19). Both the proteins
could bind the amylose resin. However, the MBP-ACPR(1-699) protein
showed several degradation products even when purified on amylose
resin. It could be the result of false termination which is
normally encountered when large sized proteins are expressed as a
fusion or the result of degradation in the host. MBP-ACPR(1-230)
was purified by passing the clarified lysate through amylose resin
column (Fig.20). The bound protein was eluted with 10mM maltose.
From 100ml cultures, the yield was 300μg for MBP-ACPR(1-230).
Factor Xa is a specific protease which recognises the amino acid
sequence Ile-Glu-Gly-Arg-Pro and cleaves specifically between Arg-
Pro (Nagia et al., 1984). When MBP-ACPR(1-230) was cleaved with
Factor Xa, several other peptide fragments were seen which were
products of cleavage within the ACPR part of the fusion. Due to the
low yield of the fusion proteins, particularly the ACPR(1-699),
attempts were made to clone the proteins in native state in an
expression vector under the control of the strong T7 RNA polymerase
promoter. pET series of vectors (Studier et al., 1990) was chosen
Fig. 18. Immunodetection of MBP fusion proteins using affinity purified proteinase antibodies and MBP antibodies. *E. coli* extracts of induced cultures expressing MBP (maltose binding protein) alone, (A); MBP-ACPR(1-230), (B); and MBP-ACPR(1-699), (C); were resolved on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and probed with affinity purified proteinase antibodies (panel A) or MBP antibodies (panel B). L and H represent low and high molecular weight markers. E, *E. coli* extract of LE392; M, MBP (maltose binding protein).
Fig. 19. Analysis of the nature of the recombinant proteins. 
E. coli crude extracts of the induced MBP-ACPR(1-230) and MBP-ACPR(1-699) fusion protein samples were analysed on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane. Lanes 1-7 of MBP-ACPR(1-230) were probed using MBP antibodies at 1:3000 dilution and lanes 9-15 of MBP-ACPR(1-699) were probed with affinity purified proteinase antibodies at 1:200 dilution. Lane 1, MBP; 2 and 9, protein purified on amylose column; 3 and 10, periplasmic fraction; 4 and 11, insoluble crude extract; 5 and 12, soluble fraction of crude extract; 6 and 13, total cell extract after induction; 7 and 14, uninduced cells; 8, low molecular standards; 15, high molecular standards.
Fig. 20. Purification of MBP-ACPR(1-230) fusion protein by affinity chromatography on amylose resin column. MBP-ACPR(1-230) fusion protein was purified from E. coli lysate and samples of the various steps of purification resolved on 10% SDS polyacrylamide gel and stained with 0.25% Coomassie Blue and destained. Lane 2, uninduced cells; lane 3, induced cells directly boiled in sample buffer; lane 4, lysate of induced cells; lane 5, lysate diluted 5 times to load on amylose column; lane 6, flow through; lane 7 & 8, fusion protein eluted with 10mM maltose and concentrated by centricon 30; lane 9 & 10, trailing fractions concentrated by centricon 30; lane 11, MBP; lane 1 & 12, high and low molecular weight markers respectively.
for the following reasons 1) It allows expression of the cloned fragments in any of the three reading frames. 2) The rate of elongation by T7 polymerase being faster than the host RNA polymerase, most of the products that accumulate are that of the cloned genes only. 3) The vector has a unique cloning site, in addition to translation initiation signals, and a transcription terminator. 4) The host used is lon protease deficient and lacks ompT gene that can degrade proteins during purification. 5) The proteins are expressed in a native state. The truncated and the whole gene were cloned into pET3c such that translation would initiate from their own ATG codon. The gene products are expressed from an inducible promoter. The level of synthesis was monitored by analysing recombinant samples after 30 min, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, and 24hrs of induction on SDS polyacrylamide gel (Fig.21). The level of protein does not increase significantly after 2hrs of induction, but the background contributed by the other proteins increased presumably due to the loss of plasmids in culture during prolonged induction. The proteins were confirmed by immunodetection on a western blot using affinity purified proteinase antibodies (Fig.22). Two hour induced samples were chosen for purification purposes. E. coli lysates were used to determine the behaviour of the overexpressed proteins. Both the proteins were compartmentalised into inclusion bodies. Lowering the temperature of growth and induction at 30°C did not solubilise the proteins. Extraction of the proteins from inclusion bodies using urea solubilised the protein which reprecipitated on slowly dialysing out the urea.
Fig. 21. Expression of ACPR(1-230) and ACPR(1-699) in *E. coli*. Cultures of BL21(DE3) harbouring ACPR(1-699) (top panel) and ACPR(1-230) (bottom panel) were analysed on 10% and 15% SDS polyacrylamide gel respectively for the expression of the proteins after induction for lane 8, 30min; lane 7, 1h, lane 6, 1.5hrs; lane 5, 2 hrs; lane 4, 3 hrs; lane 3, 4 hrs; lane 2, 5hrs; lane 1, 6hrs. Lane 9 represents uninduced sample. Lane 11 represents uninduced *E. coli* carrying only the vector; lane 12, after induction; lane 10, low molecular weight standards. The arrows on the left indicate the position of the overexpressed protein.
Fig. 22. Immunodetection of ACPR(1-230) and ACPR(1-699) expressed in *E. coli*. BL21(DE3) expressing ACPR(1-699) (top panel) and ACPR(1-230) (bottom panel) were probed with affinity purified proteinase antibodies at 1:200 dilution. **Top panel:** lane 9, uninduced cells; lane 8, induced for 30min; lane 7, 1hr; lane 6, 1.5hrs; lane 5, 2hrs; lane 4, 3hrs; lane 3, 4hrs; lane 2, 5hrs; lane 1, 6hrs. Lane 12, uninduced BL21(DE3) carrying vector only; lane 13, after induction; 10 and 11 are high and low molecular weight markers. **Bottom panel:** lane 10, uninduced cells, lane 9, induced for 30min; lane 8, 1hr; lane 7, 1.5hrs; lane 6, 2hrs; lane 5, 3hrs; lane 4, 4hrs; lane 3, 5hrs; lane 2, 6hrs; lane 1, 24 hrs; lane 13, uninduced BL21(DE3) carrying only vector; lane 14, after induction; 11 and 12 are high and low molecular markers.
4.3.2 Functional domains of ACPR

The presence of active site(s) of acid proteinase and the homology to DNA binding protein (STE12) prompted the verifications of these functions in the expressed proteins.

4.3.2.1 Peptide mapping

Since ACPR is antigenically related to secretory acid proteinase (SAP) from C. albicans, it was of interest to map the common epitopes. The protein band corresponding to the NH$_2$-terminal 1-230 amino acids, the entire coding region from 1-699 amino acids and SAP were excised from a 10% SDS page. The proteins were subject to proteolysis with trypsin and chymotrypsin within the stack of a second gel. Trypsin and chymotrypsin were used since they retain activity in the presence of SDS. The samples were subject to proteolysis with 50 $\mu$g/ml and 100$\mu$g/ml of the proteases. At 50$\mu$g/ml the digests were complete as visualised by the banding pattern on the Western blot which did not yield any more bands on using greater concentration of enzyme (Fig.23). ACPR and SAP have atleast five tryptic peptides which are antigenically related. No antigenically related peptides were seen in ACPR(1-230). The results suggest that the antigenically related epitopes lie outside the NH$_2$-terminal 1-230 amino acids (Fig.24). Due to its broad substrate specificity, chymotrypsin produces small peptides which would have lost their the epitopes and therefore could not be detected by antibodies. It is not surprising to find immunorelated peptides between ACPR and SAP since ACPR has active site(s) of acid proteinase. Attempts were made to check for the presence of enzyme activity when expressed in E. coli. MBP-ACPR(1-699) did not show any detectable activity on milk agarose plate assay. Experiments
Fig. 23. Cleveland digest peptides of ACPR and Secretory Acid Proteinase (SAP) immunodetected with affinity purified proteinase antibodies. Panel A: ACPR (lanes 3 and 4) and SAP (lanes 5 and 6) were digested with 50µg/ml (lanes 3 and 5) and 100µg/ml (lanes 4 and 6) of trypsin. Lane 2, trypsin without substrate; lane 1, low molecular weight marker. Panel B: ACPR (lanes 3 and 4) and SAP (lanes 1 and 2) were digested with 50µg/ml (lanes 1 and 3) and 100µg/ml (lanes 2 and 4) of chymotrypsin. Lane 5, chymotrypsin without substrate; lane 6, low molecular weight standards. Immunorelated peptides were detected by affinity purified proteinase antibodies at 1:200 dilution.
Fig. 24. Identification of immunorelated peptides in ACPR and Secretory Acid Proteinase by peptide mapping - Gel slices of ACPR(1-230) were filled into sample wells of lanes 2 and 5; of ACPR(1-699) into lanes 3 and 6; of SAP into lanes 4 and 7. Lanes 1 and 8 did not receive any substrate. 100μg/ml trypsin (lanes 1 through 4) and 100μg/ml chymotrypsin (lanes 5 through 8) were overlaid on the gel slices. Proteolysis was allowed to occur for 1 hour within the stack and the peptides resolved on 15% resolving gel. The peptides were electroblotted to a nitrocellulose membrane and immunodetected with affinity purified proteinase antibodies at 1:200 dilution.
could not be done with the protein expressed in native state since the protein was insoluble. It is possible that due to improper folding of the protein in E. coli the protein is not functional. It may be possible to express the functional protein in yeast, but the host proteinases can interfere with the assay. Recently an adenovirus proteinase was shown to require DNA and a peptide as cofactor for its activity (Mangel et al., 1993). Whether ACPR requires any cofactors for activity can be determined once the E. coli expressed protein is solubilised and purified.

4.3.2.2 Gel mobility shift assays

To investigate the DNA binding activities of ACPR, gel mobility shift assays (GMSA) were done. E. coli extracts expressing the MBP-ACPR(1-230) were used to bind the transcriptional control elements of Tyl and the α-pheromone receptor gene STE2 (Errede and Ammerer, 1989). Yeast extracts of a STE+ strain overproducing STE12 from a cloned copy on a plasmid (pSY2) and a ste12 mutant, wherein the STE12 gene is interrupted by a LEU2 gene, were used as controls to test binding to the same targets. Yeast extracts were partially purified by a 40% Ammonium sulphate precipitation which enriches the DNA binding proteins. Competition experiments were performed using unlabelled probe to compete out the specific protein-DNA complex and pUC19 as a nonspecific competitor. The ability of the unlabelled probe at increased molar excess to titrate out the complex indicated the specificity of the complex (Fig.25,26). The STE2 probe when bound to the extracts from a ste12 mutant strain showed a STE12 independent complex. Previous reports have suggested the occupancy of a neighbouring site on STE2 by another regulatory protein, probably MCM1 (Errede and Ammerer, 1989). The results
Fig. 25. **Competition analysis for Tyl binding.** Probe DNA in each reaction was 0.5ng of end labelled Tyl upstream fragment. Protein-DNA binding reactions were done in a 20μl volume containing 0.5ng Tyl probe and 20μg of yeast extract or 20μg of E. coli extract in binding buffer (20mM Tris Cl pH8.0, 40mM NaCl, 4mM MgCl₂, 1mM DTT, 5% glycerol). Tyl or pUC19 DNA were added as competitors at the indicated (0-100 fold) molar excess. Competitor DNA was added prior to addition of the extract from STE12 (STE⁺ strain over producing STE12 on a multicopy plasmid), ste12 (mutant strain wherein STE12 gene is interrupted by LEU2), DH5α expressing ACPR(1-230) as a fusion with MBP, or V (DH5α containing only the pMAL-c plasmid). P represents a reaction receiving no extract. Arrows indicate the complex formed and F indicates the free probe.
Fig. 26. Competition analysis for STE2 binding. Probe DNA in each reaction was 0.5ng of end labelled fragment of STE2 upstream region. Protein-DNA binding reactions were done in a 20μl volume containing 0.5ng STE2 probe and 10μg of yeast extract or 20μg of E. coli extract in binding buffer (20mM Tris Cl pH8.0, 40mM NaCl, 4mM MgCl₂, 1mM DTT, 5% glycerol). Reactions containing yeast extracts were supplemented with 2μg Salmon sperm DNA. STE2 or pUC19 DNA were added as competitors at the indicated (0-100 fold) molar excess. Competitor DNA was added prior to addition of the extract from STE12 (STE⁺ strain over producing STE12 on a multicopy plasmid), ste12 (mutant strain wherein STE12 gene is interrupted by LEU2), DH5α expressing ACPR(1-230) as a fusion with MBP, or V (DH5α containing only the pMAL-c plasmid). P represents a reaction receiving no extract. Arrows indicate the complex formed and F indicates the free probe.
indicate that the truncated ACPR(1-230) can bind PRE which are a part of the Ty1 and STE2 probes. The region of STE12 that binds DNA has been narrowed down to the NH$_2$-terminal 215 amino acids of which 40-165 are reported to be essential (Yuan et al., 1991). This region of STE12 shows >80% homology with ACPR. The region of STE12 needed for transcriptional activation extends up to 473 amino acid residues. In response to pheromone the region between 215-473 has been shown to be phosphorylated which correlates with the increased transcription of the pheromone responsive genes (Song et al., 1991). STE12 binds in a co-operative manner to at least two copies of PRE largely independent of the orientation or spacing of these elements. Single copy of PRE provides activation but to a lesser extent (Sengupta and Cochran, 1990).