Expression of mutated pea vicilins by yeast

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For My Dad.

EXPRESSION OF MUTATED PEA VICILINS BY YEAST

By

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A thesis submitted in accordance with the requirements for the degree of Master of Science in the University of Durham.

Department of Biological Sciences June 1989.

ABSTRACT

Site directed mutagenesis has been used to introduce two cysteine residues into two hydrophilic regions of the pea vicilin polypeptide previously expressed in *Saccharomyces cerevisiae*. The mutated polypeptide has been expressed. The resultant protein has been characterised by SDS-Polyacrylamide gel electrophoresis (PAGE) and the presence of both inter- and intra-polypeptide sulphydryl bonds is indicated by bands of different mobility from those of the native protein. The association of mutated vicilin polypeptides into disulphide-bonded aggregates under non-denaturing conditions is not a random process, trimers being the major species produced.

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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr John Gatehouse for advice, Mr John Gilroy for making oligonucleotides, Dr Jenny Yarwood for estimating total sulphur groups in pPE17 protein and Miss Fiona Cunningham for proof-reading this manuscript.

This project was funded by an AFRC Link Award.

MEMORANDUM

Parts of the work described in this thesis are also presented in the following publication:

Introduction of Sulphydryl Groups into Pea Vicilin: Formation of Inter- and Intra-Polypeptide Disulphide Bonds.

Peter ELVIN, Jennifer N.YARWOOD and John A.GATEHOUSE.

Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, UK.

FEBS Letters (1989).

ABBREVIATIONS

Abbreviations were those used according to the Biochemical Society Instructions to Authors, Biochem. J. (1982) 209, 1-27, with additions as given below.

The one letter notation for amino acids is given in Biochem. J. (1969) 114, 1-4.

Kb	=	Kilobase pairs
bp	=	base pairs
EtBr	=	Ethidium Bromide
CNBr	=	Cyanogen Bromide
cDNA	=	complementary DNA
A.A.	=	amino acid
SDS	=	Sodium Dodecyl Sulphate
IEF	=	isoelectric focussing
SDS-PAGE	=	Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis
X-gal	=	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
IPTG	=	Isopropyl-ß-D-thiogalactopyranoside
µg	=	micrograms
µl	=	microlitres
Ci	=	Curies
µCi	=	micro Curies

CHAPTER ONE
INTRODUCTION

1.1 Seed Storage Proteins.

The major components of total seed protein are seed storage proteins. These proteins accumulate in a tissue-specific manner in endosperm or cotyledon tissue during seed development and are localised in storage organelles called protein bodies. This packaging protects the proteins from degradation during seed development (Weber and Neumann, 1980).

Generally, these proteins supply the plant with the source of reduced nitrogen needed during germination and early seedling growth and can constitute up to 70% of total seed protein (Croy and Gatehouse, 1985).

In accordance with their function of providing the germinating seed with a source of reduced nitrogen, the legume storage proteins in particular are often very rich in the amino acids containing more nitrogen atoms: asparagine, glutamine, arginine and proline. Consequently, they have a low abundance of amino acids such as cysteine, methionine and tryptophan (Higgins, 1984).

Most of the protein component of human food is derived directly or indirectly from seed storage proteins. In developing countries seeds (beans, rice and grains) provide the major protein

component of the diet and in industrialised countries animal diets are enriched with cereal or legume seeds and seed protein concentrates. The nutritional value of legume seeds can be improved by supplementing animal feeds with the amino acids that are in low abundance - such as tryptophan. Alternatively, recombinant DNA technology could be used to improve the amino acid composition of the storage proteins themselves by changing the sequences of the storage protein genes, or for investigating effects upon seed viability or seed protein degradability during germination.
Generally, the major seed storage proteins of legumes are of the globulin class - that is they are soluble in dilute salt solutions at neutral pH, eg. phosphate-buffered saline (0.15M NaCl) at pH 7.5 (Croy and Gatehouse, 1985).

These storage globulins are usually present in the seed as oligomers. After denaturation and separation by SDS-PAGE, the globulin components are shown to be a heterogeneous mixture of constituent polypeptides in terms of amino acid composition, size and charge. An example is pea vicilin which has been shown to have up to 30 separable polypeptides under two-dimensional IEF/SDS-PAGE (Matta *et al*, 1981).

1.2 Pea Vicilin.

Pea (*Pisum sativum* L.) seed storage proteins consist of two main immunologically distinct classes: the 11S legumin and the 7S vicilin globulins. Together these contribute up to 70% of total seed protein in pea (Croy and Gatehouse, 1985). The 7S vicilin

fraction is not homogeneous but contains (in addition to vicilin) a third storage protein, convicilin. Convicilin is immunologically related to vicilin and can be purified from the 7S fraction using non-dissociating techniques (Croy *et al*, 1980).

Mature vicilin obtained from pea seeds has an Mr of 145,000 - 170,000 resulting from the trimerisation of three 50,000 Mr vicilin polypeptide subunits (Gatehouse *et al*, 1981). Following trimerisation, some of the 50,000 Mr vicilin subunits are susceptible to post-translational cleavage or "nicking" by plant proteases but the overall structural integrity of the trimer is maintained (Gatehouse *et al*, 1981). Studies on vicilin cDNAs have confirmed the presence of two potential cleavage sites in some vicilin 50,000 Mr precursors. Two sites, α:β and β:γ, have been identified (Gatehouse *et al*, 1982; Gatehouse *et al*, 1983 and Spencer *et al*, 1984). A hydrophilicity profile produced from amino acid sequence derived from vicilin cDNA implies that the α:β and β:τ sites lie on polar, surface regions of the molecule. (Croy and Gatehouse, 1985). This why the structure of the trimer can be maintained after proteolysis.

1.3 Seed Proteins as Food Additives.

In addition to their nutritional role, seed proteins are playing an increasingly important role as food additives. They are used to control physical properties of food products during food processing, for stabiising the functional properties of food

products and as substitutes for more expensive animal proteins such as casein.

The physical properties of seed storage proteins are responsible for the physical properties of many food products. The properties required are those responsible for emulsification, foam formation, foam stability and food texture.

Many functional properties of these proteins rely on the native configuration of the protein whilst others require protein denaturation followed by rearrangement of the polypeptide chains and formation of new intra- and inter-molecular bonds. All of these properties are ultimately dependent on the primary structure of the protein encoded by the genes.

Using genetic engineering, it should be possible to manipulate the structural properties of seed proteins to suit particular processing applications. This research investigates the role of mutagenesis to improve the structural properties of food proteins rather than nutritional quality.

1.4 The Role of Genetic Engineering.

As mentioned already, vicilin is one of the major storage proteins found in pea seeds. Its structure and properties are now well documented (Gatehouse *et al*, 1984 and Wright, 1988). The three 50,000 Mr subunits of each vicilin molecule, though highly homogeneous, are in fact, variable. At least 11 genes code for

the different pea vicilin subunits (Gatehouse *et al*, 1983 and Domoney and Casey, 1985).

The post-translational glycosylation and proteolysis of vicilin subunits *in vivo* add to the intrinsic heterogeneity of the protein. This heterogeneity has severely impaired *in vitro* studies attempting to relate structure to functionality. It is, however, a major objective of the food industry to exploit the functional properties of legume storage proteins such as gelation, emulsification and foaming.

The use of genetic engineering and the ability to express specific foreign genes in microorganisms have been suggested as means of producing sufficient homogeneous material to carry out the required functionality studies (Watson *et al*, 1988). By using this approach it should be possible to modify specifically vicilin primary structure by mutagenesis *in vitro*. Such alterations could be made in a predictable manner on the basis of vicilin structural data and thus allow the effect of specific changes in the primary structure on the physicochemical and functional properties to be assesed.

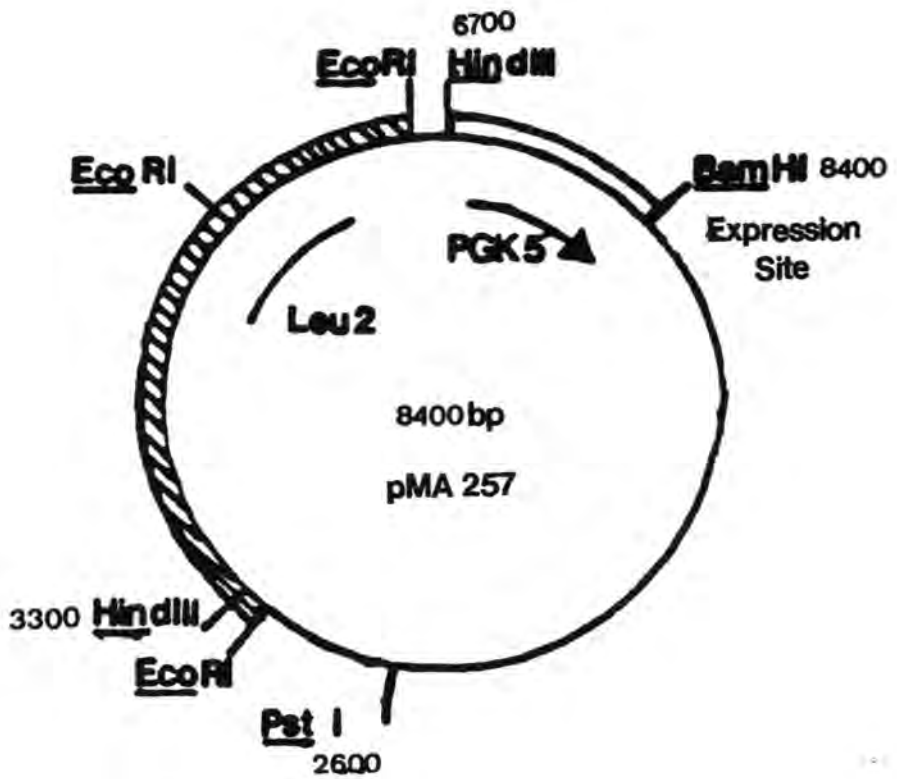
1.5 Use of Yeast as a Host Organism.

Yeast has a number of advantages over other organisms for expression of seed storage proteins. It is a eukaryote and is able to carry out post-translational processing reactions normally asociated with plant systems. It is already widely used in the food industry - notably for brewing and breadmaking; it is non-toxic and is "Generally Regarded As Safe". As a host organism, yeast has a rapid growth cycle and can be continuous or batch cultured in a fermenter. Also, it is relatively easy to purify the proteins produced.

1.6 The Yeast Expression System.

pMA257 (shown below) is an expression vector derived from the 2µm high copy number, autonomously replicating *S.cerevisiae* plasmid (Tuite *et al*, 1982). Transcription and translation from this yields a number of proteins. By the incorporation of storage seed protein cDNAs into these plasmids, it is possible to produce strains of yeast in which up to 5% of the total yeast protein is that of the encoded seed storage protein.

RESTRICTION MAP OF pMA257:



1.7 EXPRESSION OF PEA VICILIN BY YEAST.

The construct pDUB2018 contains the complete coding sequence for a 50,000 Mr vicilin polypeptide cloned into the expression site of pMA257[1]. This construct has been described previously (Watson *et al*, 1988) and when transformed into *S.cerevisiae* (MC16) expresses the vicilin cDNA. The encoded polypeptide is localized in the yeast cytosol and migrates as a single species in SDS-

[1]A restriction map of pDUB2018 is shown in figure 6, chapter three.

PAGE with an Mr of approximately 53,000. This is significantly higher than the 50,000 Mr subunit of native pea vicilin but is most probably due to the additional 22 amino acid residues of PGK (approximately 3,000 Mr) at the N-terminal end encoded by the fusion construct.

A 1 litre culture of pDUB2018 (MC16) grown in selective (minimal) medium yields around 200mg of soluble protein around 5% of which is vicilin. This homogeneous, non-glycosylated, non-proteolytically processed vicilin does not aggregate into trimers but has provided the basis for a certain amount of physicochemical characterisation and was the starting point for this piece of research.

Amino acid analysis has confirmed that vicilin contains no sulphur-containing amino acids such as cysteine or methionine (Gatehouse *et al*, 1984). This investigation sets out firstly to demonstrate that introduction of a methionine residue into vicilin will produce a polypeptide cleavable by cyanogen bromide. After testing the *in vitro* mutagenesis system in this way the system was used to introduce changes into the vicilin molecule planned on the basis of structural information about the native vicilin molecule.

Cysteine residues were to be introduced at the N-terminal of vicilin and 3 amino acids N-terminal of the potential α:ß cleavage site. The complete sequence of the vicilin cDNA (showing

the sites of oligonucleotide hybidisation) is shown in appendix I.

The regions into which cysteine residues were incorporated are both in hydrophilic areas of the vicilin polypeptide and are thus likely to be on the surface of the molecule. In addition, the proximity of the second cysteine residue to the potential proteolysis site suggests that this residue is exposed.

It was predicted that the exposed sulphydryl groups introduced into the vicilin 50,000 Mr subunit might make possible S-S bonding within the vicilin molecule and give rise to polypeptides of increased mobility (as detected by SDS-PAGE). Alternatively S-S bonding between adjacent vicilin molecules might aid aggregation of vicilin polypeptides and make possible trimerisation into molecules more like mature pea vicilin.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Chemicals.

Ampicillin, adenosine triphosphate (ATP), bovine serum albumin (BSA), dithiothreitol (DTT), ethidium bromide (EtBr), herring sperm DNA, isopropylthio-ß-D-galactoside (IPTG), 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (Xgal), lysozyme, polyvinyl pyrrolidone (PVP), rubidium chloride, MOPS, RNAase A, tricine, leupeptin, adenine, histidine, lysine, thiamine HCl, phenyl methyl sulphonyl fluoride (PMSF) and 3,3-diaminobenzidine (DAB) were from Sigma Chemical Company, Poole, Dorset, U.K.

Peroxidase-linked goat anti-(rabbit IgG) secondary antibody was from Bio-Rad Laboratories, Watford, Hertfordshire, U.K.

Affinity-purified rabbit anti-(pea vicilin) antibodies were purified as described previously (Croy *et al*, 1980).

HA-Ultrogel (hydroxyapatite) was from Life Science Laboratories, Luton, Bedfordshire, U.K.

10/10 high resolution fast protein liquid chromatography (f.p.l.c.) column and Sephadex G-50 were from Pharmacia LKB, Uppsala, Sweden.

Nitrocellulose filters (BA 85, 0.45µm) were from Schleicher and Schuell, Anderman and Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

Bacto-agar, Bacto-tryptone, yeast nitrogen base and yeast extract were from Difco Laboratories, Detroit, Michigan, U.S.A,

Whatman 3MM paper was from Whatman Ltd., Maidstone, Kent, U.K.

Restriction endonucleases and DNA-modifying enzymes were from NBL, Cramlington, Northumberland, U.K. and Boehringer Mannheim, Penzberg, FRG.

M13 DNA sequencing kit was from Boehringer Mannheim, nick translation kit, *in vitro* mutagenesis kit, Hybond-N nylon membrane and radionucleotides were from Amersham International PLC, Amersham, Buckinghamshire, U.K.

All other reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of analytical grade or the best available. Unless otherwise indicated, solutions were sterilised by autoclaving.

2.2 Bacterial and Yeast Strains.

Eschericia coli 910 (*rec*B$^-$ *rec*C$^-$) was provided by W.J.Brammar. The *Escherichia coli* strain used was TG1 (K12, (lac-pro),SupE,*thi*,*hsd*D5/F',*tra*D36,proA$^+$B$^-$,lacI^q,lacZ, M15)(Amersham, England). The yeast strain used was *Saccharomyces cerevisiae* MC16 (ade2-1,leu2-3,lys2-1,his4-712^{FS},SUF2), (Beggs, 1978).

2.3 Plasmids.

The *E. Coli* plasmids pUC18 and pUC19 and the bacteriophage M13, mp18 and mp19 were obtained from Boehringer Mannheim. The yeast expression vector pMA257 was supplied by Dr. A J Kingsman and Dr. S M Kingsman (Tuite *et al*, 1982). The expressed vicilin cDNA clone pDUB2018 was supplied by Dr. M D Watson (Watson *et al*, 1988).

2.4 Growth Media.

M9 salts (1x)

Na_2HPO_4	6g/litre
KH_2PO_4	3g
NH_4Cl	1g
NaCl	0.5g

Glucose/minimal medium

M9 salts with 15g agar	1litre
1M $MgSO_4$	1ml
1M thiamine HCl	1ml
0.1M $CaCl_2$	1ml
20% glucose	10ml

	YT Broth	2xYT Broth
Bacto tryptone	8g/litre	16g/litre
Yeast Extract	5g	10g
NaCl	5g	5g

YT Agar and 2xYT Agar: 15g/litre bacto agar were added to YT or 2xYT broth respectively to give sufficient medium for 40 plates.

H-top Agar

Bacto tryptone	10g/litre
NaCl	8g
Bacto agar	8g

YEM Broth

Yeast extract	10g/litre
Bacto peptone	20g
D-glucose	20g

Supplemented with 5µg/ml adenine (filter sterilized)

YMM Broth

Yeast nitrogen base	6.7g/litre
D-glucose	20g

YEM Agar and YMM Agar

15g/litre bacto agar were added to YEM or YMM broth respectively to give sufficient medium for 30 plates.

YMM Media were supplemented with 50µg/ml each of adenine, histidine and lysine (filter sterilized).

2.5 Buffers and Solutions.

TE buffer

10mM Tris-HCl
1mM EDTA
pH adjusted to 8.0 with HCl

10x kinase buffer

1M Tris pH8.0
100mM $MgCl_2$
Filter sterilized DTT added to 70mM and ATP to 10mM.*

*ATP omitted when used for labelling DNA with [Y-^{32}P]ATP.

25xTAE gel buffer (pH8.0)

Tris	242g/litre
Glacial acetic	acid 57.1ml
Na_2EDTA	37.2g

EtBr solution

5mg/ml stock in H_2O
used 1/5000 in agarose gels and running buffers.

10x Gel loading dye

Glycerol	2.8ml
25xTAE	0.2ml
1% SDS	1.0ml
0.5M EDTA	1.0ml
Bromophenol blue	1mg

Kenacid blue stain

0.05% kenacid blue in 50% methanol, 7% acetic acid.

Destain: 50% methanol, 7% acetic acid.

Transformation Buffer 1

RbCl	12g/litre
$MnCl_2 4H_2O$	9.9g
Potassium acetate	30ml of 1M stock, pH7.5
Glycerol	150g

Adjusted to pH5.8 with acetic acid.

Transformation Buffer 2

MOPS 20ml of 0.5M pH6.8 stock per litre.

RbCl	1.2g
$CaCl_2 2H_2O$	11.0g
Glycerol	150g

MOPS adjusted to pH6.8 with NaOH.

Transformation buffers 1 and 2 were sterilized by filtration through pre-rinsed sterile 0.22µm membrane filters.

PEG/NaCl solution

Polyethylene glycol 6000	20g/100ml
NaCl	14.6g

100xDenhardt's buffer

Ficoll	2g/100ml
PVP	2g
BSA	2g

(Filter sterilized)

30xSSC

263g NaCl and 132.3g trisodium citrate were dissolved in 800ml of distilled water and the pH was adjusted to 7.0 with conc NaOH. The volume was then adjusted to 1 litre with distilled water.

12.5% resolving acrylamide gel solution

25.8ml of 30% acrylamide/0.135% bisacrylamide, 22.5ml of tris (1M pH8.0) and 13.8ml of distilled water were mixed and degassed. 0.6ml of 10% SDS, 1.5ml of fresh ammonium persulphate (15mg/ml) and 20µl of tetramethylenediamine (TEMED) were then added and the gel was poured immediately.

Stacking acrylamide gel solution

3.0ml of 30% acrylamide/0.135% bisacrylamide and 2.5ml of tris (1M pH6.8) were mixed and degassed. 0.2ml of 10% SDS, 0.5ml of ammonium persulphate and 20µl of TEMED were then added prior to pouring.

Western Blotting Solutions:

Anode buffer 1	Anode buffer 2	Cathode buffer
0.3M tris pH10.4	25mM tris pH10.4	25mM tris pH9.0
20% methanol	20% methanol	20% methanol
0.1% SDS	0.1% SDS	0.1% SDS
		40mM 6,amino-n-hexanoic acid.

Blocking solution

5% non-fat milk powder (Marvel™, Cadbury Schweppes Ltd, Bournville, U.K.), 20mM tris pH7.2, 10.9% NaCl.

DNA Plasmid Preparation Solutions:

Solution 1		Solution 2		Solution 3
lysozyme	2mg/ml	NaOH	0.2M	Sodium acetate 0.1M
glucose	50mM	SDS	1%	tris/HClpH6.0 0.05M
EDTA	10mM			
Tris/HClpH8.0	25mM			

2.6 General Methods.

The following methods have been well documented by Maniatis *et al*, (1982) and were used without modification.

Phenol extraction and ethanol precipitation of DNA.

Use of DNA modifying enzymes.

Agarose gel electrophoresis of DNA.

2.7 Isolation of DNA Fragments from Agarose Gels.

The method used was that of electroelution (Yang *et al*, 1979).

The band of DNA was excised from the gel and placed in a narrow piece of dialysis tubing sealed at one end with a dialysis clip. The tube was topped up with 1xTAE buffer and sealed at the other end with a second dialysis clip so as to be of minimal volume and so as not to contain any air bubbles. The tube was submerged in 1xTAE buffer in a submarine electrophoresis tank perpendicular to the electric field and a current of 80mA was applied for 30

minutes. The buffer was then pipetted out of the tubing and extracted once with phenol, once with phenol/chloroform and once with chloroform. DNA was recovered from this buffer by ethanol precipitation and resuspended in TE buffer.

2.8 Preparation of *E.coli* Cells Competent for Transformation with Plasmid and Replicative Form M13 DNA.

The method for preparation of competent *E. coli* cells was based on the protocols of Hanahan (Hanahan, 1985).

A single *E. coli* TG1 or 910 colony was picked from a glucose/minimal medium plate, and used to inoculate 30ml of YT broth in a sterile 50ml polypropylene centrifuge tube. This culture was incubated overnight at 37°C, with moderate agitation. 8ml of this overnight culture was added to a 2 litre flask containing 200ml of YT broth and this was incubated at 37°C with moderate agitation to $O.D._{550}$ of 0.3. The culture was then collected in 50ml polypropylene centrifuge tubes and chilled on ice for 15 minutes. The cells were pelleted by centrifugation at 3000xg for 5 minutes at 4°C. The supernatant was removed and the pellet resuspended in 16ml of transformation buffer 1, incubated on ice for 15 minutes and the cells were pelleted as before. This cell pellet was then resuspended in 6ml of transformation buffer 2 and aliquoted as 300µl samples into 1.5ml microcentrifuge tubes which were flash freezed in liquid nitrogen and stored at -70°C until required.

2.9 Transformation of *E. coli* TG1 with M13 Replicative Form DNA.

A 300µl aliquot of frozen TG1 competent cells was thawed on ice. 1ng of M13 replicative form (RF) DNA was added in a volume of 20µl and the sample was gently mixed by pipetting. The sample was incubated on ice for 40 minutes and then heat-shocked at 42°C for 45 seconds before being returned to ice for 5minutes.

Whilst the cells were on ice the following mixture was prepared:

IPTG 100mM	40µl
X-gal 2% in dimethylformamide	40µl
Log phase *E. coli* cells ($O.D._{560}$ 0.4)	200µl

This was added to 3ml of molten (37°C) H-top agar along with the aliquot of transformed TG1 cells and poured immediately onto a prewarmed (37°C) 2xYT plate. The plate was left to set at ambient temperature and then incubated inverted at 37°C overnight.

2.10 Preparation of Single-Stranded M13 DNA.

5ml of 2xYT broth were inoculated with 50µl of a fresh overnight culture of TG1 cells. A toothpick was then used to transfer phage from a single M13 plaque into this broth. This culture was incubated at 37°C with shaking for 6 hours and poured into five separate 1.5ml microcentrifuge tubes. These tubes were centrifuged for 5 minutes at 13,000 rpm. Supernatants were transferred into five fresh microcentrifuge tubes. One of these tubes was then stored at 4°C as a phage stock. 200µl of PEG/NaCl solution was added to each of the other four tubes and these were placed at

4°C for 60 minutes. These tubes were centrifuged as before and the supernatants discarded. All visible traces of PEG/NaCl solution were removed from the pellets with a micro-pipette and the pellets were resuspended in 100µl of TE buffer. These samples were extracted once with phenol, twice with phenol/chloroform and twice with chloroform before being precipitated with ethanol. All four final DNA pellets were resuspended together in a total volume of 100µl of TE buffer. The concentration of this DNA solution was estimated by $O.D._{260nm}$ (1 O.D. of single stranded DNA being equivalent to 40µg/ml). The solution was then ethanol precipitated once more and resuspended in TE buffer to give a final DNA concentration of 1µg/µl.

2.11 Oligonucleotide-Directed *In Vitro* Mutagenesis.

The Amersham system, which is based on the method of Fritz Eckstein and his co-workers was used for this procedure (Taylor, *et al*, 1985a; Taylor, *et al*, 1985b; Nakamaye and Eckstein, 1986 and Sayers *et al*, 1988). All solutions and the disposable filters were as described in the Amersham handbook (RPN.1523). Appendix II details the result of a successful mutagenesis reaction as determined by analysis of the samples 1 - 4 taken at various stages of the reaction as described below.

An oligonucleotide was first annealed to the single-stranded DNA template. 5µl of a 1µg/µl solution of single-stranded DNA template, 2.5µl of a 1.6pmol/µl solution of phosphorylated mutant oligonucleotide, 3.5µl of buffer 1 and 6µl of water were placed

in a 1.5ml capped microcentrifuge tube in a 70°C water bath for 3 minutes. This tube was then transferred to a 37°C water bath for 30 minutes and was then placed on ice. Next the mutant DNA strand was synthesised and ligated. To the above annealing reaction were added: 5µl of $MgCl_2$ solution, 19µl of nucleotide mix 1, 6µl water, 6 units of Klenow fragment and 6 units of T4 DNA ligase. This was then mixed by pipetting and incubated at 16°C for 16 hours. A 1µl sample was removed and stored at -20°C for subsequent analysis on an agarose gel: Sample 1.

Following this incubation single-stranded (non-mutant) DNA was removed using a disposable centifugal nitrocellulose filter unit (supplied with the kit) to which the single-stranded DNA binds at high salt concentrations. 170µl of water and 30µl of 5M NaCl were added to the reaction mix. This was then centrifuged through the filter unit at 1500 rpm for 5 minutes at room temperature. The filter unit was then washed through with 100µl of 5M NaCl and the first sample and the washing combined in a fresh microcentrifuge tube. This sample was precipitated with ethanol and the DNA pellet resuspended in 25µl of buffer 2.

The non-mutant strand was then nicked using *NciI*. 10µl of the filtered sample was removed and placed in a microcentrifuge tube. 65µl of buffer 3 and 5 units of *NciI* were added. The digest was incubated at 37°C for 90 minutes and a 10µl sample was removed and stored at -20°C for subsequent analysis on an agarose gel: Sample 2.

Next the non-mutant strand was digested using exonuclease III. To the reaction mix above were added: 12µl 500mM NaCl, 10µl buffer 4 and 2µl of a 25units/µl stock of exonuclease III. This was incubated at 37°C for 30 minutes after which the enzymes were inactivated by placing in a 70°C water bath for 15 minutes. A 15µl sample was removed and stored at -20°C for subsequent analysis on an agarose gel: Sample 3.

Finally the gapped DNA was repolymerised and ligated by adding 13µl of nucleotide mix 2, 5µl of $MgCl_2$, 3 units of DNA polymerase I and 2 units of T4 DNA ligase. The sample was placed in a 16°C water bath and incubated for 3 hours. A 15µl sample was removed and stored at -20°C for subsequent analysis an an agarose gel: Sample 4.

20µl aliquots of the final reaction mix were used to transform 300µl of competent TG1 cells.

2.12 Preparation of Radioactively Labelled Oligonucleotide Probes.

The following solutions were added to a 1.5ml microcentrifuge tube (Maniatis *et al*, 1982): 1.5µl of oligonucleotide (2.5 OD_{260} units/ml), 3µl of kinase buffer, 30µCi [Y-^{32}P]ATP (3000Ci/mmol), 22µl water and 2 units of polynucleotide kinase. This reaction was incubated at 37°C for 30 minutes, transferred to a 6ml tube and diluted with 3ml of 6xSSC and then stored frozen at -20°C.

2.13 Dot-Blot Hybridisation of M13 Single-Stranded DNA.

The method detailed in the Amersham handbook was employed.

50µl of M13 phage stock (see 2.10) were applied as a dot to a dry sheet of nitrocellulose using Hybridot apparatus (Hybri-Dot™, B.R.L.). The filter was air dried and baked at 80°C for 1 hour in a vacuum oven. The filter was then pre~~hybridised~~ soaked with 10ml of (6xSSC, 10xDenhardt's solution, 0.2% SDS) at 67°C for 1 hour in a hybridisation bag. The prehybridisation solution was then discarded and replaced with 4ml of labelled probe from 2.12 above. This was left to hybridise at ambient temperature for 1 hour. The filter was then washed three times in 100ml of 6xSSC (5 minutes) and once in 50ml of 6xSSC at 5°C below Td (melting temperature) for the oligonucleotide concerned.

The Td value of a given oligonucleotide was estimated using the "Wallace rule" (Suggs *et al*): Td = 4 x (number of G,C base pairs) + 2 x (number of A,T base pairs).

Excess 6xSSC was shaken off the filter, which was then covered with plastic film and autoradiographed at -70°C with preflashed film and an intensifying screen for 2 hours.

If this procedure did not give the required discrimination between mutant and wild type phage dots then progressively higher wash temperatures were used and the filter was re-

autoradiographed. Temperatures used were 2°C below Td, then Td, and in some cases 2°C above Td.

2.14 Preparation of Plasmid DNA and Replicative Form M13 DNA from *E. coli* Cells.

The method used was a modification of that used by Maniatis *et al* (1982).

Cells were harvested from 10ml YT cultures (grown overnight at 37°C with moderate shaking, supplemented with appropriate antibiotics if required) by centrifugation (10xg for 10 minutes at 4°C). The cell pellet was resuspended in 200µl of solution 1 and transferred to a 1.5ml microcentrifuge tube. 200µl of solution 2 was added and the sample was placed on ice for 5 minutes. 150µl of solution 3 were then added and the sample was returned to ice for a further 5 minutes. The sample was then centrifuged at 13000 rpm in a microcentrifuge tube for 10 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube using a micropipette and extracted once with phenol/chloroform and once with chloroform. DNA was recovered by ethanol precipitation and resuspended in 50µl of TE buffer (a concentration of approximately 500ng/µl).

2.15 Transformation of *E. coli* 910 with Plasmid DNA.

This procedure has been detailed by Maniatis *et al* (1982).

A 300µl aliquot of frozen, competent 910 cells was thawed slowly on ice. 50ng of plasmid DNA were then added and the cells left on ice for 60 minutes. The sample was heat shocked at 42°C for 2 minutes and then returned to ice for 5 minutes. 600µl of YT broth were added and the cells were incubated at 37°C for 60 minutes. 100µl aliquots of 10^{-1} and 10^{-2} dilutions of this sample (in YT broth) were spread onto selective YT plates (ampicillin at 100µg/ml) which were incubated at 37°C overnight.

2.16 Colony Hybridisation (Grunstein and Hogness, 1975).

Colonies were replica plated from a master plate onto a gridded nitrocellulose membrane that had been placed onto a YT plate containing ampicillin at 100µg/µl. The colonies were grown at 37°C overnight. The membrane was removed from the plate and placed colony side up onto a pad of Whatman 3MM paper that had been soaked in 0.5M sodium hydroxide in order to lyse the colonies and denature the DNA. After 10 minutes the membrane was transferred to a pad of 3MM paper soaked in 1M Tris-HCl pH 7.4. The membrane was removed after 2 minutes and transferred to a fresh 1M Tris-HCl pad for a further 2 minutes. Following this the membrane was placed on a 1.5M Tris-HCl pH 7.4 pad for a final 15 minute incubation and was then air dried. Finally the membrane

was baked in a vacuum oven at 80°C for 2 hours ready for hybridisation with the appropriate ^{32}P-labelled probe DNA.

2.17 Capillary (Southern) Blotting of DNA.

The method used was that of Southern (1975) and is outlined below.

After electrophoresis in agarose, DNA in the gel was denatured by soaking in (1.5M NaCl, 0.5M NaOH) for 15 minutes. This was repeated twice, leaving in the final solution for 30 minutes. This denaturing solution was replaced with neutralizing solution (1.5M NaCl, 0.5M tris-HCl pH 7.2, 0.001M Na_2EDTA). This was left for 30 minutes and was repeated twice.

The gel was transferred to a bridge of Whatman 3MM paper set in a reservoir of 20xSSC. An Amersham Hybond-N nylon membrane was placed on top of the gel and 3 sheets of 3MM paper were placed on top of this. A stack of absorbent pads was placed on top of this and the stack was compressed with a glass plate and a 500g weight. The absorbent pads were left to wick up the 20xSSC and transfer the denatured DNA onto the nylon membrane. The transfer was allowed to proceed for 16 hours.

After blotting, the nylon membrane was carefully removed and washed in 2xSSC in order to remove any adhering agarose. The membrane was allowed to air dry. The membrane was then wrapped in cling film and placed DNA side down on a standard ultraviolet

transilluminator for 2 minutes to cross-link the DNA onto the membrane ready for hybridisation with the appropriate ^{32}P-labelled probe DNA.

2.18 Radioactive Labelling of DNA Probes by Nick Translation.

Purified DNA fragments isolated from agarose gels were radioactively labelled *in vitro* using an Amersham Nick Translation Kit (N.5000) which is based upon the method of Kelly *et al* (1970).

In order to label 1µg of DNA to a specific activity of around 1 x 10^8 dpm/µg, the following reagents were combined in a 1.5ml microcentrifuge tube: 1 µg DNA (usually 1µl), 20µl nucleotide/buffer solution, 10µl of [α-^{32}P]dCTP solution (100µCi), 10µl of enzyme solution (klenow) and sufficient water to bring the final volume to 100µl. The reaction was incubated at 15°C for 30 minutes.

The reaction mixture was then loaded directly onto a 10cm column of Sephadex G-50 suspended in buffer containing 150mM NaCl, 10mM EDTA, 0.1% SDS and 50mM Tris-HCl pH7.5 in order to stop the reaction and to separate the labelled DNA from the unincorporated nucleotides. 1ml fractions were collected and samples were monitored for radioactivity by scintillation counting. The eluted labelled double-stranded DNA was pooled and used as a probe in hybridization experiments.

2.19 Detection of Membrane-Bound DNA by Hybridization.

Membranes were placed in polythene bags and prehybridised at 65°C in 25ml of prehybridization solution (7.5ml 20xSSC, 1.25ml 100xDenhardt's solution, 1.25ml 10% SDS and 0.5mg of denatured herring sperm DNA). After 1 hour's incubation the solution was replaced with 10ml of fresh solution and 1µg of probe DNA (1 x 10^8 dpm) was added. The bag was heat-sealed and the filter was hybridised 25°C below the Td of the duplex for 16 hours (Bonner, 1973). This was usually at around 65°C.

Following hybridization, the filters were washed twice with 100ml of 2xSSC at 65°C for 15 minutes. This was followed by a 15 minute wash in 2xSSC containing 0.1%SDS (65°C, 30 minutes). The membrane was then wrapped in cling film and autoradiographed against pre-flashed film at -80°C. If the required specificity of hybridization had not been achieved at this point a 10 minute wash in 0.1xSSC at 65°C was used. The membrane was again wrapped in cling film and autoradiographed as before.

2.20 DNA Sequencing.

Sequencing of DNA inserts in M13 clones was according to the chain termination principle of Sanger et al (1977). Sequencing of DNA inserts in pUC vectors was also performed by the chain termination principle using the method developed by Chen and Seeburg (1985). In either case the procedures were performed using Boehringer Mannheim sequencing kits (1013092 and 1013106 respectively) and following exactly the protocols supplied with the kits.

Where pUC inserts were sequenced, the plasmid DNA had first to be denatured: 2µg of plasmid DNA were placed in a 1.5ml microcentrifuge tube and dried down in a vacuum centrifuge. The DNA was dissolved in 40µl denaturation buffer (NaOH, 0.2M, EDTA, 0.2mM pH8.0) and allowed to stand at room temperature. 4µl neutralising buffer (ammonium acetate 2M, pH4.5) were added. The DNA was then precipitated with ethanol immediately.

To the denatured and dried plasmid DNA were added: 1µl ≈ 2.5 pmol M13/pUC sequencing primer (solution 12), 1.5µl 10x reaction buffer (solution 10), 2µl ≈ 16 µCi [^{35}S] dATP-α-S and 9.5µl redistilled water. This mixture was incubated at 37°C for 15 minutes. 2 units of Klenow enzyme were then added and 3µl of the mixture were then pipetted into each of four labelled microcentrifuge tubes (G,A,T,C).

Where M13 inserts were sequenced, 0.5µg of single stranded M13 DNA were combined with 1µl ≈ 2.5 pmol M13/pUC sequencing primer (solution 12), 1.5µl 10x reaction buffer (solution 10) and sufficient redistilled water to bring the reaction volume to 10µl. This mixture was incubated for 10 minutes at 55°C and allowed to cool slowly to room temperature afterwards.

2µl ≈ 16 µCi [^{35}S] dATP-α-S and 1 unit of Klenow enzyme were then added to this hybridisation mixture and 2.5µl of the mixture was pipetted into each of four labelled microcentrifuge tubes (G,A,T,C).

In each case 2µl of the appropriate dNTP/ddNTP mixture were added to each tube and the tubes were incubated for 20 minutes at 37°C.

The dNTP/ddNTP mixes used were as listed in table 1 below.

Nucleotide solutions	G mixture µl	G mixture µmol/l	A mixture µl	A mixture µmol/l	T mixture µl	T mixture µmol/l	C mixture µl	C mixture µmol/l
dGTP (3)	2	5	40	100	40	100	40	100
dTTP (4)	40	100	40	100	2	5	40	100
dCTP (2)	40	100	40	100	40	100	4	10
ddGTP (5)	12	120	—	—	—	—	—	—
ddATP (6)	—	—	10	100	—	—	—	—
ddTTP (7)	—	—	—	—	50	500	—	—
ddCTP (8)	—	—	—	—	—	—	10	100
10x buffer (10)	20		20		20		20	
redist. water	86		50		48		86	
final volume	200		200		200		200	

Table 1. *Composition of dNTP/ddNTP mixes used for DNA sequencing.*

The reactions were then chased by adding 1.5µl deoxynucleotide mixture (solution 11) to each microfuge tube and incubating again at 37°C for a further 15 minutes.

The reactions were terminated by adding 4µl formamide buffer (solution 15).

Immediately before loading onto a sequencing gel the samples were denatured by incubation at 95°C for 3 minutes followed by rapid cooling on ice. 2µl of each sample were then loaded into an appropriate slot of a pre-electrophoresed and pre-heated (65°C) 5% polyacrylamide sequencing gel 0.2mm thick.

After electrophoresis, the gel was dried for 1 hour at 80°C under vacuum. An X-ray film was exposed to the dried gel overnight at room temperature. DNA sequence was then read from the developed film.

2.21 Transformation of *S.cerevisiae*.

50ml of YEM media were inoculated from a fresh overnight culture of *S.cerevisiae* MC16 and grown to $O.D._{590}$ 0.5 at 30°C. The yeast cells were harvested in a bench top centrifuge at 3000xg for 5 minutes and suspended in 5ml of 100mM lithium acetate in TE buffer. This cell-suspension was incubated at 30°C for 1 hour with agitation. The cells were harvested again by centrifugation and resuspended in a another 5ml of lithium acetate buffer

containing 15% glycerol. The cells were dispensed in 300µl aliquots in Eppendorf tubes and frozen at -70°C until required.

To transform the cells, the tubes were warmed slowly to room temperature and 10µg of DNA (10µl) were added. 0.7ml of 50% PEG 4000 was then added and the transformation mix was incubated at 30°C for 60 minutes. The cells were then heat shocked at 42°C for 5 minutes and centrifuged at 13000 rpm for 10 minutes. All the PEG solution was removed with a pipette and the cells were resuspended in 100µl of TE buffer and plated out onto selective YMM plates. The plates were then incubated inverted at 30°C for 5 days.

2.22 Small-Scale Protein Extracts.

Small quantities of protein were extracted from yeast cultures by the procedure described by Watson *et al* (1988).

Transformed yeast cultures (10ml) were grown overnight at 30°C with moderate shaking in YMM supplemented with the required amino acids. The cells were pelleted by centrifugation, cooled and immediately resuspended in 100µl of lysis buffer [1ml of 0.2M Tris/HCl, pH6.0 containing 0.2% SDS and 10% (w/v) sucrose, 10µl leupeptin (200µg/ml) and 10µl phenylmethanesulphonyl fluoride (36mg/ml in ethanol)]. An equal volume of acid-washed glass beads (450-500 µm) was added and then the cells were vortexed for five bursts of 15 seconds each. The debris was removed by

centrifugation and the supernatant was boiled for 10 minutes before being subjected to SDS-PAGE or storage at -20°C.

2.23 SDS-Polyacrylamide Gel Electrophoresis of Yeast Proteins.

Yeast protein extracts were ~~fractioned~~ analysed by 12.5% SDS-PAGE under reducing conditions and non-reducing conditions (ie in both the presence and absence of 2-mercaptoethanol respectively). The procedure used was as described by Laemmli, 1970.

2.24 Western Blotting.

Proteins fractioned by SDS-PAGE were either stained with kenacid blue stain (0.05% w/v in 50% methanol 7% acetic acid) or were transferred to nitrocellulose membranes by electroblotting followed by immunological detection.

After electroblotting (Burnette, 1981), nitrocellulose membranes were blocked overnight at 4°C in 50ml of blocking solution. The blocked filter was probed with rabbit anti-vicilin as primary antibody and peroxidase-linked goat anti-(rabbit IgG) as secondary antibody. Proteins were visualised by staining for 2-3 minutes with 50ml of a fresh solution of 1x tris/salt buffer containing 50mg DAB, 3ml 1% cobalt chloride and 100µl H_2O_2. Filters were then photographed.

2.25 Large Scale Preparation of Yeast Proteins.

10ml stationary-phase cultures of yeast cells grown in YMM were used to inoculate 500ml YMM cultures in 2 litre baffled conical flasks. These were supplemented with the appropriate amino acids and incubated at 30°C with shaking for 48 hours. The cells were harvested by centrifugation (5000xg for 10 minutes) after this time (corresponding to an OD_{200} of approximately 3.0).

Yeast cells were resuspended in 30ml of lysis buffer [100ml of 0.2M Tris/HCl buffer pH6.0 containing 10% w/v sucrose, 1ml of leupeptin (200µg/ml) and 1ml of phenylmethanesulphonyl fluoride (36mg/ml in ethanol)]. An equal volume of acid washed glass beads (450-500µm) was added and the cells were vortexed for 5 bursts of 15 seconds, placing the cells on ice for 1 minute between vortex mixes. The extract was centifuged at 12000xg for 30 minutes at 4°C and the pellet, containing intact yeast and cell debris was discarded. The supernatant (yeast soluble extract) was placed on ice.

Solid $(NH_4)_2SO_4$ was added slowly to the above extract with stirring to a final concentration of 95% relative saturation (for 30ml of supernatant at 0°C this was 22g of solid $(NH_4)_2SO_4$). After 15 minutes the extract was centrifuged at 12000xg for 30 minutes and the supernatant was discarded.

The 95% saturation $(NH_4)_2SO_4$ pellet was dissolved in 10ml of 150mM sodium phosphate buffer pH7.5 and dialysed against 10mM sodium

phosphate buffer pH7.5 for 24 hours with several changes of buffer.

This extract was then fractionated on a hydroxyapatite column as described below.

2.26 Fast Protein Liquid Chromatography (F.P.L.C.).

Dialysed protein extracts were loaded onto a hydroxyapatite ultrogel column packed into a 10/10 glass f.p.l.c. column, interfaced to an LKB f.p.l.c. system with a UV1 ultraviolet monitor and a 280nm filter as a detection unit (Wright *et al*, unpublished).

The column was pre-equilibrated in 150mM sodium phosphate buffer and the yeast protein was loaded in 10ml aliquots with the aid of a 10ml Superloop.

Unbound material was eluted with 150mM sodium phosphate buffer.* Bound material (vicilin) was eluted with a linear gradient of increasing sodium phosphate concentration (up to 750mM). The flow rate throughout was 1ml/minute and all buffers used were filtered through 22µm pore-size filters before use. The purified vicilin was freeze dried and stored at -20°C.

*Unless stated otherwise, buffers for F.P.L.C. at pH 7.5.

2.27 Protein Assay.

Protein was estimated by ultraviolet absorption using an extinction coefficient E_{280} 1cm of 0.8 for a 1 mg/ml solution of vicilin.

2.28 CNBr Cleavage of Proteins.

50µg of vicilin were resuspended in 150µl 70% formic acid, transferred to a plastic 10ml scintillation vial and 200µl of a 20% solution of CNBr in acetonitrile were added. The vial was sealed and kept at room temperature in the dark for 24 hours. 6ml of distilled water was then added and the sample was freeze dried. This procedure ensured the removal of all CNBr from the sample. Samples were stored dry at -20°C.

2.29 Vicilin Gelling.

The turbidity of heat-induced gels of vicilin was determined using an adaptation of the method that Nakamura *et al* (1984) used for glycinin gels.

0.3 OD_{280} units of vicilin were dissolved in 500µl of 150mM sodium phosphate buffer pH7.5. Each protein solution was heated at a rate of 1°C per minute to a final temperature of 75°C and the formation of the protein gel was noted by following the change in OD_{450}.

CHAPTER THREE
THE METHIONINE MUTANT OF PEA VICILIN

3.1 Introduction of a Methionine Residue into Pea Vicilin.

A single base change was made to the vicilin cDNA construct pDUB2018 at position 819 of the cDNA insert (see appendix I). The base T in pDUB2018 was changed to an A by *in vitro* mutagenesis. The mutant vicilin gene was then fused to the yeast phosphoglycerate kinase (PGK) promoter in the yeast expression vector pMA257. This mutant construct (pPE18) was expressed in *S.cerevisiae* to yield a vicilin fusion peptide containing the first 16 amino acid residues of PGK in addition to the vicilin sequence, identical to the pDUB2018 peptide except that the leucine residue 282 has been converted to a methionine residue.

The vicilin cDNA was isolated from the construct pDUB2018 by restriction with *Bam* HI, gel electrophoresis and electroelution of the 1.4 Kb vicilin *Bam* HI fragment. This fragment was ligated into the polylinker *Bam* HI site of the M13 vector mp18 which was then transformed into *E. coli* TG1 (see Figure 1). Single-stranded DNA was purified from a number of recombinant plaques and these were checked by dideoxysequencing to ensure that the vicilin sequence was correct. A recombinant containing the vicilin cDNA in the 5' - 3' orientation was selected and used to make the single-stranded template DNA required for the *in vitro* mutagenesis.

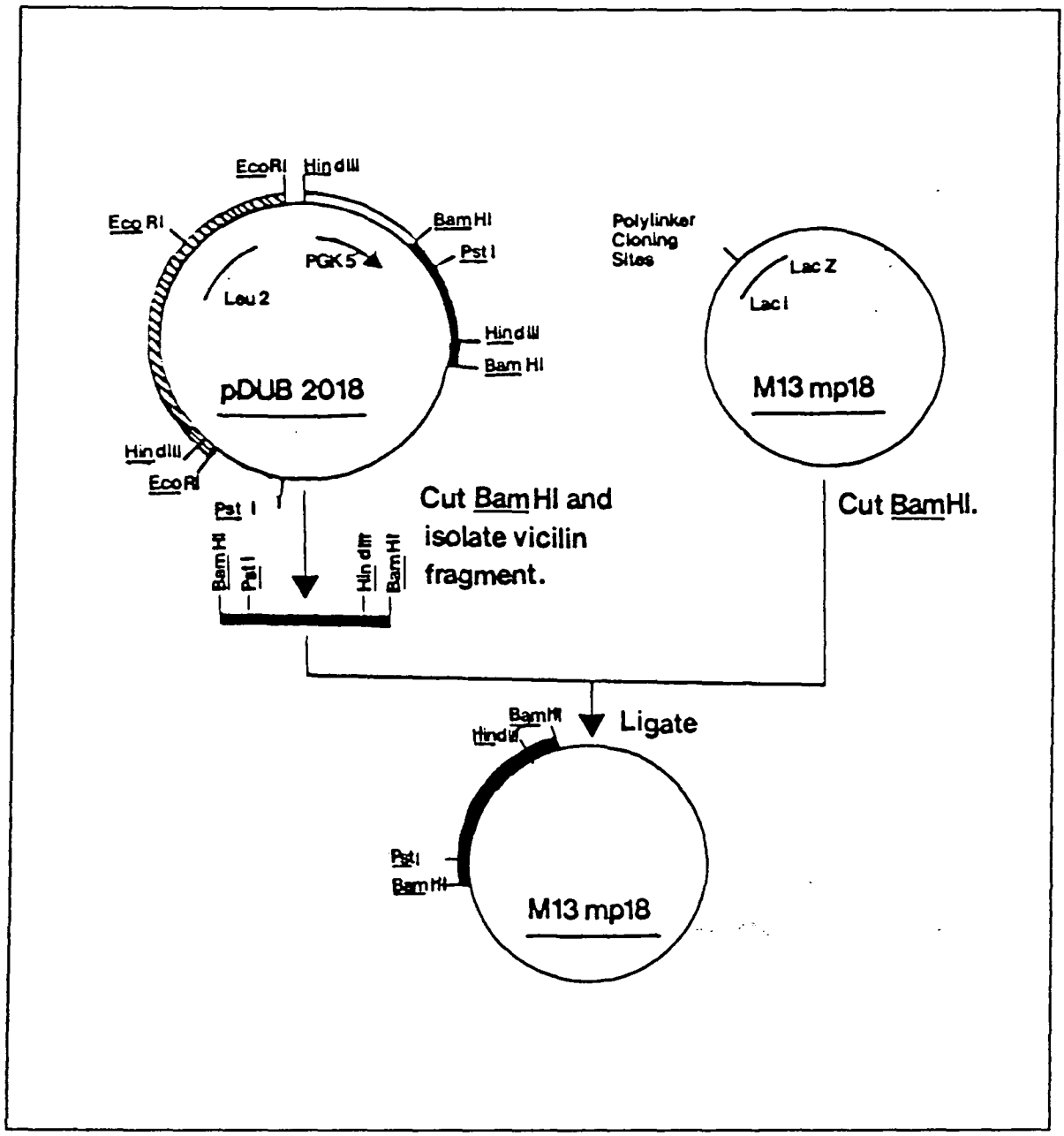


Figure 1. *Cloning of the vicilin cDNA into M13 mp19.*

An oligonucleotide (oligo 1) of part of the antisense strand of vicilin was made by the phosphoramidite method on an automatic DNA synthesiser (Applied Biosystems model 381A) by Mr John Gilroy (see acknowledgements). After synthesis, the oligonucleotide was purified by high pressure liquid chromatography (h.p.l.c.) and

phosphorylated using T_4 polynucleotide kinase (Berkner and Folk, 1977). The sequence of oligo 1 is shown in figure 2.

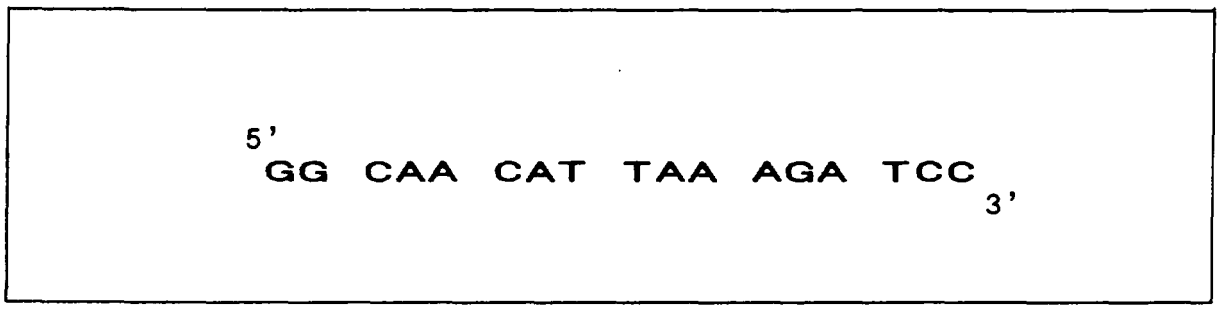


Figure 2. *Sequence of Oligonucleotide 1.*

The oligonucleotide was annealed to the single-stranded vicilin template and was used to prime the *in vitro* mutagenesis reactions described in chapter 2.

After *in vitro* mutagenesis and transformation into *E. coli* TG1, a number of plaques were screened for the methionine mutation. Putative mutant phage were grown up in 2ml 2xTY broth cultures overnight and 100µl samples of phage supernatant were spotted onto a nitrocellulose filter using dot blot apparatus. 15pmol of oligo 1 was end-labelled with 30µCi[$Y^{-32}P$]ATP using polynucleotide kinase and this was used to probe the filter as described in chapter 2. Figure 3 shows a 24 hour autoradiograph of a filter with 8 plaques after washing to 47°C (Td + 2°C) in 6xSSC.

Replicative form DNA was prepared from the phage showing strongest hybridisation (sample 8, figure 3). The mutant vicilin cDNA was isolated from this construct by restriction with *Bam* HI, gel electrophoresis and electroelution of the 1.4 Kb vicilin *Bam*

HI fragment. This was then ligated into the *Bam* HI site of the yeast expression vector pMA257 to create pPE18 which was transformed into *E. coli* 910.

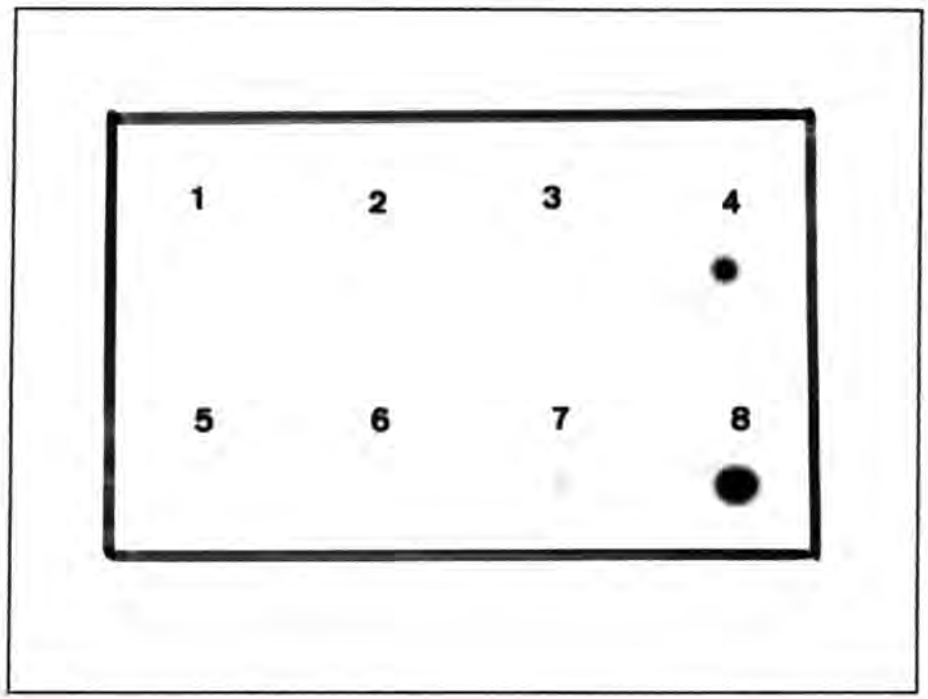


Figure 3. *Dot blot hybridisation of putative methionine mutants of vicilin in M13 probed against oligo 1. Eight samples are shown. The sample shown top left (sample 1) is of non-mutant vicilin phage supernatant and serves as a negative control. The sample shown bottom right (sample 8) was selected as being a probable methionine mutant of vicilin as it showed strongest hybridisation to oligo 1.*

The scheme for construction of the methionine-containing vicilin mutant pPE18 is summarized in figure 4.

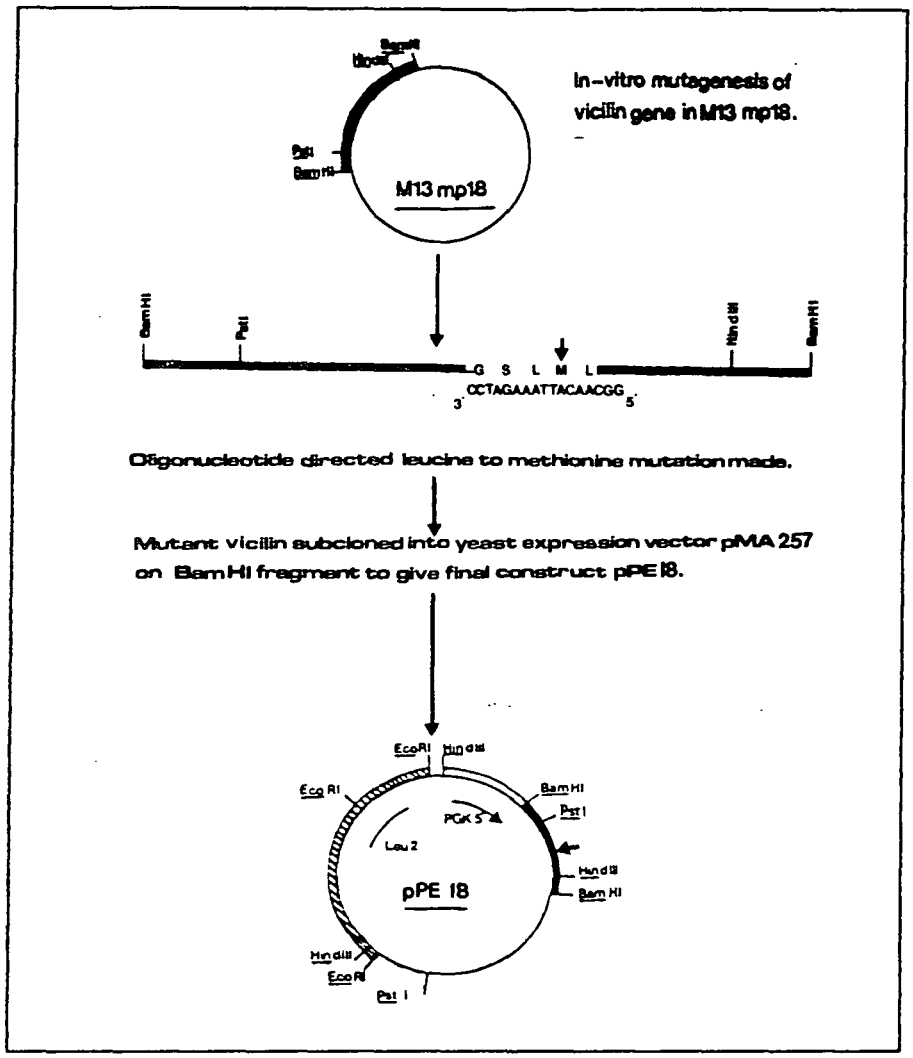


Figure 4. *Construction of methionine mutant of vicilin pPE18.*

Plasmid DNA was prepared from a number of potential pPE18 transformants and this was restricted with either *Bam* HI or *Hin*dIII. 500ng samples of restricted DNA were then subjected to agarose gel electrophoresis on an 0.7% (w/v) agarose gel, Southern blotted onto a nylon membrane and probed using the radioactively labelled oligo 1 described above. The blot was washed in 1xSSC at 42°C. Figure 5 shows a photograph of the ethidium bromide-stained gel and an autoradiograph of the Southern blot after 48 hours exposure.

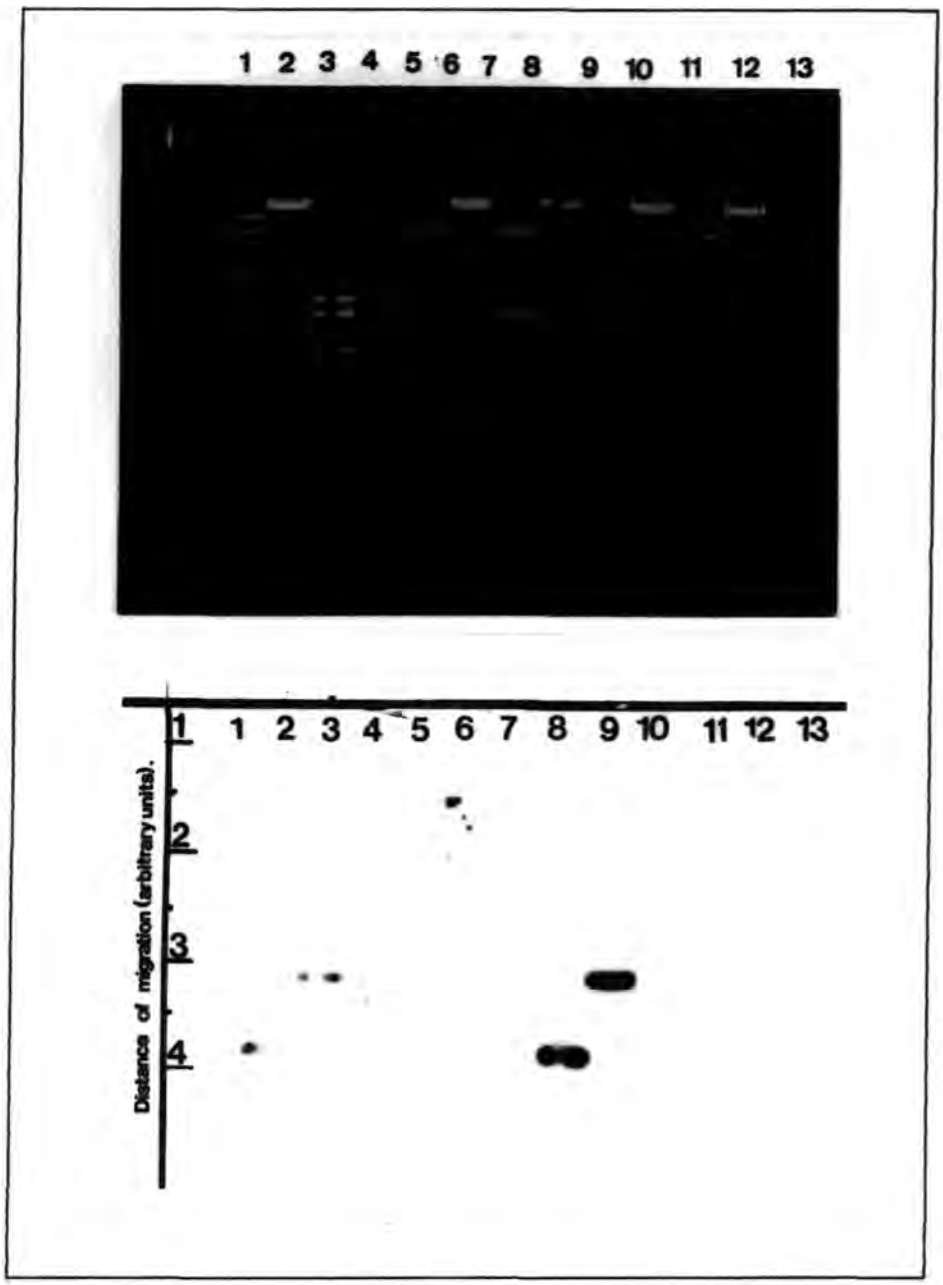


Figure 5. *Gel electrophoresis and corresponding autoradiograph of Southern blot used to screen for pPE18 mutant. Lane 1, molecular weight markers (phage lambda digested with BstEII). Lane 2, pDUB2018 BamHI. Lane 3, pDUB2018 HindIII. Lanes 4 - 13, potential pPE18 mutants (methionine mutants of vicilin): even numbers are BamHI digests and odd numbers are HindIII digests.*

Only the construct shown in lanes 8 and 9 contains the vicilin insert in the correct orientation for expression from the PGK promoter; that is to say, it yields a 1.4kb *Bam* HI fragment (lane

8) and a 2.7kb *Hind* III fragment (lane 9) similar to pDUB2018 (lanes 2 and 3). Lanes 8 and 9 also show a positive hybridisation of the 1.4kb *Bam* HI and 2.7kb *Hind* III fragments against oligo 1 in the Southern blot. Figure 6 shows a restriction map of pDUB2018 and indicates the sizes of the DNA fragments resulting from restriction of pDUB2018 with *Bam* HI and *Hind* III and similar restriction of a construct containing the vicilin insert in the opposite orientation.

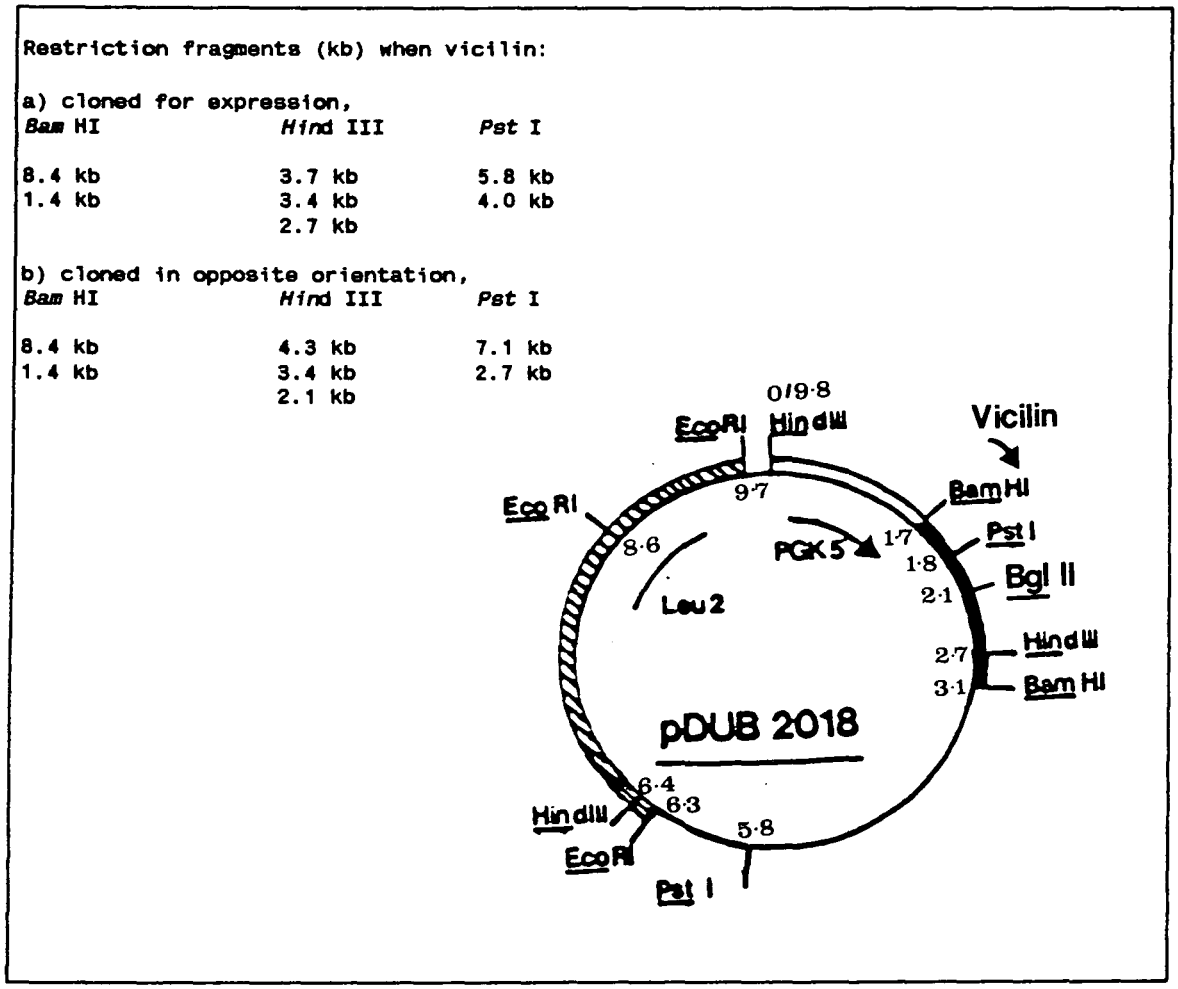


Figure 6. *Restriction map of pDUB2018.*

The construct shown in lanes 8 and 9 of figure 5 was, therefore, designated pPE18, the vicilin insert of which was checked by

dideoxy sequencing in M13 to confirm the methionine mutation and to check that the clone was otherwise identical to pDUB2018.

3.2 Introduction of pPE18 into *S.cerevisiae*.

5µg of pPE18 DNA was used to transform 300µl of competent *S.cerevisiae* MC16. 50 transformant colonies (selected on YMM plates) were streaked onto nitrocellulose membranes along with a pDUB2018(MC16) colony and a pMA257(MC16) colony. The membranes were placed on fresh YMM plates and incubated overnight at 30°C. One such plate was kept as a replicate whilst the yeast colonies on the nitrocellulose membrane of the second plate were lysed by placing the membrane on filter paper soaked in [0.1% SDS, 0.2M NaOH, 0.5% 2-mercaptoethanol] for 30 minutes. This filter was then probed with [rabbit anti-vicilin/goat anti-rabbit peroxidase complex] as described in chapter 2, section 2.24. A number of yeast transformants were found to be expressing vicilin at an apparently similar level to the pDUB2018(MC16) positive control. The pMA257(MC16) colony serves as a negative control. A photograph of this filter is shown in figure 7. A construct containing the mutated vicilin in the opposite orientation for expression was similarly used to transform *S.cerevisiae* and was similarly screened. In this case no transformants were seen to be expressing vicilin.

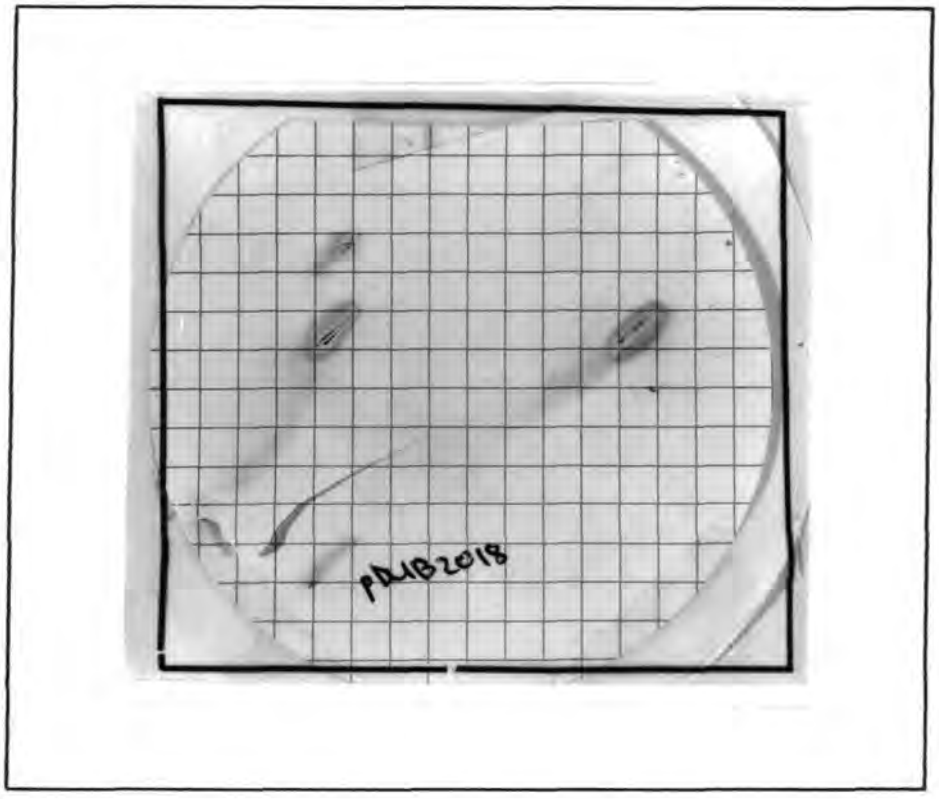


Figure 7. *Western probe of potential pPE18 MC16 S.cerevisiae transformants against anti-(pea)vicilin. Three transformants can be seen to be expressing vicilin at a similar level to pDUB2018.*

3.3 Expression of Vicilin Methionine Mutant by Yeast.

20ml cultures of pMA257(MC16), pDUB2018(MC16) and pPE18(MC16) were grown overnight at 30°C and small scale protein extracts were prepared as described in chapter 2. Aliquots of each protein extract were subjected to CNBr cleavage then all protein samples were analysed by SDS-PAGE (in the presence of 2-mercaptoethanol) and Western blotting against anti-(pea)vicilin. A photograph of this Western blot is shown in figure 8.

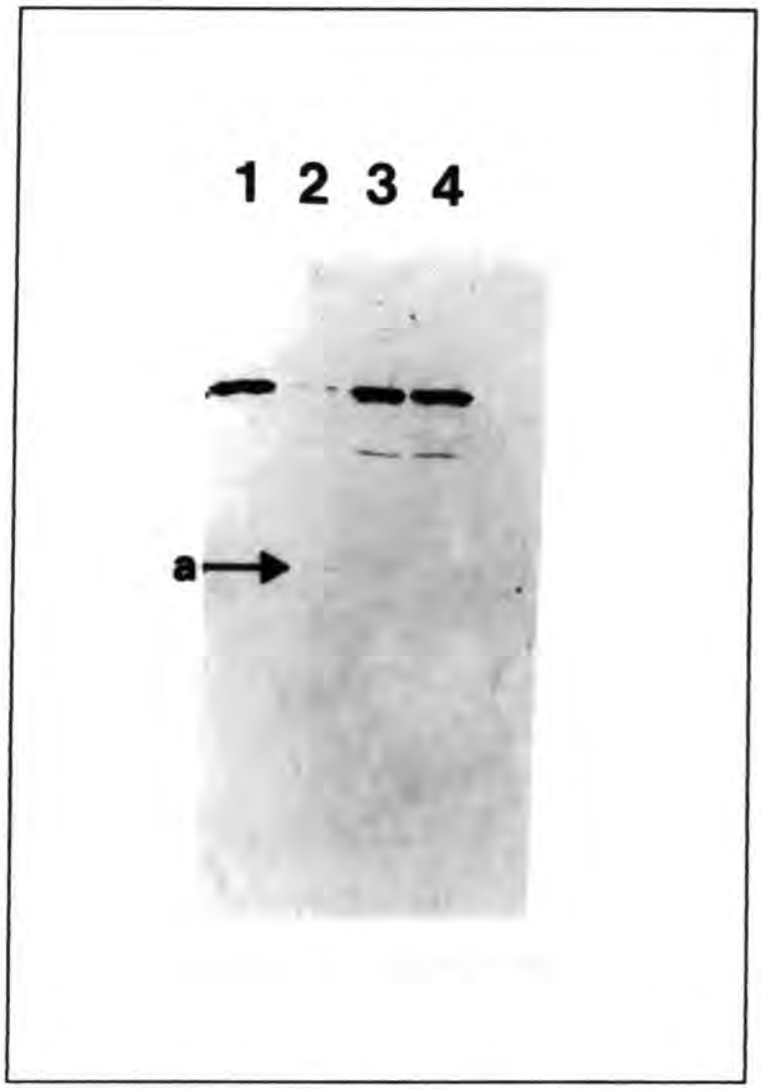


Figure 8. *Western blot analysis of pPE18(MC16) vicilin (methionine) extracts. Lane 1, pPE18(MC16) protein extract (untreated). Lane 2, pPE18(MC16) protein extract cleaved with CNBr. Lane 3, pDUB2018(MC16) protein extract (untreated). Lane 4, pDUB2018(MC16) protein extract cleaved with CNBr. Lane 5, pMA257(MC16) protein extract (untreated). All lanes contain 20µl protein (approximately 10µg total soluble protein). All lanes contain 2-mercaptoethanol. The presence of a cleaved vicilin product in lane 2 of approximately Mr 27,000 is shown by arrow a.* The concentration of protein solution in lane 2 is unfortunately lower than in other lanes.

Figure 8 shows that the methionine mutant of vicilin produced by yeast is cleavable by CNBr. Non-mutant vicilin is not cleaved and no vicilin is produced by clones not containing the vicilin insert (or by clones containing the insert in the opposite orientation for expression).

CHAPTER FOUR

THE CYSTEINE MUTANTS OF PEA VICILIN

4.1 Introduction of Two Cysteine Residues into Pea Vicilin.

Serine residue 2 and serine residue 183 of pDUB2018 were converted to cysteine residues by the *in vitro* mutagenesis procedure to produce the intermediate constructs pPE9 and pPE11 respectively. The mutagenesis was performed using the oligonucleotides oligo 2 and oligo 3 which were prepared as described in chapter 3. The sequences of oligos 2 and 3 are shown in figure 9 and the subcloning procedure used to construct pPE9 and pPE11 is shown in figure 10. The vicilin cDNA sequence showing the sites of hybridisation for oligos 2 and 3 is given in appendix I.

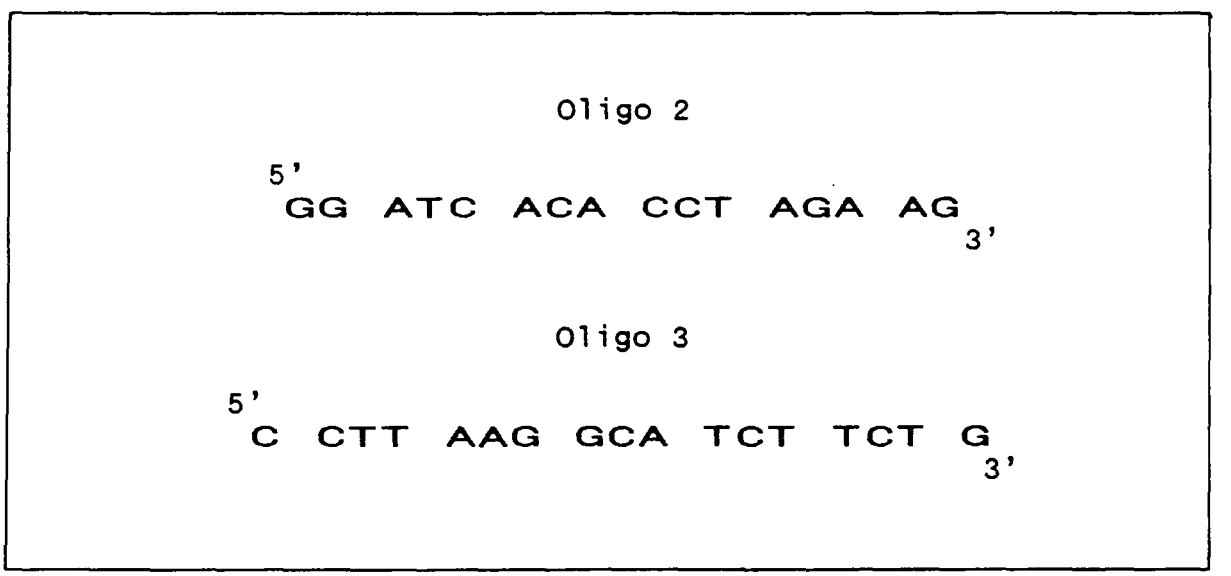


Figure 9. *Sequence of oligonucleotides 2 and 3.*

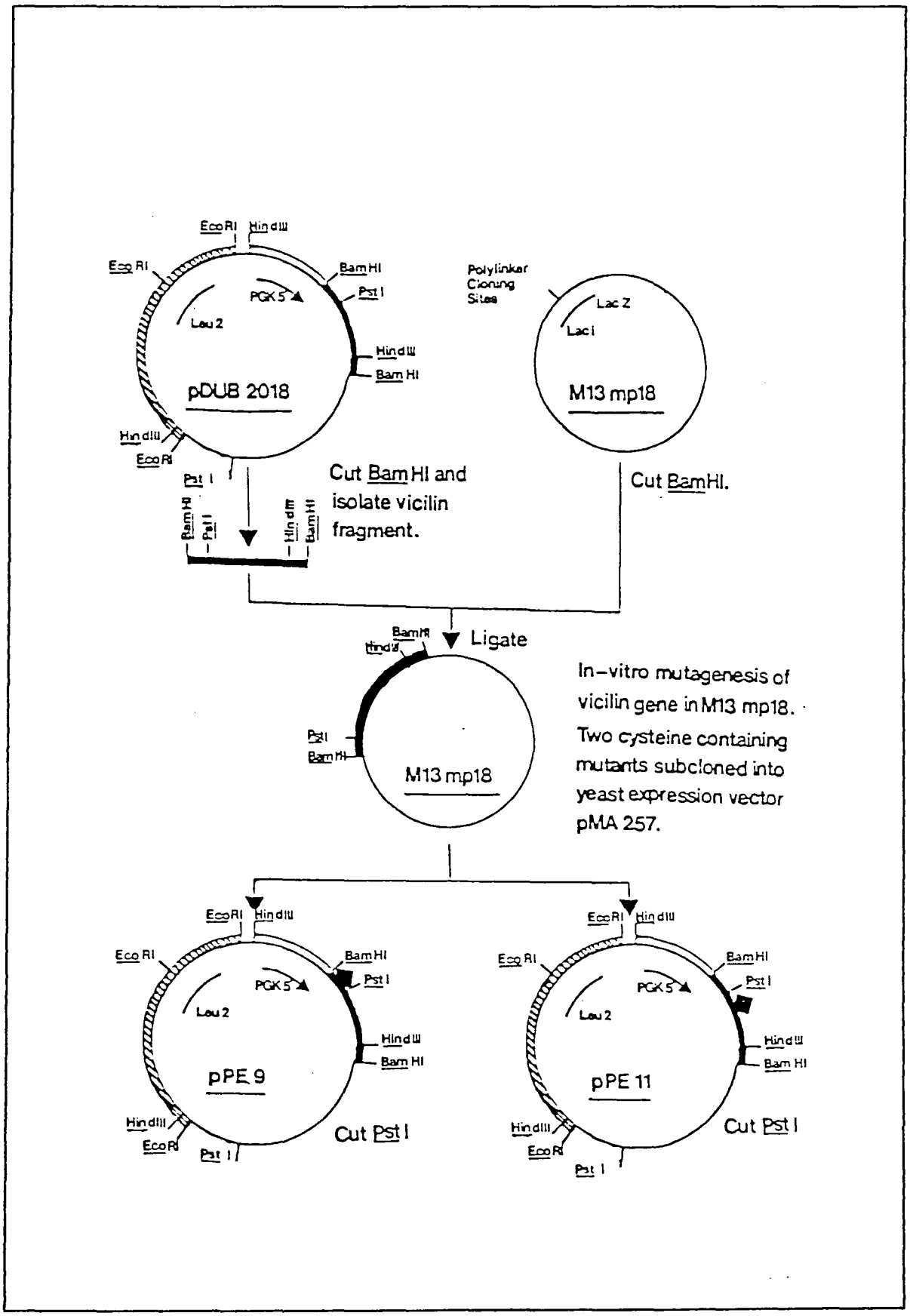


Figure 10. *Construction of pPE9 and pPE11.*

After *in vitro* mutagenesis of the cDNA (by the same procedure used for the methionine construct) plaques were screened by dot-blot hybridisation against labelled oligonucleotides 2 and 3 as described in chapters 2 and 3. A dot-blot of putative cysteine mutants is shown in figure 11.

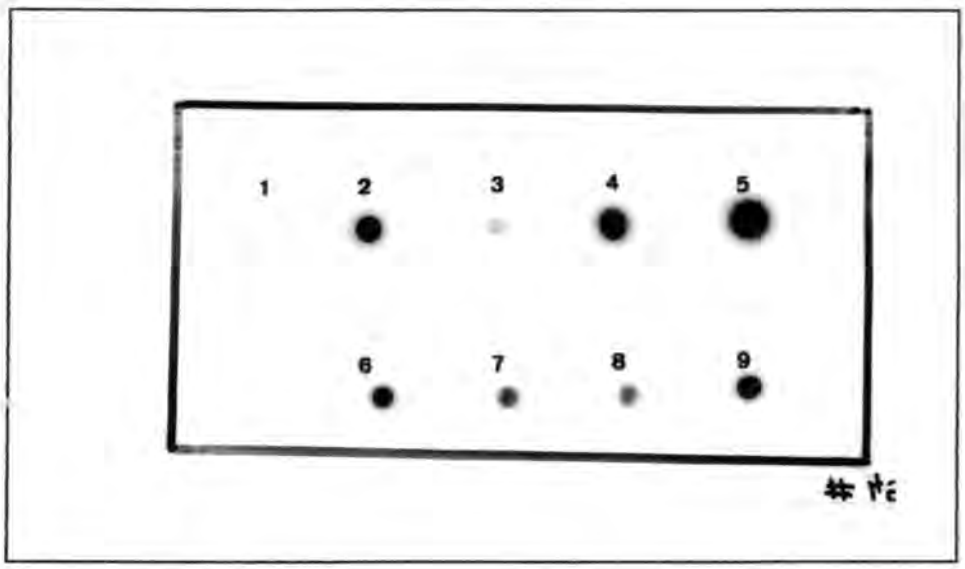


Figure 11. *Dot-blot of putative cysteine mutants. Dot 1 is M13 mp19 and serves as a negative control - it has been probed against both oligo 2 and oligo 3. Dots 2-5 are potential ser 2 mutants probed against oligo 2. Dots 6-9 are potential ser 183 mutants probed against oligo 3. 50µl of phage supernatant was loaded in each case.*

Replicative form DNA was prepared from the phage showing strongest hybridisation: phage 5 in the case of the oligo 2 mutant and phage 9 in the case of the oligo 3 mutant. The mutant vicilin cDNAs were isolated from these constructs by restriction with *Bam* HI, gel electrophoresis and electroelution of the 1.4 Kb vicilin *Bam* HI fragments. These were then ligated into the *Bam* HI site of the yeast expression vector pMA257 and transformed into *E. coli* 910 to create pPE9 and pPE11. Constructs containing

the mutant vicilins in the opposite orientation to that required for expression by the PGK promoter were also obtained and were designated pPE10 and pPE12 respectively. pPE9 and pPE11 constructs were checked by restriction, agarose gel electrophoresis and Southern blotting against oligos 2 and 3 as shown in figure 12 below.

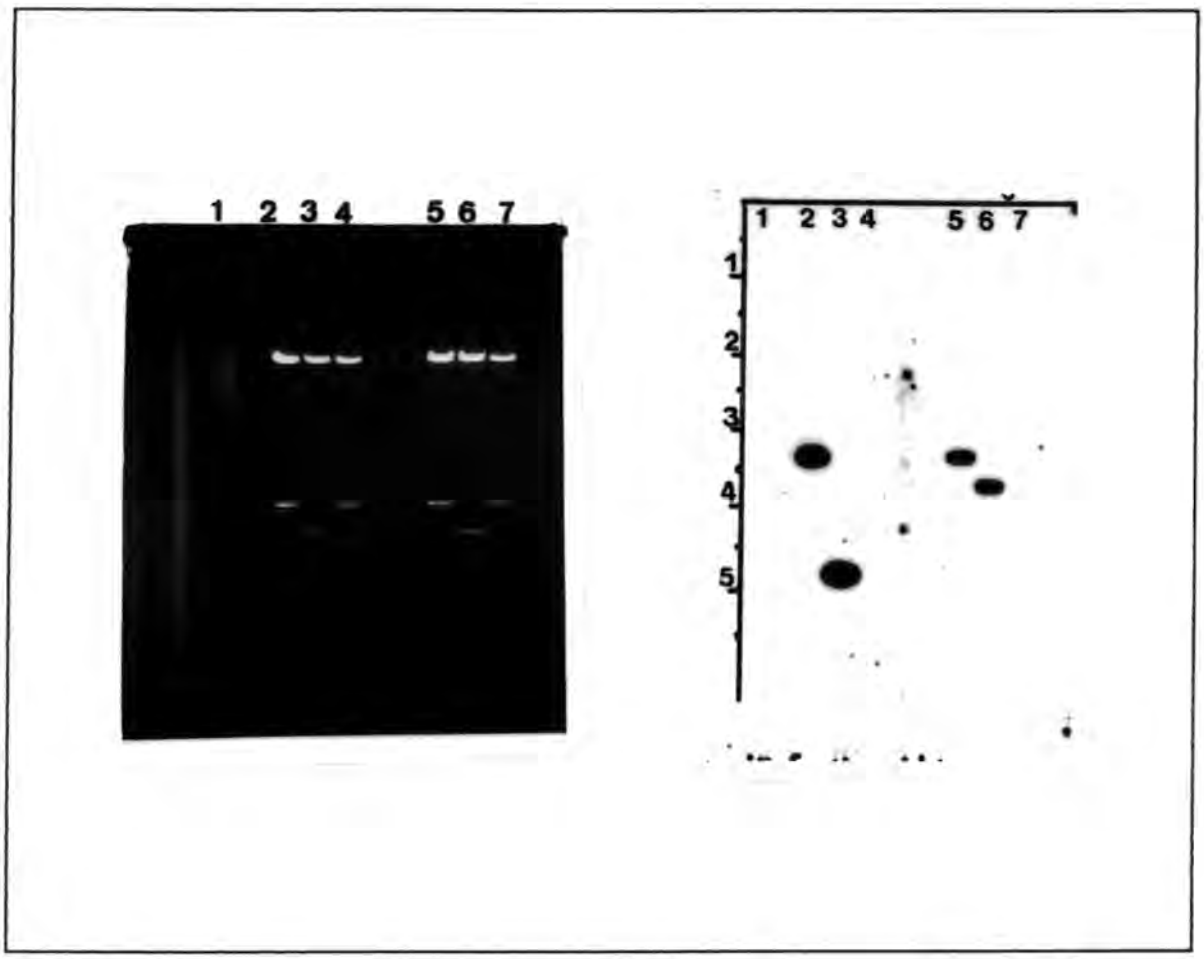


Figure 12. *Agarose gel electrophoresis and Southern blot of pPE9 and pPE11. Lane 1, DNA size markers (Lambda restricted with Bst EII). Lane 2, pPE9 Bam HI. Lane 3, pPE9 Bam HI/Bgl II. Lane 4, pDUB2018 Bam HI. Lane 5, pPE11 Bam HI. Lane 6, pPE11 Bam HI/ Bgl II. Lane 7, pDUB 2018 Bam HI. Lanes 2, 3 & 4 are probed against oligo 2. Lanes 5, 6 & 7 are probed against oligo 3.*

Digests of pPE9, pPE11 and pDUB2018 with *Bam* HI have yielded the expected fragments of 8.4kb and 1.4kb. Digests of pPE9 and pPE11 with *Bam* HI and *Bgl* II together have yielded the expected fragments of 8.4kb, 1.0kb and 0.4kb. With pPE9, the 1.4kb *Bam* HI fragment and the 0.4kb *Bam* HI/*Bgl* II fragment hybridised against oligonucleotide 2 (lanes 2 and 3 of figure 12). With pPE11, the 1.4kb *Bam* HI fragment and the 1.0kb *Bam* HI/*Bgl* II fragment hybridised against oligonucleotide 3 (lanes 5 and 6 of figure 12). This reflects the position of binding of the oligonucleotides within the vicilin insert[2]. No hybridisation of oligonucleotide 2 to pPE11 or of oligonucleotide 3 to pPE9 was detected. No hybridisation of the oligonucleotides to pDUB2018 was detected (lanes 4 and 7 of figure 12).

4.2 Expression of Cysteine Mutants by *S.cerevisiae*.

pPE9, pPE10, pPE11 and pPE12 were transformed into *S.cerevisiae* MC16 as described in chapter 3. 10ml transformant yeast cultures were grown up overnight in YMM broth in each case. Similar cultures of pDUB2018(MC16) and pMA257(MC16) were also produced. Small scale protein extracts of all these cultures were prepared under the denaturing conditions described in chapter 2 and 20µl aliquots of these extracts were analysed by SDS-PAGE and Western blot. The results of this analysis are shown in figure 13.

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[2]See also figure 6 in chapter three.

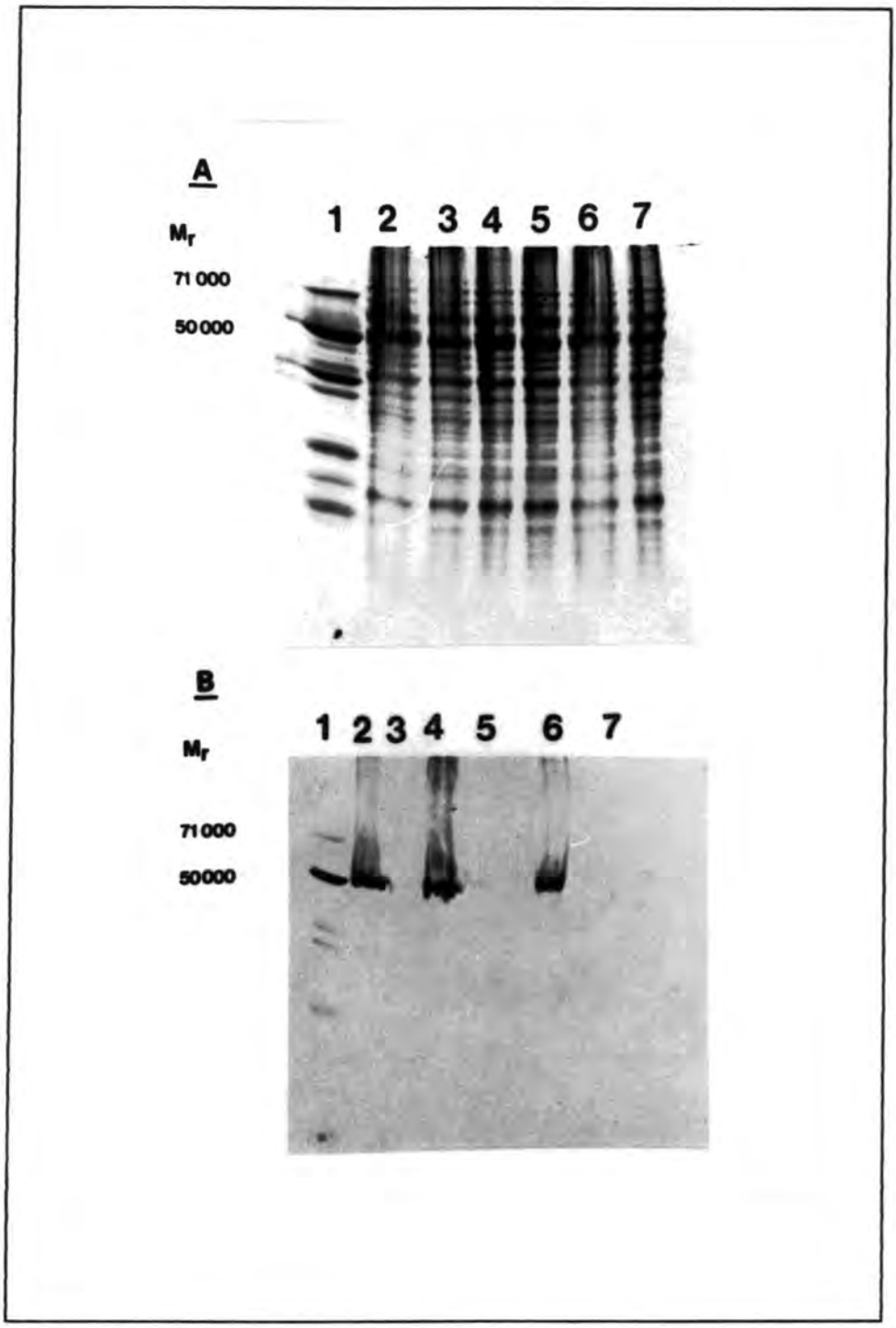

Figure 13. *SDS-PAGE and subsequent western blot analysis of protein extracts from cysteine-containing vicilin mutants produced by yeast. Lane 1, Vicilin/Convicilin Standards. Lane 2, pPE9(MC16). Lane 3, pPE10(MC16). Lane 4, pPE11(MC16). Lane 5, pPE12(MC16). Lane 6, pDUB2018(MC16). Lane 7 pMA257(MC16).*

Plate A shows a Kenacid blue stained gel.

Plate B shows a Western blot analysis of a similar gel.

The constructs pPE9 (MC16), pPE11 (MC16) and pDUB2018 (MC16) produce a 53,000 Mr vicilin (lanes 2, 4 and 6 of figure 13 respectively). Constructs pPE10 (MC16), pPE12 (MC16) and pMA257 (MC16) do not produce a vicilin product (lanes 3, 5 and 7 of figure 13).

In figure 14, a similar Western blot of pPE9 and pPE11 protein extracts is shown but in this case only lanes 1 to 5 contain the reducing agent 2-mercaptoethanol. Lanes 6 to 10 were run under non-reducing conditions (in the absence of 2-mercaptoethanol). No difference can be seen between lanes containing 2-mercaptoethanol and lanes not containing 2-mercaptoethanol. This will be discussed later.

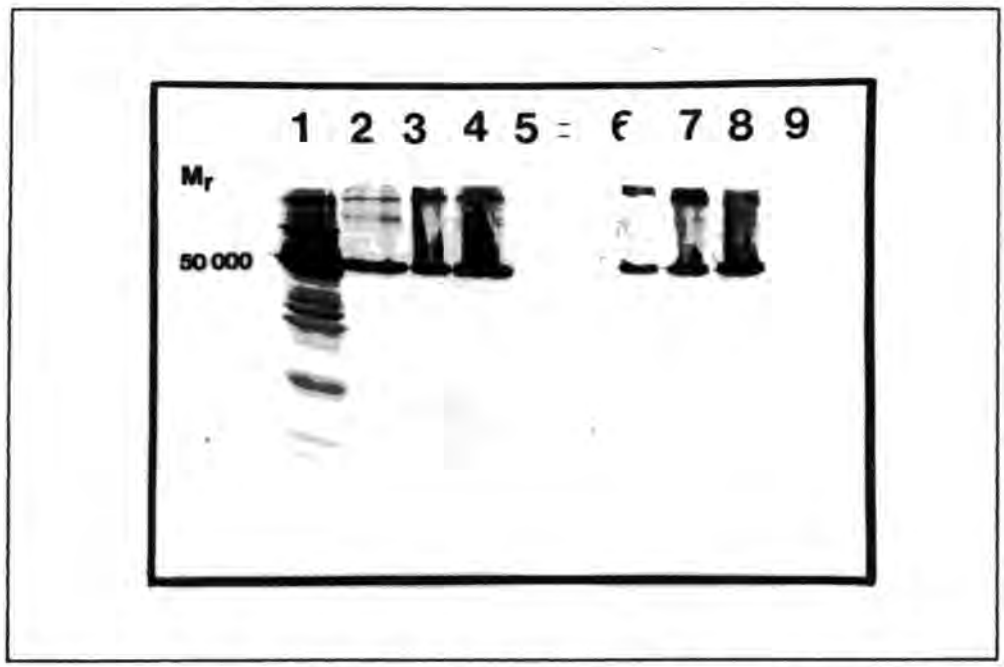

Figure 14. *Western blot of pPE9 and pPE11 proteins separated by SDS-PAGE under both reducing and non-reducing conditions. Lane 1, Vicilin/Convicilin Standards. Lane 2, pPE9(MC16) + 2-mercaptoethanol. Lane 3, pPE11(MC16) + 2-mercaptoethanol. Lane 4, pDUB2018(MC16) + 2-mercaptoethanol. Lane 5, pMA257(MC16) + 2-mercaptoethanol. Lane 6, pPE9(MC16). Lane 7, pPE11(MC16). Lane 8, pDUB2018(MC16). Lane 9, pMA257(MC16). Lanes 6 to 9 were run in the absence of 2-mercaptoethanol.*

4.3 pPE17: A Construct Containing Both Cysteine Mutations.

A hybrid vicilin construct pPE17, containing both of the serine to cysteine mutations, was made by ligation of the 5' coding region from pPE9 to the 3' region from pPE11 through a *Pst* I site in the vicilin cDNA sequence and a *Pst* I site in the vector (see figure 15).

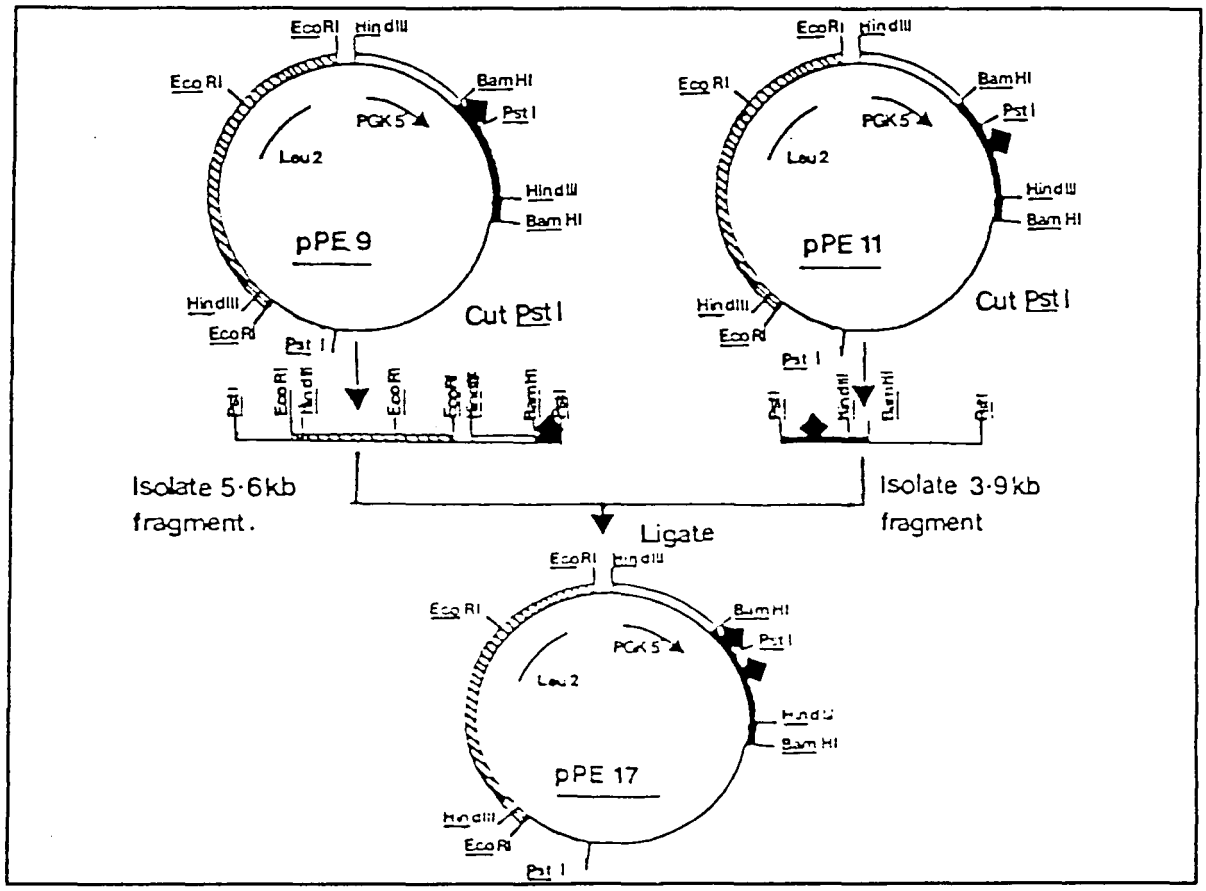

Figure 15. *Construction of pPE17.*

After transformation into *E. coli* 910 a number of transformants were analysed by restriction analysis and Southern blotting using labelled oligos 2 and 3 as probes. Figure 16 shows one such Southern blot from which sample 1 was selected as being a correct cysteine double mutant of vicilin (sample 4 is similar). Sample 1 shows a positive hybridisation of the 1.4kb *Bam* HI fragment against oligonucleotides 2 and 3, positive hybridisation of the 5.8kb *Pst* I fragment against oligonucleotide 2 and positive hybridisation of the 4.0kb *Pst* I fragment against oligonucleotide 3[3]. This construct was designated pPE17 and was fully sequenced to confirm that the mutations, the rest of the vicilin sequence and the PGK promoter sequence were correct.

The location of the hybridisation sites for oligonucleotides 2 and 3 within the final vicilin construct, the alteration to the amino-acid sequence of the vicilin produced and the extent of the sequencing carried out on pPE17 has been summarised in figure 17.

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[3]See also figure 6 in chapter three.

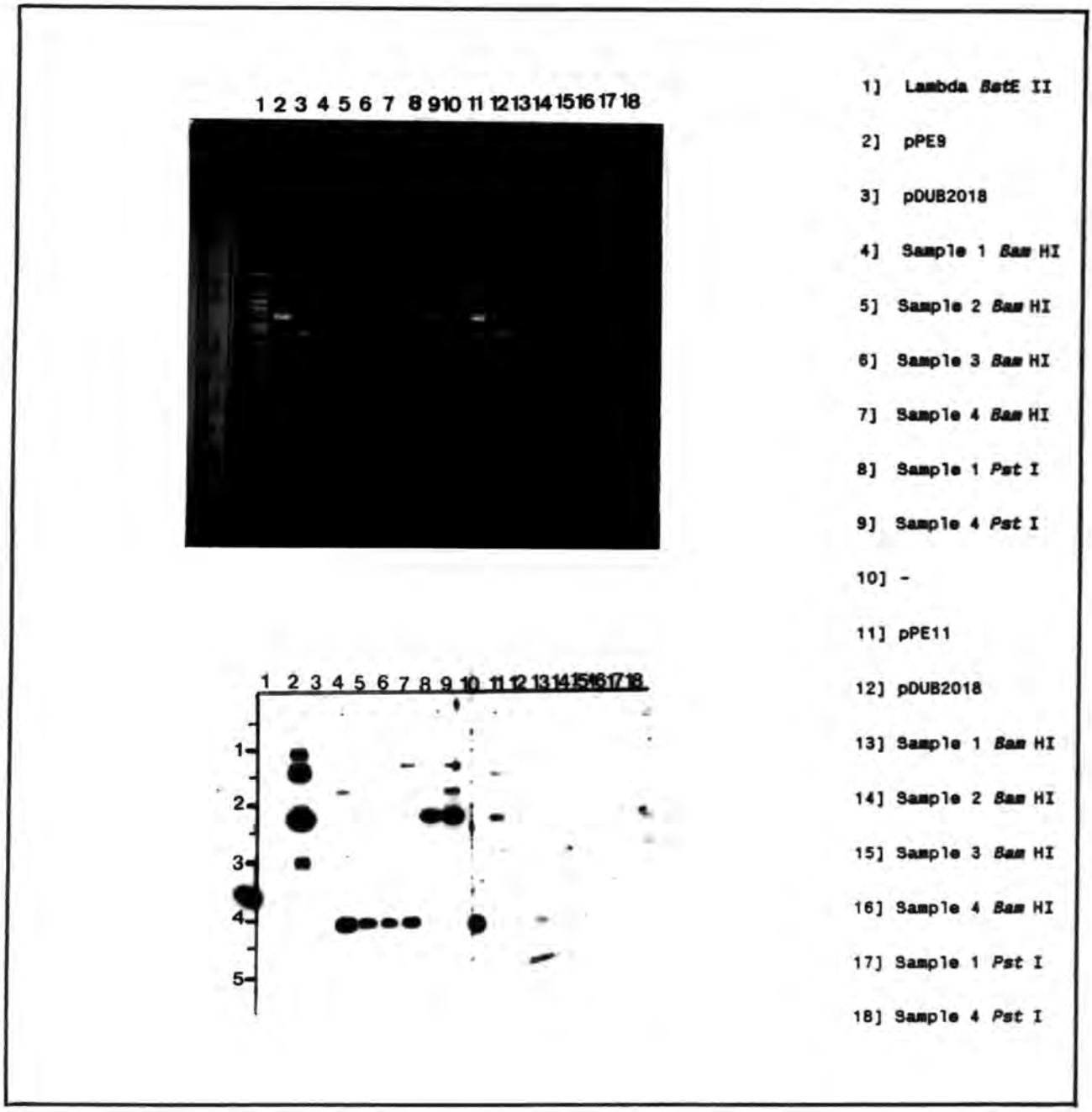


Figure 16. *Agarose gel and subsequent Southern blot used to identify pPE17. Lanes 1 to 9 of the Southern blot were probed with labelled oligonucleotide 1 and lanes 11 to 18 were probed with labelled oligonucleotide 2. Sample 1 was selected and designated pPE17.*

10µg of pPE17 plasmid DNA was prepared from a 200 ml culture of and used to transform an aliquot of competent *S.cerevisiae* MC16 cells as described in chapter 2. The transformed *S.cerevisiae*

were again found to express vicilin as determined by an antibody screen.

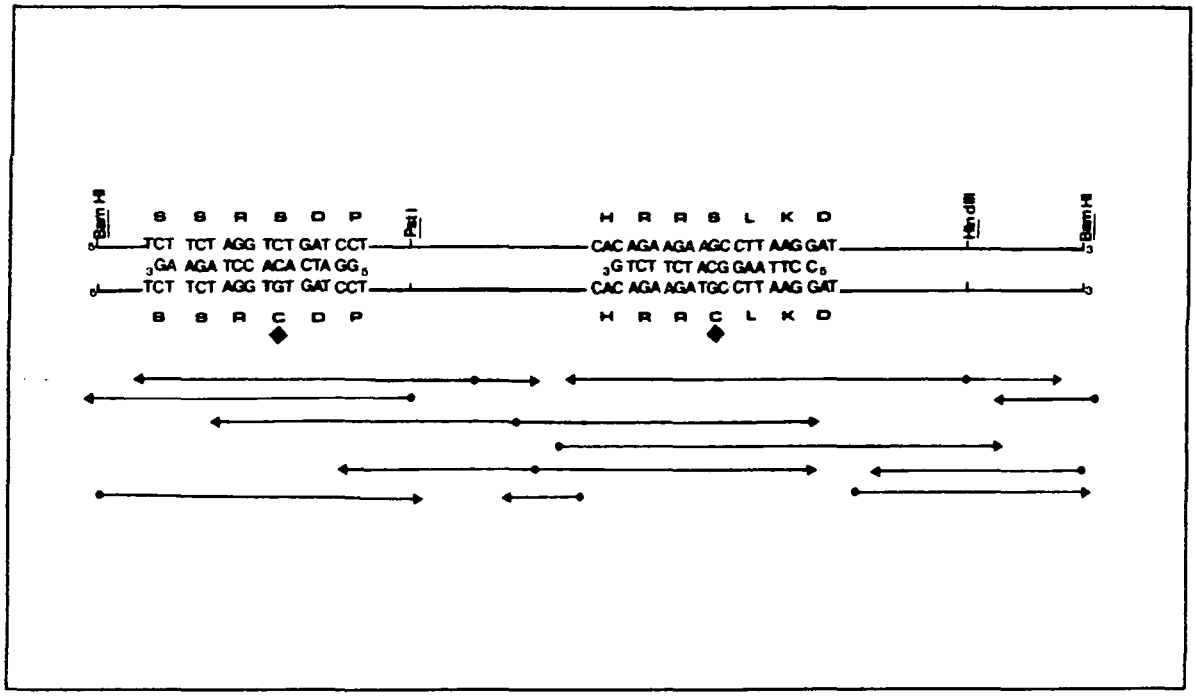


Figure 17. *Position of oligonuceotides 2 and 3 within the 1.4 Kb Bam HI insert of pPE17 and sequencing strategy used to confirm construct. Not to scale.*

Small scale protein extracts of pPE17 protein were prepared under denaturing conditions and were analysed by SDS-PAGE and Western blotting as shown in figure 18. Vicilins were only synthesised by constructs containing the cDNA in the 5'-3' orientation (construct pPE19 is identical to pPE 17 but with the vicilin cDNA cloned in the 3'-5' orientation with respect to the PGK promoter). Under non-reducing conditions an additional vicilin component of greater mobility was seen with protein produced by the pPE17 construct. This was not seen under reducing conditions and was not seen with protein produced by the pDUB2018 construct.

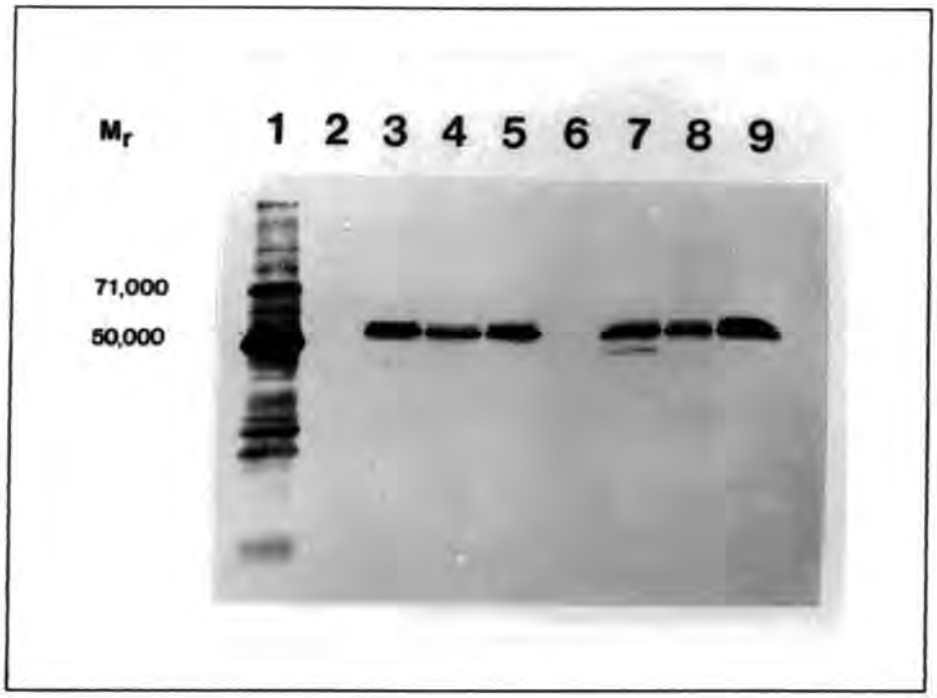


Figure 18. *Western blot analysis of various small scale protein extracts from yeast. Track 1, vicilin and convicilin Mr standards. Tracks 2,3,4 and 5 were run in the presence of 2-mercaptoethanol. Tracks 6,7,8 and 9 were run under non reducing conditions in the absence of 2-mercaptoethanol. Each of tracks 2-9 contains 50µg of yeast soluble extract. Tracks 2 and 6, pPE19. Tracks 3 and 7, pPE17. Tracks 4 and 8, pPE9. Tracks 5 and 9, pDUB2018.*

The soluble extracts of 500ml cultures of pDUB2018 and pPE17 were subjected to a 45-95% relative saturation $(NH_4)_2SO_4$ fraction as described in chapter 2. In this case the protein was extracted under non-denaturing conditions. The 95% saturation $(NH_4)_2SO_4$ pellets were dissolved in 10 ml of 150 mM sodium phosphate buffer pH7.5 and dialysed against 10 mM sodium phosphate buffer pH7.5 for 24 hours. These extracts were then fractioned on a hydroxyapatite column. Under the conditions employed, vicilin was the only component in the extracts to bind to the column and was eluted with a phosphate gradient (see figure 19). Protein was

estimated by ultraviolet absorption using an extinction coefficient E_{280} 1cm of 0.8 for a 1 mg ml^{-1} solution of vicilin.

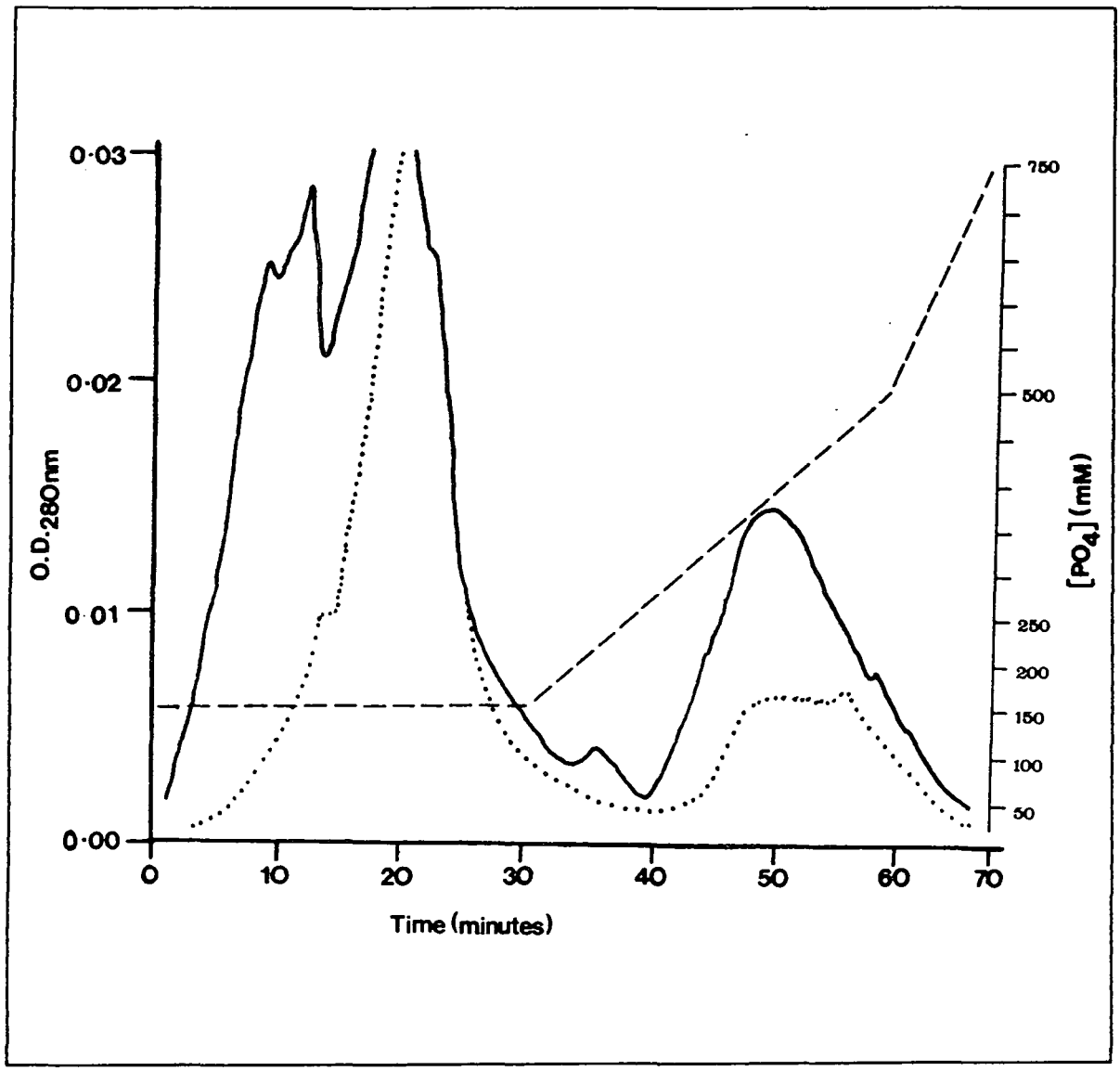


Figure 19. *Purification of expressed pPE17 and pDUB2018 vicilin by HA-Ultrogel column chromatography. See text for details.*

———, A_{280} pDUB2018 eluate; ·····, A_{280} pPE17 eluate;

----, concentration of sodium phosphate.

Both pDUB2018 and pPE17 vicilin were eluted as the phosphate concentration was increased to around 250mM (the second peak in figure 19 in each case). The first peak in each case represents unbound material.

Purified pDUB2018 and pPE17 vicilins were shown to be homogeneous in each case by SDS-PAGE, migrating as single species with an Mr of approximately 53,000 as shown in figure 20. It was estimated by Dr Jenny Yarwood that 50% of the total sulphur containing groups in pPE17 vicilin were present as free sulphydryl groups under strongly denaturing conditions by the method of Thannhauser *et al*, 1987.

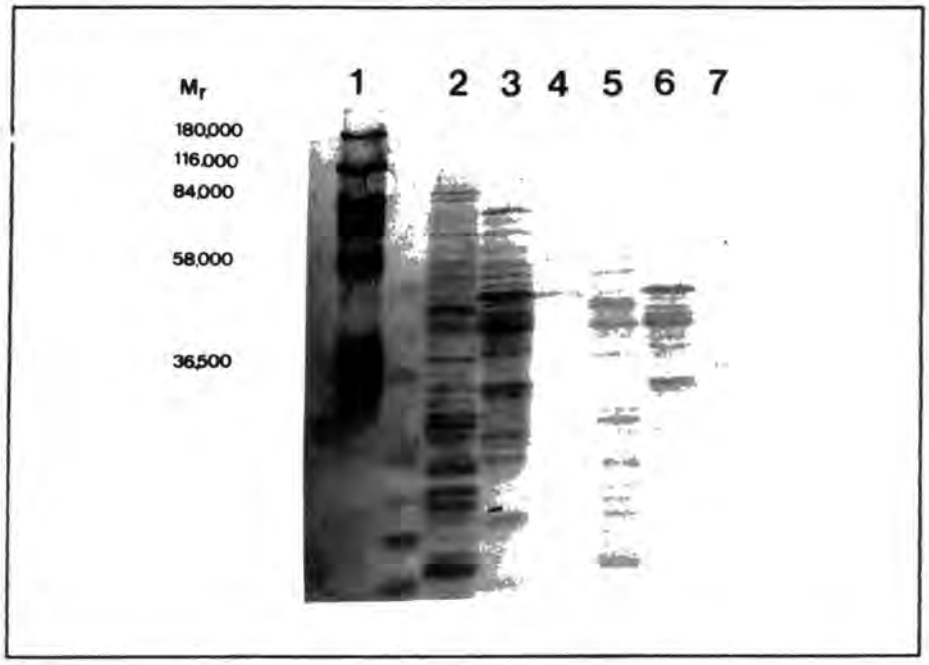


Figure 20. *SDS-PAGE analysis of fractions from the purification of vicilin from yeast. Samples were analysed in the presence of 2-mercaptoethanol as indicated in the text. Track 1 contains Mr standard proteins (10µg of each). Track 2, pDUB2018 yeast soluble extract (100µg). Track 3, 45-95% saturation ammonium sulphate fraction of pDUB2018 protein. Track 4, bound peak from HA-Ultrogel column of pDUB2018 protein. Track 5, pPE17 yeast soluble extract (100µg). Track 6, 45-95% saturation ammonium sulphate fraction of pPE17 protein. Track 7, bound peak from HA-Ultrogel column of pPE17 protein.*

Analysis of these protein samples by SDS-PAGE and Western blot analysis under non-reducing conditions revealed the formation of specific high molecular weight aggregates by the pPE17 protein as shown in figure 21. These aggregates, present in the yeast soluble extract, had estimated Mr values of 150,000 and approximately 300,000; further species of Mr > 300,000 and between 150,000 and 300,000 were present in purified protein. No such aggregates were seen with protein samples from pDUB2018.

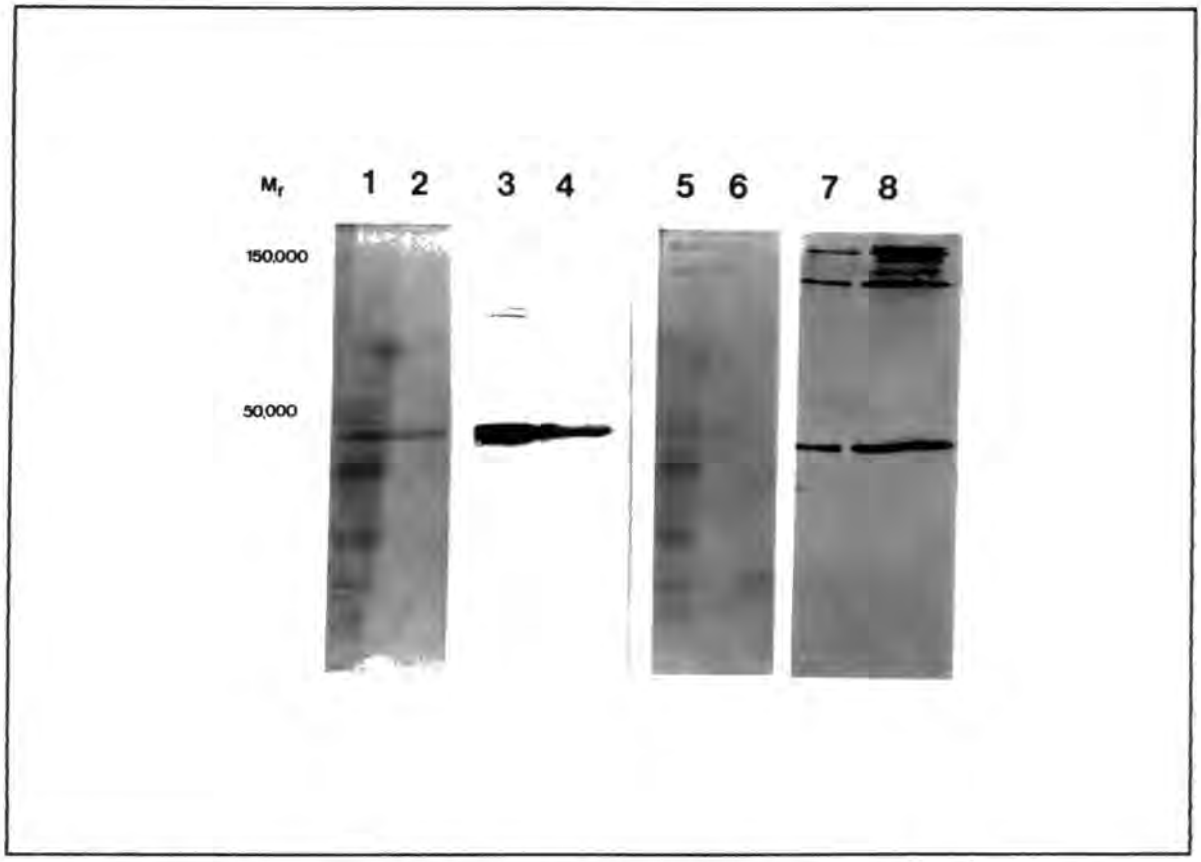


Figure 21. *SDS-PAGE and western blot analysis of pDUB2018 and pPE17 vicilin under non reducing conditions. Track 1, SDS-PAGE of pDUB2018 total soluble extract (100µg). Track 2, SDS-PAGE of HA-Ultogel purified pDUB2018 protein (30µg). Track 3, western blot of pDUB2018 total soluble extract (100µg). Track 4, western blot of HA-Ultrogel purified pDUB2018 protein (30µg). Track 5, SDS-PAGE of pPE17 total soluble extract (100µg). Track 6, SDS-PAGE of HA-Ultogel purified pPE17 protein (30µg). Track 7, western blot of pPE17 total soluble extract (100µg). Track 8, western blot of HA-Ultogel purified pPE17 protein (30µg).*

Finally, the gelling properties of pPE17 and pDUB2018 vicilins were compared by following the gel turbidity of gelling solutions of vicilin at increasing temperatures as described in chapter 2. It can be seen from figure 22 that the gelling profiles of the two proteins are similar.

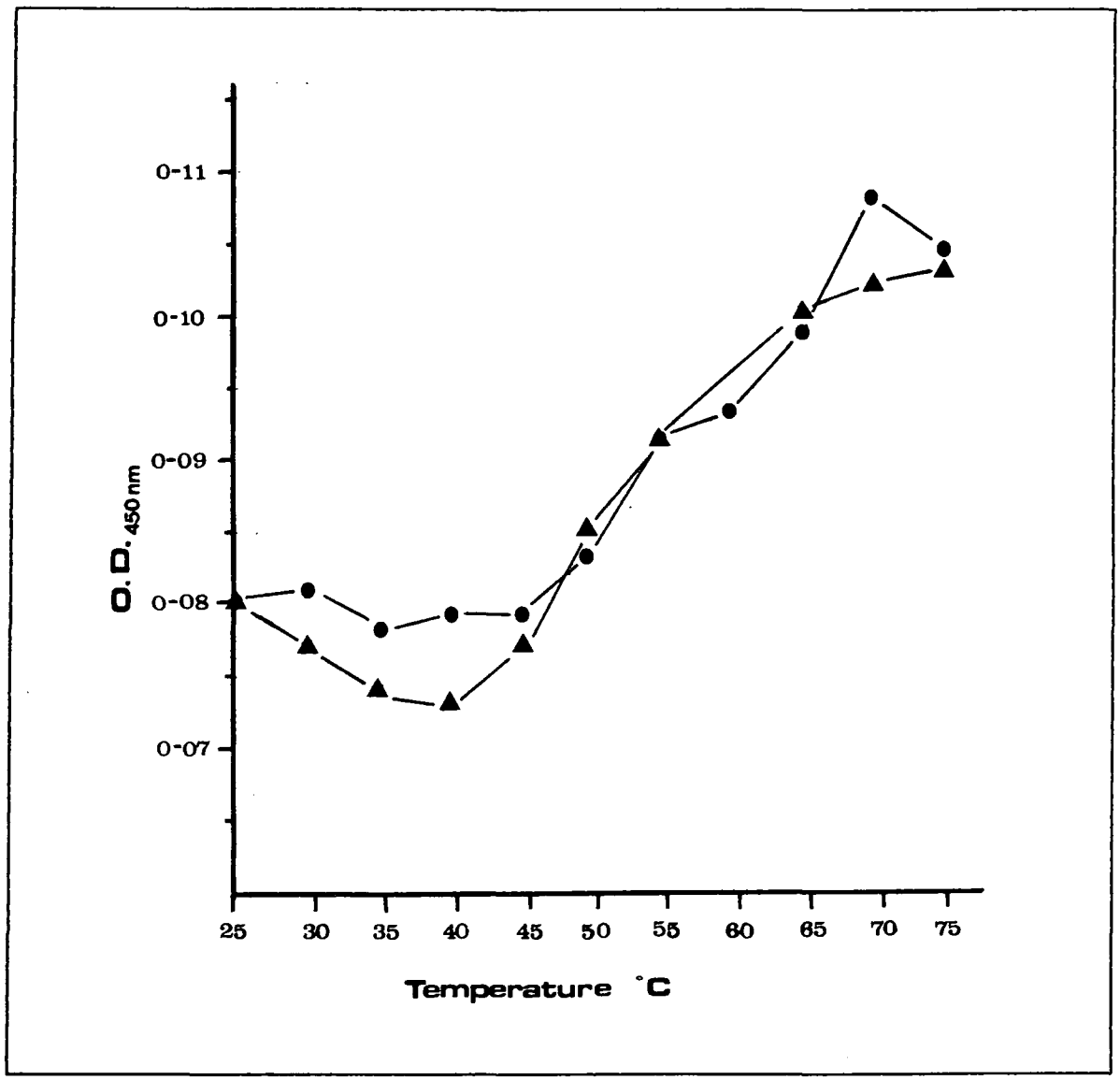


Figure 22. *Comparison of Gelling Characteristics of pDUB2018 and pPE17 Vicilins. The turbidity of 1mg/ml solutions of vicilin has been recorded for increasing temperatures as described in chapter 2.*

● pDUB2018 vicilin. ▲ pPE17 vicilin.

CHAPTER FIVE

DISCUSSION

5.1 Expression of Mutant Vicilins by *S.cerevisiae*.

A vicilin cDNA has been mutated to contain a methionine residue, one of two cysteine residues or two cysteine residues. These constructs have been expressed in yeast using the PGK expression vector pMA257. The encoded proteins migrated as single species with Mrs of approximately 53,000 in the presence of 2-mercaptoethanol as did the non-mutant vicilin produced by pDUB2018. The level of vicilin expression in both cases was approximately 5% of soluble protein as determined by direct protein assay. This is a similar level of expression to other heterologous proteins produced from the PGK promoter in yeast (Tuite *et al*, 1982).

5.2 The Methionine Mutant of Vicilin.

The purpose of the pPE18 construct was to produce a vicilin clone with an easily identified physical change from native vicilin. This was to test the application of the mutagenesis system for producing altered homogeneous proteins. Thus the chances of success for studying structure/function relationships using this technique could be assessed. CNBr is known to cleave polypeptides after methionine residues. As already mentioned, amino acid analysis has confirmed that vicilin contains no sulphur-containing amino acids such as cysteine or methionine (Gatehouse *et al*, 1984). The incorporation of a single methionine residue into vicilin should render the protein cleavable by CNBr and provide evidence at the protein level that the mutagenesis has been successful.

The incorporation of a single methionine residue at amino acid position 282 of pea vicilin in pPE18 did not alter the level of vicilin expression by transgenic *S.cerevisiae*. Leucine to methionine changes are not, in fact, uncommon in nature and these residues have similar properties. The mutated vicilin cDNA was successfully expressed in yeast using the PGK expression vector pMA257. The mutant construct (pPE18), like the original construct pDUB2018 and the cysteine mutants pPE9, pPE11 and pPE17, produced vicilin that was localised in the cytosol of the yeast and which migrated as a single species in SDS-PAGE with an M_r of approximately 53,000. This is higher than the 50,000-M_r subunit of pea vicilin but is most probably due to the additional 22

amino acid residues (approximately 3,000 M_r) at the N-terminal end encoded by the fusion constructs (Watson *et al*, 1988).

The subcloning and mutagenesis route used to construct the mutant along with the restriction analysis and DNA hybridisation carried out is illustrated by figures 1 to 6. The expression of vicilin by pPE18 was first detected by the antibody screen shown in figure 7.

The successful mutation was first indicated by the dot-blot hybridisation and Southern blot shown in figures 3 and 5 respectively. This was later confirmed by dideoxy sequencing.

After demonstrating production of vicilin by pPE18 transformed *S.cerevisiae* (figure 7) the effect of CNBr on protein extracts was tested. It was found that the vicilin produced by pPE18 was cleaved into two subunits ~~of approximate M_r 27,000~~ by CNBr (figure 8). Such cleavage did not occur with pDUB2018 vicilin which, other than the absence of a methionine residue at position 282, was identical to pPE18 vicilin. Vicilin was not produced by the construct not containing the vicilin insert (pMA257) or by constructs containing the vicilin insert cloned in the opposite orientation for expression by the PGK promoter.

Having demonstrated the successful site-directed mutation of vicilin, both at the gene level and by its effect on the protein expressed, the introduction of amino acids thought likely to

effect a change on the physical properties of vicilin was considered.

5.3 The Cysteine Mutants of Vicilin.

The incorporation of cysteine residues into vicilin provides the basis for cross-linking via S-S bonds. Serine to cysteine changes were made as these required only single base changes at the DNA level and because serine and cysteine residues are of similar size.

In the intermediate construct pPE9 the single serine residue at position 2 of the mature vicilin polypeptide was mutated to cysteine whilst in pPE11 the alteration was made to the serine at position 183, 3 amino acids N-terminal to the potential α:ß cleavage site. Both serine 2 and serine 183 are located in hydrophilic regions of the vicilin polypeptide, which are likely to be on the surface of the vicilin molecule; in addition the proximity of serine 183 to the potential proteolysis site suggests that this residue is exposed. The hybrid construct pPE17 contains both mutations and was constructed by ligation of the 5' coding region from pPE9 to the 3' coding region of pPE11 through a *Pst* I site in the vicilin cDNA sequence and a *Pst* I site in the vector.

The construction of pPE9, pPE10, pPE11 and pPE12 is illustrated by figures 9 - 12. Once again vicilin was produced only by constructs containing the vicilin insert in the correct

orientation for expression from the PGK promoter. The 53,000 M_r vicilin produced by pPE9, pPE11 and pDUB2018 can be seen on the protein gel shown in figure 13. The western blot (also shown in figure 13) shows this more clearly. This figure also illustrates that pPE10, pPE12 and pMA257 transformants do not produce vicilin.

As the two cysteine residues were placed in (hopefully) exposed regions of the vicilin peptide it was thought possible that cysteine residues might enable sulphydryl bonding between neighbouring vicilin molecules and so lead to dimers of vicilin being formed. However, no such dimers have been seen by SDS-PAGE under non-denaturing, denaturing, non-reducing or reducing conditions. Figure 14 shows a western blot of pPE9, pPE11 and pDUB2018 separated by SDS-PAGE under both reducing and non-reducing conditions (+/- 2-mercaptoethanol). It can be seen that all the vicilin molecules behave in a similar manner and that the introduction of single cysteine residues has had no apparent effect on the vicilin produced.

The hybrid construct, pPE17, was made in order to determine whether or not intra-molecular sulphydryl bonds could be formed by a mutant vicilin. Figures 15, 16 and 17 show this construction.

SDS-PAGE analysis of pPE17 yeast soluble extracts produced under denaturing conditions revealed a vicilin component of greater mobility produced only by the pPE17 construct (figure 18). This

component was seen only under non-reducing conditions and is thus produced by intramolecular disulphide bonding within the vicilin polypeptide. However, this component was only a small proportion of the total vicilin (<10%) reflecting the reducing environment inside the yeast cell. The vicilins produced by transgenic yeast have previously been shown to be located in the yeast cytoplasm.

pPE17 and pDUB2018 vicilins from non-denatured extracts of 500 ml YMM cultures were successfully purified by hydroxyapatite column chromatography. The binding and elution conditions for both proteins were similar and a shouldered elution peak characteristic of vicilin was seen in both cases (figure 19). In both cases purification was to a single band of M_r 53,000 as seen after SDS-PAGE. It was thought that the non-denaturing conditions used for protein extraction in this case would maximise the chances of seeing sulphydryl bonding in the purified protein.

When vicilin was extracted from yeast under these non-denaturing conditions, formation of disulphide-bonded molecular aggregates of vicilin polypeptides was observed on non-reducing SDS-PAGE gels (Figure 21 tracks 5 and 7). Aggregation is clearly not a random process since in the extract only aggregates corresponding to trimers and hexamers of the vicilin polypeptides were observed. No band corresponding to the dimer of vicilin polypeptides (Mr approximately 100,000) was observed in the yeast extract or the purified protein. The purification protocol employed to yield pure vicilin was oxidative and formation of

additional disulphide bonded aggregates was observed after purification (Figure 21 tracks 6 and 8). However, trimers were still the major aggregated components present. In both extract and purified vicilin approximately 40% of the protein was present as disulphide-bonded aggregates. Pea vicilin *in vivo* exists as a trimeric molecule (and as aggregates of these trimers). Although the vicilin polypeptides produced in yeast have not been shown to have the same conformation as vicilin purified from pea seeds, they are nevertheless capable of forming trimeric molecules.

It was hoped that the introduction of cysteine residues into vicilin would affect the gelling characteristics of the protein. Evidence has been given that the content and reactivity of sulphydryl groups in glycinin relate to the strength and turbidity of glycinin gels (Nakamura *et al*, 1984). However, turbidity studies on the purified vicilin subunits produced by yeast have not revealed any significant difference between the non-mutant vicilin produced by pDUB2018 and the cysteine-containing vicilin produced by pPE17 (figure 22). Nor were any differences detected between the gelling characteristics of pPE9, pPE11 and pDUB2018 vicilins.

It may be that the cysteine residues introduced into the vicilin subunit are not well placed on the surface of the molecule to be reactive, thus they only result in small differences in gel turbidity.

This project has shown that *in vitro* mutagenesis can be used successfully to alter the amino acid sequence of a cloned protein and change its physical properties as a result. A mutant vicilin has been produced which contains two cysteine residues and is capable of aggregation via disulphide bonding. The door has been opened for further physical investigation of this protein and comparison with the non-mutant vicilin. Gel-filtration studies will more accurately reveal the nature of the oligomers produced. Circular dichroism analysis may give insights into the way the mutant vicilin molecule is folded and the technique of scanning tunnelling microscopy could be used to compare the actual physical appearance of mutant and non-mutant vicilin molecules.

Facilities for this work are not available at Durham Universiy but it is hoped that these studies may be carried out at the AFRC Research Institute in Norwich. Certainly *in vitro* mutagenesis is a viable technique for studying the relationship between a protein's physical properties and its primary structure.

APPENDIX I

SEQUENCE OF VICILIN CDNA INSERT AND CORRESPONDING AMINO ACID SEQUENCE. ALSO SHOWS SITES OF HYBRIDISATION OF OLIGONUCLEOTIDES 1, 2 AND 3 USED FOR THE METHIONINE AND CYSTEINE MUTATIONS DESCRIBED IN THE TEXT.

```
                                  oligonucleotide 2
                 Bam HI           GA AGA TCC ACA CTA GG
cDNA     GG ATC CGG TTT TCT TCT AGG TCT GAT CCT CAA AAT CCT TTT ATC TTC AAG TCT AAC AAG TTT CAA ACT CTT TTT GAG      68
A.A.                S  S  R  S  D  P  Q  N  P  F  I  F  K  S  N  K  F  Q  T  L  F  E
                                 N-terminus ▼
                                            C
                                        Pst I
cDNA     AAT GAA AAT GGG CAC ATT CGA CTT CTG CAG AAA TTT GAC CAA CGT TCT AAA ATT TTC GAG AAT CTA CAA AAC TAC CGT     146
A.A      N  E  N  G  H  I  R  L  L  Q  K  T  D  Q  R  S  K  I  F  E  N  L  Q  N  Y  R

cDNA     CTT TTG GAA TAT AAG TCC AAA CCT CAC ACA ATA TTT CTT CCA CAG CAC ACC GAT GCC GAT TAC ATC CTT GTT GTA CTC     224
A.A.     L  L  E  Y  K  S  K  P  H  T  I  F  L  P  Q  H  T  D  A  D  Y  I  L  V  V  L

cDNA     AGT GGA AAA GCT ATA CTC ACA GTG TTG AAA CCC GAT GAT AGA AAC TCC TTC AAC CTT GAG CGC GGA GAT ACG ATA AAA     302
A.A.     S  G  K  A  I  L  T  V  L  K  P  D  D  R  N  S  F  N  L  E  R  G  D  T  I  K

                                                                          Bgl II
cDNA     CTT CCT GCT GGC ACA ATT GCT TAT TTG GTT AAC AGA GAT GAC AAC GAG GAG CTT AGA GTA TTA GAT CTC GCC ATT CCC     380
A.A.     L  P  A  G  T  I  A  Y  L  V  N  R  D  D  N  E  E  L  R  V  L  D  L  A  I  P

cDNA     GTA AAT AGA CCT GGC CAA CTT CAG TCT TTC TTA TTG TCT GGA AAT CAA AAC CAA CAA AAC TAC TTA TCT GGG TTC AGT     458
A.A.     V  N  R  P  G  Q  L  Q  S  F  L  L  S  G  N  Q  N  Q  Q  N  Y  L  S  G  F  S

cDNA     AAG AAC ATT CTA GAG GCT TCC TTC AAT ACT GAT TAT GAA GAG ATA GAA AAG GTT CTT TTA GAA GAG CAT GAG AAA GAG     536
A.A.     K  N  I  L  E  A  S  F  N  T  D  Y  E  E  I  E  K  V  L  L  E  E  H  E  K  E

                                  oligonucleotide 3
                                  G TCT TCT ACG GAA TTC C
cDNA     AAA GAG ACA CAA CAC AGA AGA AGC CTT AAG GAT AAG AGG CAG CAA AGT CAA GAA GAG AAT GTA ATA GTA AAA TTA TCA     608
A.A.     K  E  T  Q  H  R  R  S  L  K  D  K  R  Q  Q  S  Q  E  E  N  V  I  V  K  L  S
                               ▼
                               C
                                  α:β proteolysis site

cDNA     AGG GGA CAA ATT GAG GAA TTG AGT AAA AAT GCA AAG TCT ACC TCC AAA AAA AGT GTT TCC TCT GAA TCT GAA CCA TTC     686
A.A.     R  G  Q  I  E  E  L  S  K  N  A  K  S  T  S  K  K  S  V  S  S  E  S  E  P  F

cDNA     AAC TTG AGA AGT CGC GGT CCT ATC TAT TCC AAC GAG TTT GGA AAA TTC TTT GAA ATC ACC CCA GAG AAA AAT CCA CAG     764
A.A.     N  L  R  S  R  G  P  I  Y  S  N  E  F  G  K  F  F  E  I  T  P  E  K  N  P  Q
```

oligonucleotide 1

CTT AGA AAT TAC AAC GG

cDNA CTT CAA GAC TTG GAT ATA TTT GTC AAT TCT GTA GAG ATT AAG GAG GGA TCT TTA TTG TTG CCA CAC TAC AAT TCA AGG 842
A.A. L Q D L D I F V N S V E I K E G S L L L P H Y N S R

▼
N

cDNA GCC ATA GTA ATA GTA ACA GTT AAC GAA GGA AAA GGA GAT TTT GAA CTT GTG GGT CAA AGA AAT GAA AAC CAA CAA GAG 920
A.A. A I V I V T V N E G K G D F E L V G Q R N E N Q Q E

cDNA CAG AGA AAA GAA GAT GAC GAG GAA GAG GAA CAA GGA GAA GAG GAG ATA AAT AAA CAA GTG CAA AAT TAC AAA GCT AAA 998
A.A. Q R K E D D E E E E Q G E E E I N K Q V Q N Y K A K

B:τ protgolysis site

Hind III

cDNA TTA TCT TCA GGA GAT GTT TTT GTG ATT CCA GCA GGC CAT CCA GTT GCC CTA AAA GCT TCC TCA AAT CTT GAT TTG CTT 1076
A.A. L S S G D V F V I P A G H P V A L K A S S N L D L L

cDNA GGG TTT GGT ATT AAT GCT GAG AAC AAT CAG AGG AAC TTT CTT GCA GGC GAT GAG GAT AAT GTG ATT AGT CAG ATA CAG 1154
A.A. G F G I N A E N N Q R N F L A G D E D N V I S Q I Q

cDNA CGA CCA GTG AAA GAG CTT GCA TTC CCT GGA TCA GCT CAA GAG GTT GAT AGG ATA CTA GAG AAT CAG AAA CAA TCC CAC 1232
A.A. R P V K E L A F P G S A Q E V D R I L E N Q K Q S H

cDNA TTT GCA GAT GCT CAA CCT CAA CAA AGG GAG AGA GGA AGT CGT GAA ACA AGA GAT CGT CTA TCT TCA GTT TGA AATGTTT 1311
A.A. F A D A Q P Q Q R E R G S R E T R D R L S S V *

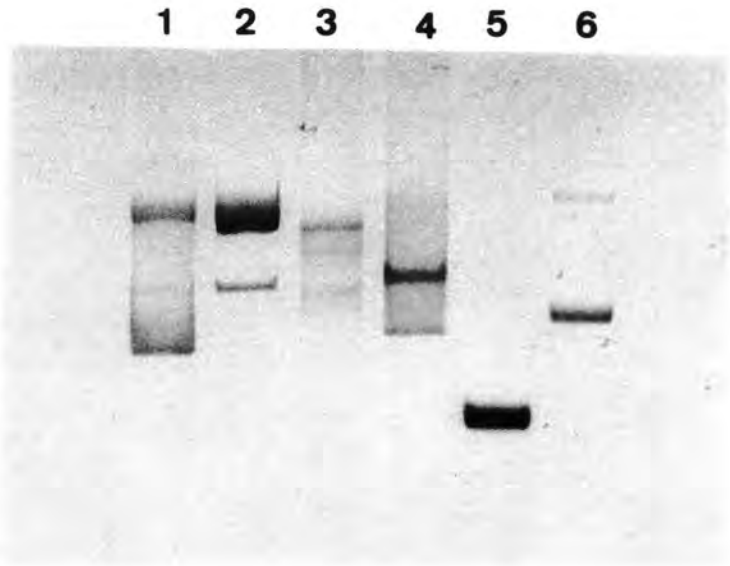
cDNA CTTAATGAGTGGACAAAATACTATGTATGTATGCTATCAAGAGATATATCTCACGGGGAGCAATGAATAAAACAATGTTATCTTATAACTATAATTATATATC 1414

Bam HI

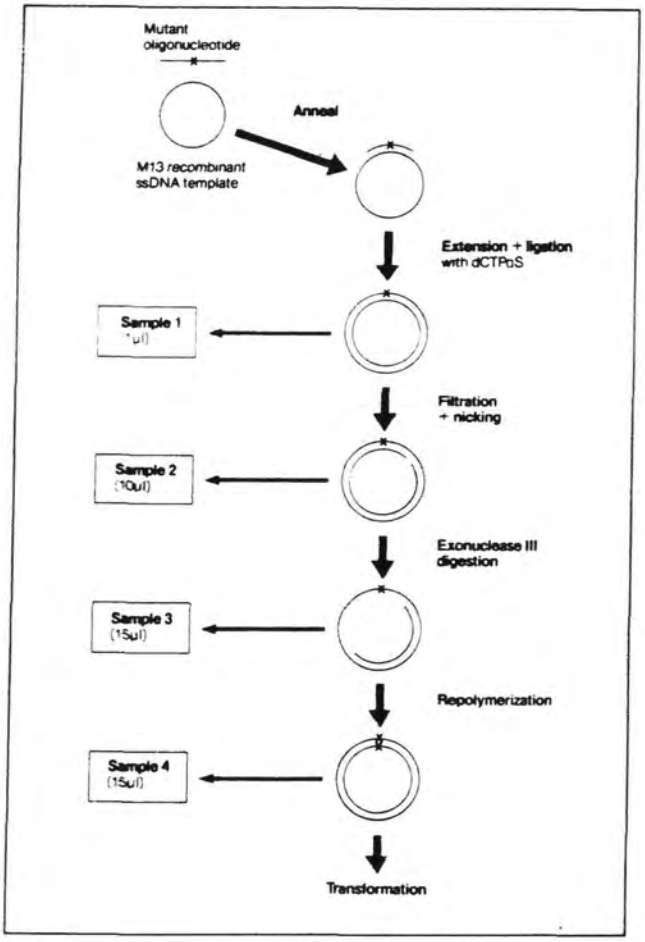
cDNA CACTTTTCTACTATGAATA 1433.

APPENDIX II

EXAMPLE OF A SUCCESSFUL *IN-VITRO* MUTAGENESIS REACTION (SEE SECTION 2.11 FOR DETAILS).



KEY:



Lane 1, SAMPLE 1; shows covalently closed circular (ccc) DNA and a smear of nicked DNA.

Lane 2, SAMPLE 2; shows heavy band of nicked DNA and some ccc DNA.

Lane 3, SAMPLE 3; shows DNA after exonuclease digestion.

Lane 4, SAMPLE 4; shows DNA after repolymerisation.

Lane 5, Single-stranded M13 DNA.

Lane 6, Double-stranded M13 DNA.

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