The synthesis and processing of pea legumin in saccharomyces cerevisiae

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THE SYNTHESIS AND PROCESSING OF PEA LEGUMIN IN *SACCHAROMYCES CEREVISIAE*

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DISSERTATION SUBMITTED IN PARTIAL
FULFILLMENT OF REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BOTANY
UNIVERSITY OF DURHAM
OCTOBER 1987

ABSTRACT

An expression vector utilising the promoter from the yeast phosphoglycerate kinase gene and a complete protein-encoding sequence, was used to direct the expression of the pea seed storage protein, legumin, in yeast (*Saccharomyces cerevisiae*). Levels of expression of between 1.95 and 2.24% total cellular protein were obtained. The legumin polypeptide expressed in yeast has an estimated M_r. of 59,000. In plants, the legumin precursor polypeptide is processed by co-translational proteolysis to remove a "leader" sequence and by post-translational proteolysis to generate the mature disulphide-linked α + β polypeptides (approximate M_r.'s of 38,000 and 21,000 respectively).

Although "leader" sequence removal in yeast may take place, post-translational proteolysis to generate the two smaller sub-units does not occur.

CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. Jenny Yarwood, for introducing me to the Western blotting technique, for providing me with the transformed yeast, for her helpful advice throughout this project and for reading my early drafts.

My special thanks to Dr. John Gatehouse, for critical readings of my early drafts.

I also wish to thank Dr. Angray Kang, for demonstrating the ELISA technique and John Gilroy for the protein purification and amino acid sequencing techniques.

Finally, thanks to N. Appleby and the members of laboratory D2 for their help.

This work is dedicated to my parents and my sister, Alice, for her constant encouragement and support.

ABBREVIATIONS

The one-letter notations for amino acids are given in Biochemical Journal (1969) 113, 1-4.

The following abbreviations were used:

ABTS	2'2'Azinobis 3'-Ethyl benze-Thiozoline sulpfonic acid
cDNA	complementary DNA
DAB	3'3'Diaminbenzidine
DABITC	4-N'N'-dimethylamino azobenzene 4-isothiocyanate
DABTC	4-N'N'-dimethylamino azobenzene 4' Thiocarbonyl
DABTZ	4 N'N'-dimethylamino azobenzene 4'Thizolinol
DABTH	4 N'N'-dimethylamino azobanzene 4'Thiohydantoin
DAF	Day after flowering
ELISA	Enzyme-linked immunosorbent assay
E.coli	*Escherichia coli*
GADPH	glyceraldehyde-3P dehydrogenase gene
HPLC	High performance liquid chromatography
M_r	Relative molecular weight
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PGK	Phosphoglycerate kinase
PMSF	Phenylmethyl sulphonylfluoride
PITC	Phenylisothiocynate
SDS	Sodium docedyl sulphate
TEMED	Tetramethylenediamine

TLC	Thin-layer chromatography
TFA	Trifluoroacetic acid
uv	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
YEM	Yeast-enriched medium
YMM	Yeast minimal medium

CHAPTER ONE

1 Introduction

1.1 Introduction to seed storage protein

Seed storage proteins are generally defined as:

(1) Proteins which are synthesized at a specific stage of seed development.

(2) Proteins stored in membrane-bound organelles ie. protein bodies.

(3) Proteins that survive desiccation during seed maturation and are subsequently hydrolysed during germination to provide carbon and nitrogen sources for the developing seed.

These proteins usually accumulate to a level which constitutes up to 50% of total seed protein. The majority of proteins are globulin-soluble in dilute salt solutions. There is a significant amount of water-soluble albumin protein present. The globulin fraction has been extensively characterised, particularly in nutritionally important legume and oil seeds. The storage proteins of the legumin family *Faboideae* make up one of the most complex protein systems, mainly by virtue of their extensive post-translational modifications (Croy and Gatehouse,

(9)), as exemplified by pea (*Pisum savitum L.*) and broad bean (*Vicia faba L.*).

Much of the early work on seed storage proteins was done because of their economic value as a major source of proteins for the human diet. Since the elucidation of the basis of genetic inheritance and the advent of techniques for the manipulation of DNA, seed proteins have become the ideal systems to investigate the control of gene expression in eukaryotes. Each protein is the product of a single or small family of genes, which are under strict developmental control, so that these genes are only transcribed in the developing seed at certain stages of development, in specific tissues.

One strategy for investigating the control of gene expression is to isolate a particular storage gene, preferally one known to be efficiently expressed, and to introduce the gene into a suitable host which will continue to recognise the indigenous control system. Mutated genes with altered control sequences can also be produced and used to test the effects of the alteration.

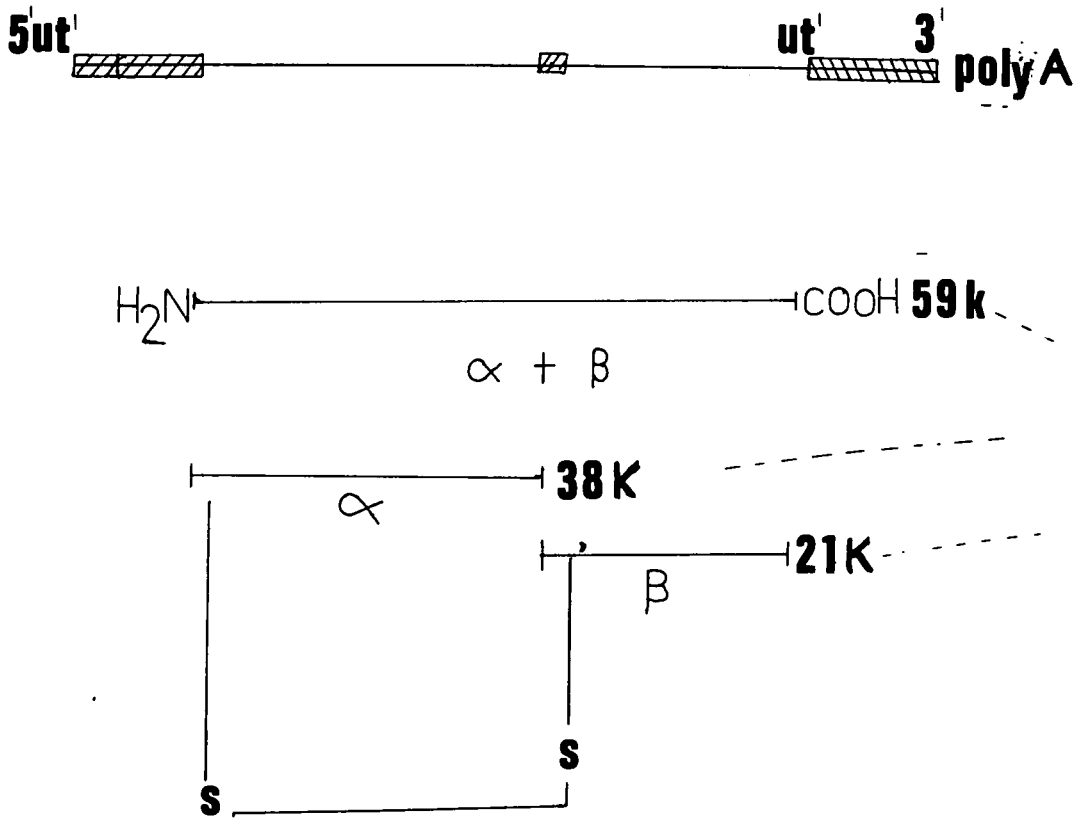
1.2 Legumin

Legumin, a seed storage protein in pea (*Pisum savitum L.*) makes an ideal model. Its properties, synthesis and genetics have been reviewed (Gatehouse *et al*, (16), Ersland *et al*, (15), Casey and Pomorey, (3)).

Futhermore, legumin-encoding genes have been isolated and fully characterised (Croy *et al*, (9), Lycett *et al*, (24), Nielson, (27)).

Legumin is a hexameric protein of M_r. 380,000 - 410,000, consisting of six subunits. Legumin is synthesised as a precursor polypeptide which undergoes co- and post-translational proteolysis. The major legumin species are derived from the Leg A gene sub-family. The precusor polypeptides (M_r. 58,998) are synthesised on polyribosomes attached to endoplasmic recticulum. They undergo post-translational removal of a signal peptide of 21 amino acids, and co-translational sequestration into the lumen of endoplasmic recticulum, followed by formation of oligomers. Transport of these oligomers to the golgi complex may occur via tubular connections between the endoplasmic reticulum and the golgi bodies. From the golgi complex, the storage proteins are transported to the protein bodies. It is in these protein bodies, where proteolytic processing takes place on the C-terminal side of the asparagine 322 to generate the mature disulphide-linked α and β polypeptides (M_r. s of approximately 38,000 and 21,000). Processing occurs within 2 hours of systhesis; therefore the amounts of precusor present in <u>vivo</u> is small (Figure 1 shows the biosynthesis of legumin).

Figure 1 Biosynthesis of legumin Precusor



1.3 Yeast : *Saccharomyces cerevisiae* as a host/vector system

With the advent of recombinant technology, the expression of eukaryotic gene products have been largely concentrated around *Escherichia coli (E. coli)*. However *E. coli* proved to be an unsuitable host vector system. It contains a number of pyrogenic factors that must be eliminated before any potentially useful pharmaceutical products can be synthesized. Higher eukaryotic proteins are not processed or modified accurately by prokaryotic cells. There is now great interest for alternative vector systems. Yeast (*Saccharomyces cerevisiae*) is one of the most useful eukaryotic organisms for the study of the regulation of gene expression and for the expression of introduced foreign DNA sequences. The generation time of yeast is short (a few hours) and its genome is small (1.4×10^4kb). Therefore yeast can be experimentally manipulated as easily as most prokaryotes. At the same time it can be used to study some extremely complex phenomena specific to eukaryotes, including chromosome structure, mitotic and meiotic cell division, RNA splicing, post-translational modification, phosphorylation and glycosylation. There now exist simple and general methods for isolating and amplifying virtually any yeast gene, although these methods generally require an intermediate step in *E. coli*. Futhermore there is the availability of transformation

procedures for introducing exogenous DNA into yeast. This has allowed the development of a variety of plasmid vehicles for cloning (Kingman *et al*, (19), Struhl *et al*, (30), Beggs *et al*, (1)). Powerful and sentitive hybridisation methods have been developed that allow direct physical analysis of any chromosomal regions containing a gene that has been integrated.

A variety of selectable marker systems, for example Leu 2+, His +, Ura 3+ and Trp 1+, have been developed. These markers can be used readily to select for transformants which complement the corresponding mutation in yeast. These technological advances have combined to make truly feasible, molecular, as well as classical genetic manipulations and analyses in yeast. Yeast has recently been used to make significant advances in our understanding of the molecular biology of eukaryotes.

Beggs *et al*, (1) introduced the rabbit β globin gene into yeast on a 2 μm based recombinant molecule. Transcription started at a position downstream from the normal initiation site and terminated prematurely in the second intron. Also, the Herpes simplex virus (Hsv) under the thymidine kinase gene is not expressed in yeast unless it is under the control of the yeast promoter (Kiss *et al*, (20)). Therefore in order to express a higher eukaryotic gene in yeast, the coding sequence lacking introns must be placed under the

control of a yeast promoter.

The yields of heterologous proteins are determined both by the choice of promoter and the type of vector. For example, the "promoter" from the efficiently expressed yeast for PGK (Holland and Holland, (17)) directs the expression of heterologous genes with at least a 500-fold greater efficiency than from inefficient TRP7 promoter (Dobson *et al*, (12), Tuite *et al* (31)). However, a high copy number 2 μm plasmid-based shuttlevector such as PMA 230 or PMA 301 (Tuite *et al*, (31), Mellor *et al* (25)), gives 100-fold higher yield than the unstable copy number ARS-based plasmid (Hitzeman *et al*, (18)) or the low copy number ARS CEN plasmid.

Several plant proteins have been shown to undergo signal peptide cleavage, glycosylation or both both when synthesized in *S. cerevisiae* (Eden *et al*, (14), Cramer *et al*, (7), (8)). The main advantage of using this system for expression of heterologous eukaryotic protein is the presence of well-characterized pathways for the post-translational alterations and transports of proteins, offering the potential for correct modifications and for intra- extracellular targeting of foreign gene products.

By expressing legumin in microbial eukaryotes, it is possible to examine and compare co-translational and post-translational proteolysis in pea plants and yeast

systems, and to investigate the effects of changes in the sequences of proteins, on transport and processing.

1.4 Construction of PJY 28 Construct

A yeast expression vector containing the complete legumin coding sequence is derived from several short cDNA species. Previous studies have failed to produce a full length LegA cDNA species. The construction of a hybrid sequence designated PJY 28 is shown in Appendix I.

The region of amino acid 3 to 67 was derived by excision of an appropriate fragment from a genomic subclone of the Leg A gene pRC 6.2 (Lycett *et al*, (23)). The region of amino acid 68-231 was derived from part of the insert of a cDNA, pAD 10.5 (Lycett *et el*, (24)). These fragments were assembled in pairs to a final ligation to produce the construct PJY 28, a PUC 18 subclone. This construct lacks the first 7 bases of the Leg A coding sequence, which is supplied by addition of a suitable pair of oligonucleotide sequences, containing the missing sequence plus a BAM HI linker in the the appropriate reading frame. The integrity of the coding sequence across the ligation point was checked by DNA sequences. BAM HI was used to cut the insert from PJY 25 and it was ligated into the BGL II expression site of vector pMA 91 (Mellor *et al*, (25)) to produce PJY 28. This construct contains the

yeast phospoglycerate kinase (PGK) promoter including the 5' and 3' untranslated region and 94 base pairs of 3' coding sequence.

PJY 28 was grown in *E. coli*, before transformation into yeast. The construct was checked by restriction analysis. Transformants were identified by selection on yeast minimal media, in the absence of additional leucine and were tested for correct auxotrophy. Single colonies were grown and examined for legumin expression.

1.5 Contruction of pMA expression vector

The sandwich expression plasmid pMA 91 contains sequences from both the 5' and 3' regions of the PGK gene (Mellor *et al*, (26)). The 5' regions im pMA 91 was derived from pMA 30 (Appendix IIa). This plasmid contains about 1.5 kb of the PGK 5' flanking regions, extending to include the nucleotide of position -2 upstream from the initiator ATG. A unique Bgl II expression site was introduced via the synthetic linker 5' -GAAAAGATCTTTTG- 3'. The 3' PGK region was derived from pMA 27 (Appendix IIb). In the contruction of pMA 91, (Appendix IIc) the small Bgl II-Pst I fragment from pMA 301 (Appendix IIa) was replaced with the small Bgl II-Pst I fragment from pMA 27 (Appendix IIb). Bgl II cleaves within the PGK coding sequence. pMA 91 contains 94 bp of coding sequence and 281 bp of 3'

flanking sequence, which has all the signals for efficient transcription termination. In pMA 91 the 5' and 3' regions of the PGK gene are linked via a Bgl II site. The nulceotide sequence around this junction is shown in (Appendix IId). The PJY 28 was ligated into the Bgl II expression of pMA 91 and is sandwiched between the efficient signal for the identification of transcription and translation and for transcription termination.

1.6 Aims of the project

1) To demonstrate the expression of pea legumin in transformed yeast by western blotting.

2) Quantitative determination of legumin produced in transformed yeast using ELISA and western blotting.

3) To investigate the biosynthesis of legumin in different media and at different stages of growth in yeast.

4) Characterization of yeast legumin.

5) To investigate the processing of pea legumin in yeast.

CHAPTER TWO

2 Materials and methods

2.1 Materials

2.1.1 Biochemical and biological reagents

All reagents, with the exception of those listed below, were obtained from BDH Chemical Ltd., Poole, Dorset, U.K. and were of analytical grade or the best avaliable.

Nitrocellulose membrane (BA 85 0.45 uM), from Schleicher and Schull and Co. Ltd., Kingston upon Thames, Surrey, U.K.

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

Bacto-trypton, Bacto-agar and yeast extract, from Difco Laboratories, Detriot, Michigan, USA.

Phenylmethyl sulfonyl fluoride (PMSF), 3,3,Diaminobenzidine, Tricine, glass beads type V (450-500 microns), Bovine serum albumin, L-leucine, D-L histidine, Adenine and 2,2,Azinobis 3-ethyl-benzthiozoline sulfonic acid, from Sigma Chemical Company, Poole, Dorset, U.K.

Micrototer plate (NUNC Immunoplates), from GIBCO, Washington Road, Abbotsinch Industrial Estate, Paisley PA3 4EP.

Dialysis membrane and tubing, from Amersham International PLC, White Lion Road, Amersham, Buckinghamshire HD6LL, U.K.
Sodium chloride from Koch Light Ltd., Haverhill, Suffolk, U.K.
Guanidine isothiocyanate and Guanidine hydrochloride, from Fluka, Switzerland.
DEAE-sepharose, from Pharmacia Fine Chemicals, Uppsala, Sweden.
Goat anti-rabbit IgG horseradish peroxidase conjugate, from Biorad Laboratories, 32nd Griffin Avenue, Richmond, California.

All heat stable solutions were sterilized by autoclaving (15 minutes at 120°C).

PJY 28 yeast, supplied by Dr. J. Yarwood, Durham University Botany Department.
Anti-legumin, was raised in rabbit and purified as described by Croy *et al*, 1980.

2.1.2 Sterilization

All glasswares, plastic-wares, cocktail sticks and media were autoclaved prior to use.

2.1.3 Growth media

Yeast-enriched medium:

Yeast extract	1%
Baoto-peptone	2%
D-glucose	2%

Amino acid supplements

Mc 16 control yeast and PJY 28: Adenine

Yeast minimal medium:

Yeast N-base	0.67%
D-glucose	2%

Amino acid supplements

Mc 16 control yeast: Adenine, Lysine, Histidine and Leucine.

PJY 28 yeast: Adenine, Lysine and Histine.

All amino acids were added to a final concentration of stock solution of 5 μg/ml from a stock solution of 5 mg/ml.

Small scale culture

20 ml of medium in a 100 ml flask were inoculated by using a sterile cocktail stick to pick up a colony of yeast from an agar plate and placing it in the flask containing the medium. The appropriate amino acid supplements as mentioned above were added. During

inoculation, aseptic techniques were used whenever possible. Forceps were flamed with ethanol before being used to pick up any sterile material. Stock solution bottles, flasks containing the sterile media and sterile cocktail sticks were flamed at the neck before and after opening. All transfers to and from agar plates were carried out with minimal contamination possible. After inoculation, the culture flask was attached to a rotating shaking platform (250 rpm) at 30°C for 48 hrs.

2.1.4 Storage

PJY 28 yeast was plated out onto yeast minimal agar and Mc 16 control yeast were plated onto yeast enriched agar. YM agar is yeast minimal medium and yeast enriched-medium both with 2% bacto-agar added (20 g/l) with the appropraite amino acid supplements described in section 2.1.3. Yeast cells grown in agar plates were stored at 4°C sealed with "Nescofilm".

2.2 Biochemical techniques

2.2.1 Extraction of protein

2.2.1.1 Yeast protein

Yeast cells (20 ml) were grown for 48 hours at 30°C in minimal medium until stationary phase was reached. The cells were pelleted by centrifugation (8,000g). The cells were washed once with extractant buffer (50mM Tricine / 20mM NaCl, pH8). The cell pellet was suspended in 200 µl of extractant buffer with 1 µl of leupeptin (3 mg/ml) and 15 µl PMSF (36 mg/ml in dry ethanol). Supernatant protein was extracted by vortexing with equal volumes of acid washed 450 - 500 micron glass beads three times for one minute at 0°C. Insoluble materials were removed by centrifugation for 5 minutes in an Eppendorf micro-centrifuge. To the supernatant, 1/4 volume of 5 x SDS buffer (1M Tris / 10% SDS / 50% sucrose, pH 6.8) was added. Samples were boiled for 2 minutes at 100°C. Pellets associated with the remaining pellet were extracted by boiling with 100 ul of 1 x SDS buffer (0.2M Tris / 2% SDS / 10% sucrose) for 15 minutes. Insoluble materials were removed by centrifugation for 10 minutes in an Eppendorf micro-centrifuge.

Yeast cells were also extracted with extractant buffer containing 6 M urea and 6 M guanidine

isothiocyanate. The procedure of extraction was similar to that described previously, except that after vortexing the cells with acid washed beads, the lysed yeast cells in the Eppendorf tube were allowed to stand in ice for 30 minutes before extracting the supernatant protein. Pellet protein was extracted with 1 x SDS under the same conditions. The above procedures of extraction were in the absence of reducing agents. Yeast proteins were also extracted under reducing conditions. 1% B-mercapethanol was added along with the extractant buffer (50 mM Tricine / 20 mM NaCl) and the same procedures were used.

2.2.1.2 Protein extraction at different stages of growth

200 ml of YMM and YEM were inoculated with 500 ul of YMM culture at stationary phase and grown at 30°C on a shaking platform. At time intervals between 2 and 12 hours, 1 ml of medium was removed under aseptic conditions. The optical density of the cell culture was measured at 590 nm. When the optical density was more than 0.8, 20 ml of culture medium was removed. Supernatant and pellet proteins were extracted as described in section 2.2.1.1.

2.2.1.3 Extraction of pea protein

50 mg of finely ground pea meal from 13-17 DAF were weighed and extracted in 500 µl borate buffer (50 mM

Borate, pH 8) at 4 C with constant agitation for 10 hours. The debris was removed by centrifugation for 10 minutes (8,000g).

2.2.2 Separation of protein by SDS-PAGE

The protein extracts were fractionated by 12.5% and 17% SDS-PAGE under non-reduced and reduced conditions. The recipes for acrylamide are shown in table 1 and 2.

Table 1 - Resolving gel

	Final acrylamide concentration	
Ingredients	12.5%	17%
Main gel (30% acrylamide/ 0.135% bisacrylamide)	25.8 ml	43.5 ml
Tris (1M, pH 8)	22.5 ml	22.5 ml
Distilled water	13.8 ml	0.9ml
10% SDS	0.6 ml	0.6 ml
Ammonium persulphate (15 mg/ml) freshly made	1.5 ml	1.5 ml
TEMED	20 µl	20 µl

Table 2 - Stacking gel

Ingredients	Volume used
Stacking gel 30% acrylamide/ 0./.435% bisacrylamide)	3.0 ml
Tris (1M pH 8)	2.5 ml
10% SDS	0.2 ml
Ammonium persulphate (freshly made)	0.5 ml
TEMED	20 µl

2.2.2.1 Construction of acrylamide gel

A gel of area 15 x 18 x 0.15 cm was contructed, using a gel slab shown in figure 2. The gel comb was slided on top of the gel slab and the position 2 cm above the comb marked. The appropriate volume of the main gel solution and Tris (as shown in table 1) was placed in a Buchnner flask and degassed under vacuum. The remaining ingredients were added into the flask, mixed well and poured onto the gel slab to the marked position. It was overlaid with a layer of distilled water and left for 30 minutes to polymerise.

The stacking gel was prepared by placing the appropriate volume of stacking gel solution and Tris into a Buchnner flask and degassed as before, followed by the addition of the remaining ingredients (as shown in table 2). The layer of water was removed and gel solution was added to the top of the gel slab. The comb was slided on top, taking care that no bubble formed. It was left for 30 minutes to polymerise. The position of each well was marked before removing the gel comb. The bottom spacer was extracted. The gel slab was clamped onto a vertical electrophoresis tank (Studier type gel apparatus). Bubbles were removed from the base of the tank using a syringe with a bent needle. 500 ml of reservior buffer (192 mM glycine / 22mM Tris, 0.1% SDS) was added to the top and bottom tank.

2.2.2.2 Loading of samples

50 µl of yeast protein was added to each well. In the same gel, 15 µl of legumin standard (3 mg/ml) and 15 ul of convicilin and vicilin standard (3 mg/ml) were added to individual wells. Under reducing conditions, a drop of β-mercapethanol was added. Three drops of tracking dye (1% w/v bromophenol blue in ethanol) were added to the buffer in the top tank prior to start of electrophoresis. The gel was run at 8 mA overnight or at 25 mA until the dye marker reached the bottom of the gel.

2.2.3 Detection of protein

On the completion of electrophoresis, the fractionated protein was visualised by 2 methods.

2.2.3.1 Staining with a dye

The gel was stained with kenacid blue stain (0.05% w/v kenacid blue R in 50% methanol and 1% acetic acid). Excess stain was removed by soaking the gel with 2 - 3 changes destain solution (50% methanol / 7% acetic acid) over a period of 12 hours. The gel was covered with dialysis membrane, which was previously soaked in distilled water. The gel was then placed in a gel drier for 3 - 4 days.

Western blotting

The fractionated protein from the acrylamide gel was transferred to the nitrocellulose paper and followed by immunological detection.

2.2.3.2.1 Electroblotting

The procedure is described in the Sartorius manual. The graphite plates were rinsed with distilled water. Two layers of 3 MM Whatman paper (16 x 16 cm), soaked in anode buffer 1 (0.3M Tris / 20% methanol / 0.1% SDS pH 10.4) were placed on top of the graphite plates. Next, one layer of 3 MM Whatman paper, soaked in anode buffer 2 (25 mM Tris / 20% methanol / 0.1% SDS pH 10.4) was placed on top, followed by a nitrocellulose paper which had been previously soaked in distilled water. The acrylamide gel was then placed on top of the membrane, making sure no air bubbles were trapped in between. The left-hand side of the gel and positions of each well were marked on the membrane. An additional layer of 3 MM Whatman paper, soaked in cathode buffer (40 mM 6,amino-n-hexanoic acid / 20% methanol / 0.1% SDS/ 25mM Tris pH 9) was placed on top of the gel followed by a dialysis membrane. Finally the stack was covered with two layers of 3MM Whatman paper which were soaked in cathode buffer. The lid to which the cathode plate was attached was then placed in position. The electroblotter was connected to a power

supply and was run for 2 - 3 hours at 0.3 Amp.

2.2.3.4 Probing legumin polypeptides with anti-legumin

Nitrocellulose membrane was blocked overnight in blocking solution (5% non-fat dry milk / 20mM Tris / 10.9% NaCl pH 7.2). Blot was then incubated for 2 hours with affinity purified anti-legumin IgG, diluted 1:256 in blocking solution. The blot was then extensively washed in blocking solution and incubated for 2 hours with goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:250 in blocking solution, followed by Tris / NaCl buffer.

2.2.3.5 Detection of legumin by immunolocalisation

25 - 30 mg of Diaminobenzidine (DAB) was dissolved in 100 mls Tris / NaCl buffer, pH 7. 3 ml of 1% cobalt chloride and 100 µl of 30% H_2O_2 was added. In the presence of cobalt chloride the colour product generated from DAB changed from brown to black. Therefore the presence of legumin was visualised as a black band.

2.3 Methods of quantitative analysis

2.3.1 Quantitative estimation of total yeast protein by Microbuiret assay (Leggette-Bailey 1967)

2.3.1.1 Contruction of a standard curve

A standard curve was set up using Bovine serum albumin (BSA 1 mg/ml). 62.5 µl, 125 µl, 187.5 µl and 250 µl of BSA was added to individual Eppendorf tube and made up to 250 µl with distilled water. To each tube, 750 µl of 1M sodium hydroxide was added, followed by 50 µl of Benedict's solution (17.3% w/v Trisodium citrate.$2H_2O$ / 10% w/v sodium carbonate.$10H_2O$ / 1.73% w/v copper sulphate.$10H_2O$). A blank with 250 µl of distilled water was also set up. The mixture was mixed by vortexing and left for 15 minutes. The absorbance of each sample was read at 330 nm using a PYE unicam uv/vis spectrophotometer.

2.3.1.2 Determintion of yeast total protein

25 µl of yeast protein was placed in an Eppendorf tube and made up to 250 µl with distilled water. The procedure mentioned above was used. A blank solution of Tricine / NaCl and 1 x SDS buffer was set up. The former was used as the blank for measuring supernatant protein, while the latter was used as a blank for measuring pellet protein.

2.3.2 Estimation of yeast legumin by quantitative western blotting

17% acrylamide gel was constructed as described in section 2.2.2.1. Volumes of 10 µl, 25 µl and 50 µl of yeast protein were loaded into individual wells. Legumin standard ranges of between 0.5 and 50 µg were loaded into the remaining wells. The gel was subjected to electrophoresis and followed by western blotting as described in section 2.2.3.2. The intensity of the yeast legumin band was compared to the intensity of known standard legumin bands.

2.3.3 Quantitative estimation of yeast legumin using ELISA

ELISA was performed in 96 wells microtiter plates (NUNC Immunoplate). 2'2' Azinobis 3-ethyl benzthiazoline sulfonic acid was used as the substrate in the detection system. It gives a green colour reaction which can be measured at 414 nm, using Multiscan MCC, filter 1, mode 1.

2.3.3.1 Preliminary antibody binding curve

Three different anti-sera were used as primary antibodies.

(1) Preimmune serum = serum obtained befored immunisation of rabbit.

(2) $R_{28}B_{28}$ serum = crude serum obtained after immunisation with legumin protein.

(3) Affinity purified anti-legumin antibody.

The preliminary ELISA was to investigate the best type and dilution of primary antibody that would give the lowest possible non-specific binding, but with the highest detectable colour reaction.

2.3.3.1.1 Coating of antigen

A portion of total PJY 28 protein was diluted 1:1,000. 100 µl of the diluted protein was added to each microtiter well. The plate was covered with aluminium foil to avoid desiccation and was incubated overnight at 4°C. The coated wells were washed with PBST (phosphate buffer saline, pH 7.2, in 0.1% w/v Tween) and were then washed once with distilled water. The wells were dried by inversion over absorbent paper. 100 µl of 1% BSA in phosphate buffered saline (8% w/v NaCl / 0.2% w/v KCl / 0.1% Thiomersol pH 7.2) was added to each well and incubated for 1 hour at room temperature. Wells were washed and dried as before.

2.3.3.1.2 Binding of primary antibody

To the first row of duplicate wells, 100 µl of preimmune serum in dilutions of 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 were added. In the second and

third rows of duplicate wells, similar dilutions of $R_{rb}B_{rb}$ serum and $R_{rb}B_{rb}$ IgG were added in the same order. The plate was incubated at room temperature for 2 hours. The wells were washed and dried as before.

2.3.3.1.3 Binding of secondary antibody

100 µl of goat anti-rabbit antibody IgG peroxidase conjugate (1:1,000 dilution) was added to all wells. The plate was incubated at room temperature for 2 hours. The wells were washed and dried as before.

2.3.3.1.4 Detection system

100 µl of ABTS (0.75 mg/ml ABTS in citrate buffer : 2.3% w/v citrate monohydrate; 0.05 µl 33% w/v hydrogen peroxidase, pH 4) was added to all wells and incubated for 15 minutes. The samples were read at 414 nm, using Multiscan MCC. Filter 1 and mode 1 were used.

2.3.4 To investigate the effects of yeast protein on a standard curve

In order to standardise an assay to measure the level of legumin, a standard curve was constructed using legumin in the presence of yeast protein. It was necessary to consider the possibility of interference resulting from the presence of yeast protein. The following amount of pure legumin were set up:

1) 0.68ng

2) 6.8 ng

3) 68 ng

4) 680 ng

5) 6,800 ng

6) 68,000 ng

Sets of the above dilutions in triplicate were prepared. In the first set, the legumin standard was diluted with Tris / NaCl buffer (20 mM Tris / 30mM NaCl, pH9). In the second set, pure legumin standard was diluted with supernatant protein of Mc 16 control yeast (1:1000 dilution with Tris / NaCl buffer). For the third set, pure legumin standard was diluted with pellet protein of Mc 16 control yeast (in 1:1,000 dilution with Tris / NaCl buffer).

100 µl of the prepared legumin range of 0.68 - 68,000 ng was loaded into each well in triplicate. The same conditions were used for the coating of antigen. In the process of binding primary antibody, $R_{\infty}B_{\infty}$ IgG of dilution 1:5,000 was used. The binding of antibodies and detection system were similar to the procedure described previously.

2.3.5 Estimation of yeast protein by ELISA

Yeast supernatant protein was diluted by 1:5,000 and pellet protein in a 1:1,000 dilution with Tris / NaCl buffer. Serial dilutions were made by placing 40 µl, 60 µl , 80 µl and 100 µl of the diluted supernatant and

pellet protein into individual wells in triplicate. The final volume of each well was made up to 100 µl with Tris / Nacl buffer. The sample in each well was subjected to ELISA as described in section 2.2.3. $R_{\infty}B_{\infty}$ IgG was used as primary antibody in a 1:5,000 dilution. At the same time, a standard curve of pure legumin standard, diluted with Mc 16 control yeast was set up, as described in section 2.3.4. The standard curve was in the range of 17.2 - 82.5 ng.

2.4 Purification of yeast protein

2.4.1 DEAE-sepharose

2.4.1.1 Packing of the column

A suspension of DEAE-sepharose was made by mixing equal volume of Tris buffer (50 mM, pH 7.5) and DEAE-sepharose to form a fairly thick slurry. The column (1.5 x 30 cm) was mounted vertically on a suitable stand. Air was eliminated from the dead space by flashing the end pieces with 50 mM Tris buffer, pH 7.5. The column outlet was then closed. The ion-exchanger suspension was poured into the column down the side of the chromatographic tube to avoid bubble formation. The column outlet was opened and the gel allowed to settle into the column. This was repeated until a gel column of 20 cm was formed.

2.4.1.2 Loading of yeast protein

200 ml of YMM was inoculated with a 10 ml YMM stationary PJY 28 yeast culture and was grown at 30°C until it reached early stationary phase (O.D.>1.50). Yeast cells were harvested and extracted with extractant (50 mM Tricine / 20 mM NaCl) containing 6M urea. Approximately 4 ml of supernatant protein was yielded.

Using a syringe, the protein sample was carefully

loaded onto the gel surface and was allowed to drain into the gel. When all the samples had entered the gel bed, the top column was washed with aliqouts of Tris buffer and was connected up for elution.

A linear gradient of 0 - 0.5 M NaCl at a flow rate of 8 ml/h was set up and eluted fractions were collected by an automated collector. Appromately 2 ml of each fraction were collected. The absorbance of each fraction was measured at 280 nm using PYE unicam uv/vis spectrophotometer.

2.4.2 Analysis of DEAE-sepharose yeast protein

2.4.2.1 To determine the fraction that consists M_r 59,000 legumin polypeptide.

200 µl of each peak fraction was dialysed overnight and freeze-dried. 100 µl of 1 x SDS buffer was added to each to redissolve the protein. The solution was boiled for 3 minutes.

2.4.2.2 Identification of M_r 59,000 polypeptide by 17% SDS-PAGE and western blotting

50 µl of each peak fraction and 15 µl of legumin, vicilin and convicilin were loaded onto individual wells of 17% polyacrylamide gel. The gel underwent electrophoresis and stained as described in section 2.2.2. Another identical gel was set up and was

subjected to western blotting as described in section 2.2.3.2.

2.4.2.3 Quantitative analysis of DEAE-sepharose purified protein by ELISA

200 µl of fraction 34 was dialysed overnight and freeze-dried. 100 µl of Tris / NaCl buffer was added and the sample was subjected to ELISA as described in section 2.3.5.

2.5 Futher purification of legumin M_r 59,000 polypeptide by reverse phase HPLC.

After determination of the fraction which consisted of M_r. 59,000 legumin polypeptide, the fractions falling under the whole peak were dialysed. The dialysis tubing was boiled in distilled water for 5 minutes with a spatula of sodium bicarbonate. The tubing was rinsed and a knot was tied at one end. Using a syringe, the fractions (32-37) were collected and placed inside the tubing, and a knot was tied at the other end, allowing sufficient room for water to accumulate during dialysis. The dialysis tubing was placed in a beaker with 2 litres of distilled water and constantly agitated to allow maximum dialysis. This was left for 24 hours with at least four changes of water. The dialysed sample was transferred to an Eppendorf tube and freeze-dried under vacuum.

The sample was dissolved in 500 µl of 6M Guanidine hydrochloride / 0.1% Trifluoro-acetic acid (TFA). The solution was mixed by vortexing and was spun in an Eppendorf microcentrifuge for 1 minute to get rid of bubbles. 500 ul of the solution was injected into a column of vydac C_{18} (0.5 x 25 cm, Technical, Stockport). A gradient of 0 - 30% increase of TFA for the first 10 minutes and a gradient of 10% increase of TFA for the rest (110 minutes). The sample components which emerged from the base of the column were detected

by measuring the absorbance at 215 nm. The results were presented as a series of peaks on the chart recorder. The gradient was also recorded on this chart. A sample was collected from the outlet where a peak was recorded on the chart. The fraction collected was freeze-dried under vacuum.

2.6 N-terminal sequencing using a manual Edman method (Chang *et al*, 1979)

Protein was placed in an acid-washed tube fitted with a Quickfit glass stopper, and was dissolved in 80 ul aqueous pyridine (50% v/v) and treated with 40 µl DABITC solution (10 nmol/µl, 2.82 mg/ml), which was freshly prepared. The tube was flushed with nitrogen for seconds, sealed with a glass stopper and placed in a heating block at 52°C for 15 minutes.

After the first coupling, 10 µl PITC was added and the second coupling reaction was allowed to proceed at 52°C for 30 minutes. After the reaction, the excess reagents and by-product were removed by mixing the reaction mixture with two portions of 0.5 ml heptane / ethyl acetate (2:1 v/v) on a vortex mixer and centrifuged.

The organic phase was removed with a fine pipette and discarded. The mixture was evaporated in a vacuum desiccator. The dried residue was dissolved in 50 µl of anhydrous trifluoroacetic acid, flushed with

nitrogen, and sealed with a glass stopper and heated in a heating block at 52°C for 15 minutes. TFA was removed in a vacuum desiccator and the sample was dissolved in 50 µl for dissolved in 50 µl distilled water. Extraction of cleared DABTZ-amino acid (and PTZ-amino acid) was performed by mixing one portion of 200 µl butyl acetate extract, the peptide in the water phase was evaporated in a desiccator and subjected to the next degradation cycle. The butyl acetate extract, containing the released thiazolinones, was evaporated and the residue was redissolved in water (20 µl) and 40 µl acetic of 50% TFA added. Conversion of thiazoline of amino acid into thiohydantion was carried out at 80° C in a heating block for 50 minutes. The sample was dried and redissolved in 20 µl of ethanol and was used for TLC identification.

2.6.1 Identification of DABTH-amino acid by thin layer chromatography

DABTH-amino acid can be identified, by 2-dimensional chromatography, on polyamide sheet (2.5 x 2.5 cm). Acetic acid and water, (1:2 by volume) were used for first dimensional separation. Toluene, hexane and acetic acid (2:1:1 by volume) were used as solvents for the second dimensional separation. DABTH-Leu could only be identified on gel plates, using chloroform / ethanol (100:3 v/v) as solvents for one dimensional

separation.

After TLC separation, the plate was dried and exposed to hydrochloric acid vapour to visualise the coloured DABTH-amino acid. Each amino acid was identified by the distinct spot pattern produced. The pattern of the spots for each amino acid is shown in Appendux III.

CHAPTER THREE

3 Results

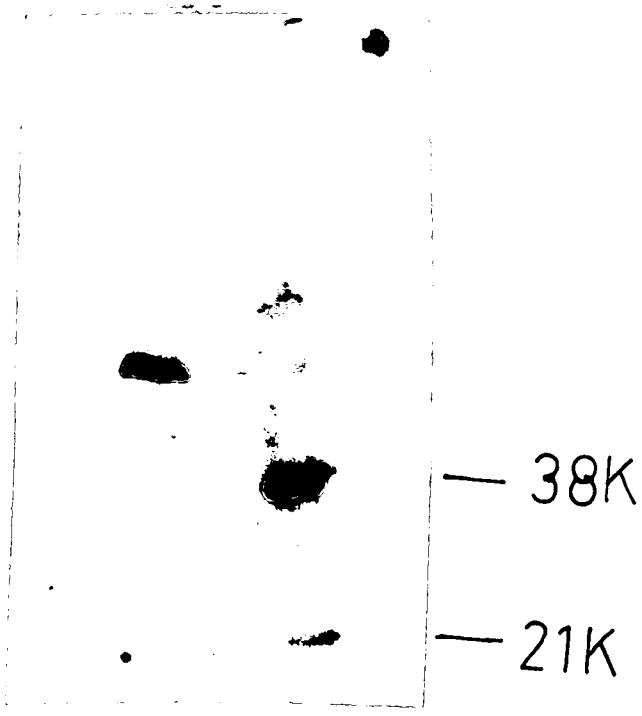
3.1 Demonstration of legumin expression in PJY 28 yeast

Yeast protein was extracted from yeast transformed with PJY 28 construct (Leg A in pMA 91) and a control yeast strain, Mc 16. The supernatant was extracted under non-denaturing conditions and the pellet was under denaturing conditions. Yeast proteins were subjected to 17% SDS-PAGE (reduced), followed by elelectroblotting onto nitrocellulose paper and reacted with anti-legumin antibodies (as described in the methods).

Results are shown in Figure 3. Legumin polypeptides were detected only in transformed yeast and not in the control yeast strain. The legumin polypeptides were detected mainly in the pellet fraction. A minute amount, which was only just detectable, was observed in the supernatant fraction. The legumin polypeptide has an indicated M_r. of 59,000 and was not reduced to smaller polypeptides of M_r 38,000 and 21,000 as expected in pea legume.

Fig: 1

Western-blot of Leg A in PMA 91 (PJY 28) and Mc 16 yeast protein fractions



A = 50µl Mc 16 Pellet protein

B = 50µl Mc 16 supernatant protein

C = 50µl PJY 28 pellet protein

D = 50µl PJY 28 supernatant protein

E = 20µg legumin standard

3.2.2 PJY 28 supernatant protein extracted in 6 M urea extractant and pellet protein extracted in 1 x SDS buffer

Legumin polypeptides were detected mainly in supernatants, with a small detectable amount in the pellet fractions. Under all conditions, M_r. 59,000 polypeptides were detected. Traces of polypeptides ranged from M_r. 30,000 to 45,000 were detected (Tracks B, C, D, I and J.) Detection of higher molecular weight polypeptides (over 130,000 to 160,000) were located in Track C and D.

3.2.3 PJY 28 supernatant protein extracted in Tricine / NaCl extractant and pellet extracted in 1 x SDS

Result are shown in Figure 6. The majority of legumin was detected in the pellet fraction. Under all conditions, M_r. 59,000 polypeptide legumins were detected. The presence of higher molecular weight polypeptides were detected in Track B (unreduced) and Track G (in the presence of reducing agents).

Figure 4 - Western blot photograph of yeast supernatant protein extracted in 6 M guanidine isothiocyanate and pellet protein extracted in 1 x SDS in non-reducing and reducing conditions.

A) 15 µg legumin standard

B) 50 µl pellet protein extracted with 1% B-mercapethanol

C) 50 µl pellet protein + 1 µl B-mercapethanol

D) 50 µl pellet protein

E) 50 µl supernatant protein extracted with 1% B-mercapethanol

F) 50 µl supernatant protein + 1 µl B-mercapethanol

G) 50 µl supernatant protein

Fig:4

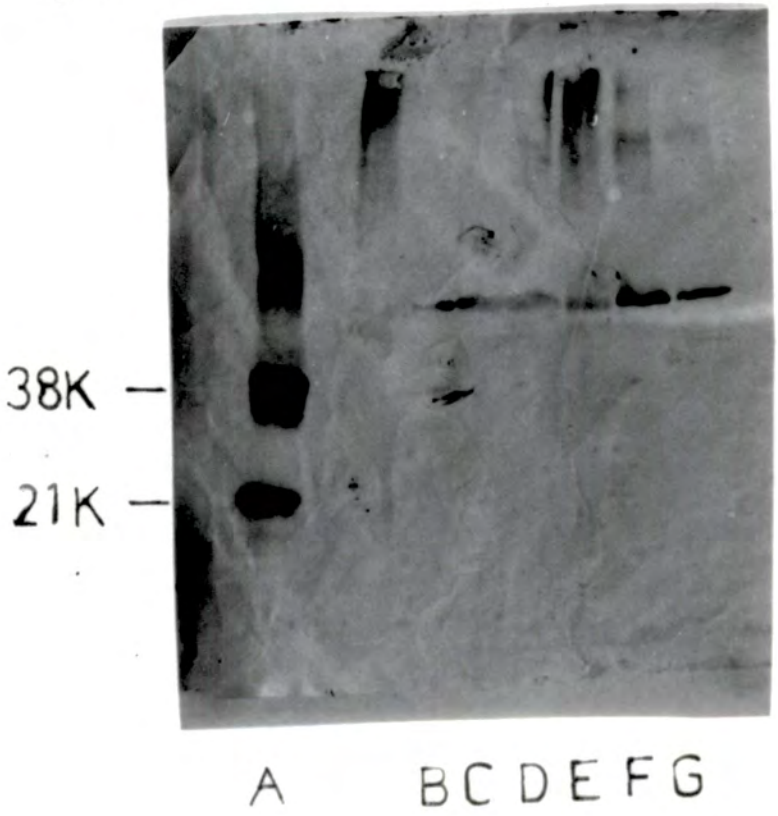


Figure 5 - Western blot photograph of PJY 28 yeast supernatant protein extracted in 6 M urea and the pellet protein extracted in 1 x SDS in non-reducing and reducing conditions.

A) 15 µg convicilin and vicilin standard

B) 50 µl pellet protein extracted with 1% B-mercapethanol

C) 50 µl pellet protein + 1 µl B-mercapethanol

D) 50 µl pellet protein

E) 50 µl supernatant protein

F) 50 µl supernatant protein + 1 µl B-mercapethanol

G) 50 µl supernatant protein extracted with 1% B-mercapethanol

H) 15 µg legumin standard

Figure 6 - western blot photograph of PJY 28 yeast supernatant protein Tricine/NaCl extractant and pellet extracted in 1 x SDS in non- and reducing conditions.

A) 15 µg legumin standard

B) 50 µl supernatant protein

C) 50 µl supernatant protein extracted with 1% B-mercapethanol

D) 50 µl supernatant protein +1 µl B-mercapethanol

E) 50 µl pellet protein

F) 50 µl pellet protein extracted with 1% B-mercapethanol

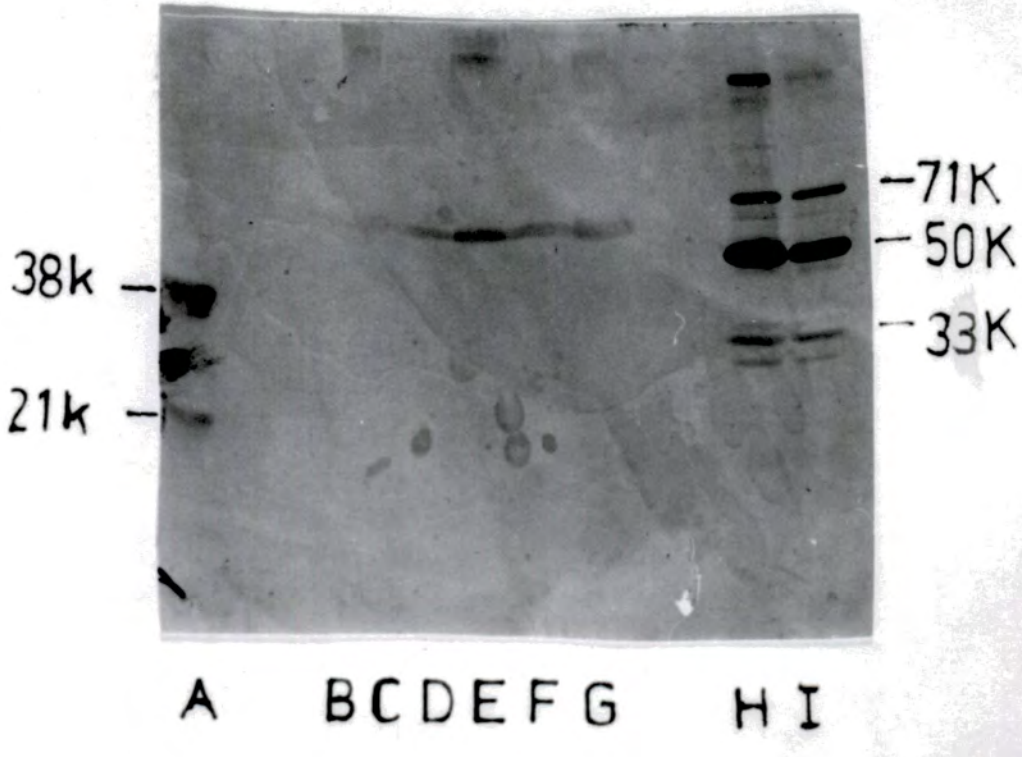
G) 50 µl pellet protein + 1 µl B-mercapethanol

H) 15 µg convicilin and vicilin standard

I) 15 µg convicilin and vicilin standard

Fig : 5

Fig : 6



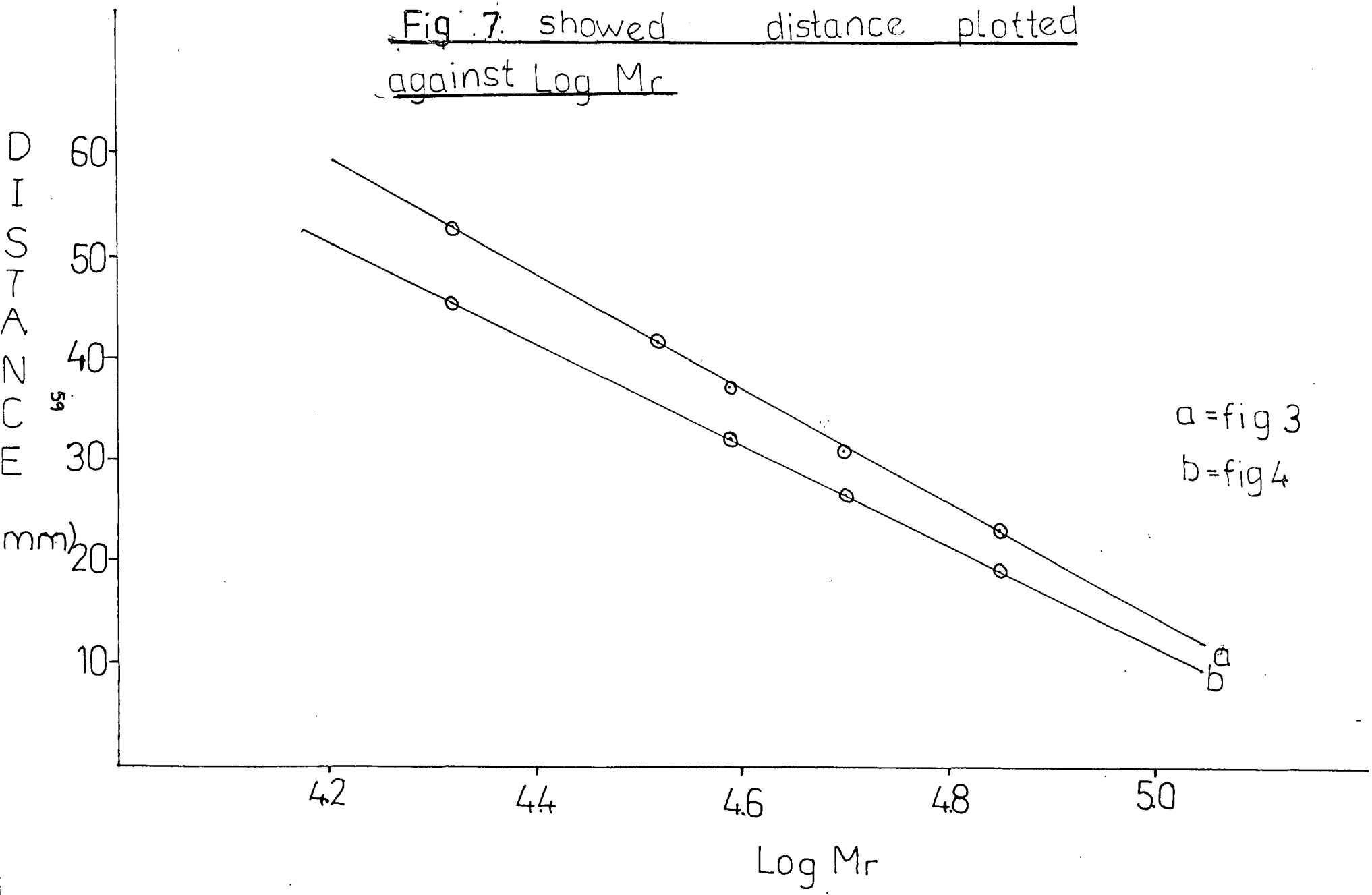
3.3 Estimation of the M_r. of yeast legumin polypeptide.

Using the results from figure 5 and 6, the distances travelled by yeast legumin polypeptide and known molecular markers were measured. A graph of distances travelled by proteins was plotted against the log of M_r. (figure 7). From graph 7, the M_r. of yeast polypeptides can be estimated.

	Track	distance travelled	M_r.
Figure 5	A-E	2.50	59,566
Figure 6	A-C	2.30	58,884
	D-F	2.25	58,210

Using Graph 7, the M_r. of yeast legumin polypeptide lies in the range 58,210 - 59,566.

Fig 7. showed distance plotted against Log Mr



3.4 Expression of legumin in PJY 28 yeast in minimal and enriched media at different stages of growth

Yeast supernatant protein was extracted in non-denaturing conditions and the pellet was extracted in denaturing condition. Yeast protein was extracted at time intervals of 2 to 6 hours. Before each extraction, the optical density of medium was measured at 590 nm. The proteins were subjected to 17% SDS-PAGE followed by western blotting.

3.4.1 Expression of legumin in PJY 28 yeast in minimal medium

The results are shown in Figure 8 and 9. The results showed that yeast expression was switched on at all stages of growth. The yield of legumin polypeptide (M_r. 59,000) increased with an increase of cell density and gave maximum yield at early stationary phase. There was also a build up of high molecular weight legumin polypeptides of indicated M_r. 130,000 - 150,000.

Figure 8 - Photograph of western-blot of PJY 28 supernatant protein at different stages of growth in minimal medium.

A) 50 µl supernatant protein extracted at O.D. 0.69
B) 50 µl supernatant protein extracted at O.D. 0.88
C) 50 µl supernatant protein extracted at O.D. 1.25
D) 50 µl supernatant protein extracted at O.D. 1.39
E) 50 µl supernatant protein extracted at O.D. 1.56
F) 50 µl supernatant protein extracted at O.D. 1.70
G) 50 µl supernatant protein extracted at O.D. 1.87
H) 50 µl supernatant protein extracted at O.D. 1.98

Figure 9 - Photograph of the master blot of PJY 28 pellet protein at different stages of growth in minimal medium.

A) 50 µg legumin standard
B) 50 µl pellet protein extracted at O.D. 0.75
C) 50 µl pellet protein extracted at O.D. 1.15
D) 50 µl pellet protein extracted at O.D. 1.55
E) 50 µl pellet protein extracted at O.D. 1.76
F) 50 µl pellet protein extracted at O.D. 1.80
G) 50 µl pellet protein extracted at O.D. 1.89
H) 15 µg legumin standard

Western-blot of transformed yeast extracts at different stages of growth in YMM medium

Fig 8 : Supernatant fraction

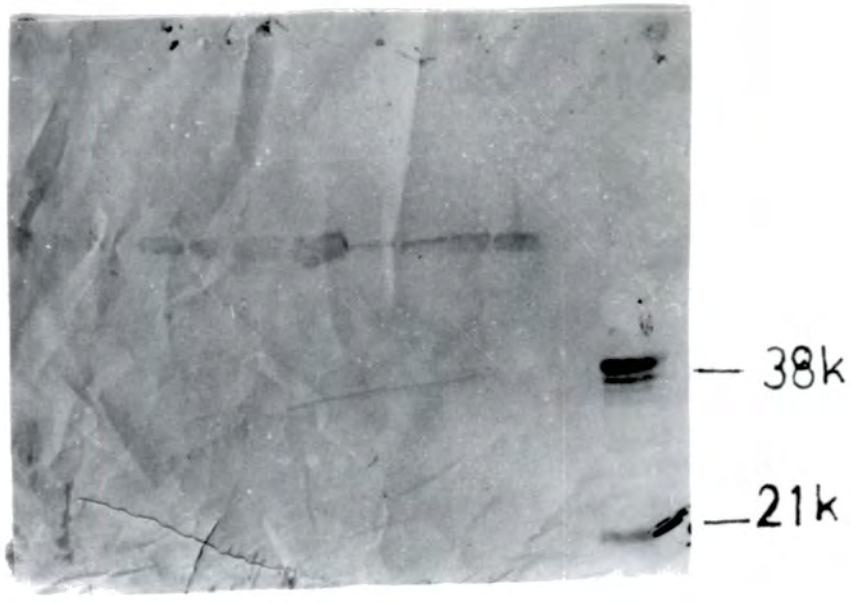
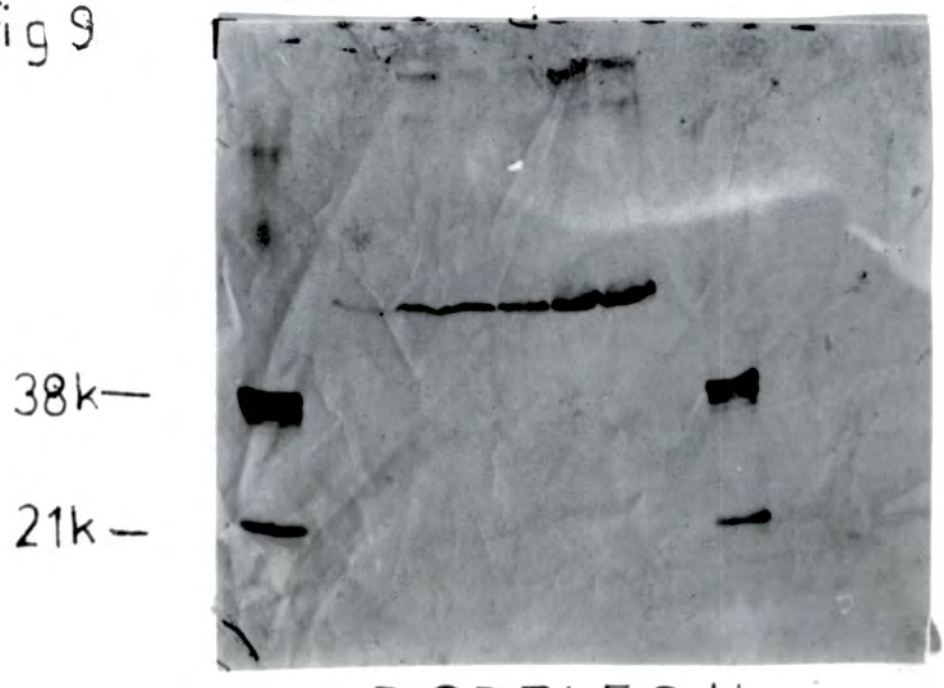


Fig 9

Pellet fraction



3.4.2 Yeast PJY 28 supernatant and pellet protein at different stages of growth in enriched-medium

The results are shown in Figure 10 and 11. In supernatant and pellet fractions, there was an apparent cessation of legumin synthesis at O.D. 1.42. The apparent lack of legumin polypeptides suggested turnover of previously synthesized polypeptides.

Figure 10 - Western blot photograph of PJY 28 yeast supernatant protein at different stages of growth in enriched-medium.

A) 15 μg *legumin standard*

B) 50 μl yeast supernatant protein extracted at optical density 0.89

C) 50 μl yeast supernatant protein extracted at optical density 1.15

D) 50 μl yeast supernatant protein extracted at optical density 1.42

E) 50 μl yeast supernatant protein extracted at optical density 1.65

F) 50 μl yeast supernatant protein extracted at optical density 1.71

G) 50 μl yeast supernatant protein extracted at optical density 1.84

H) 50 μl yeast supernatant protein extracted at optical density 1.90

I) 50 μl yeast supernatant protein extracted at optical density 1.99

Fig 10: Supernatant fraction

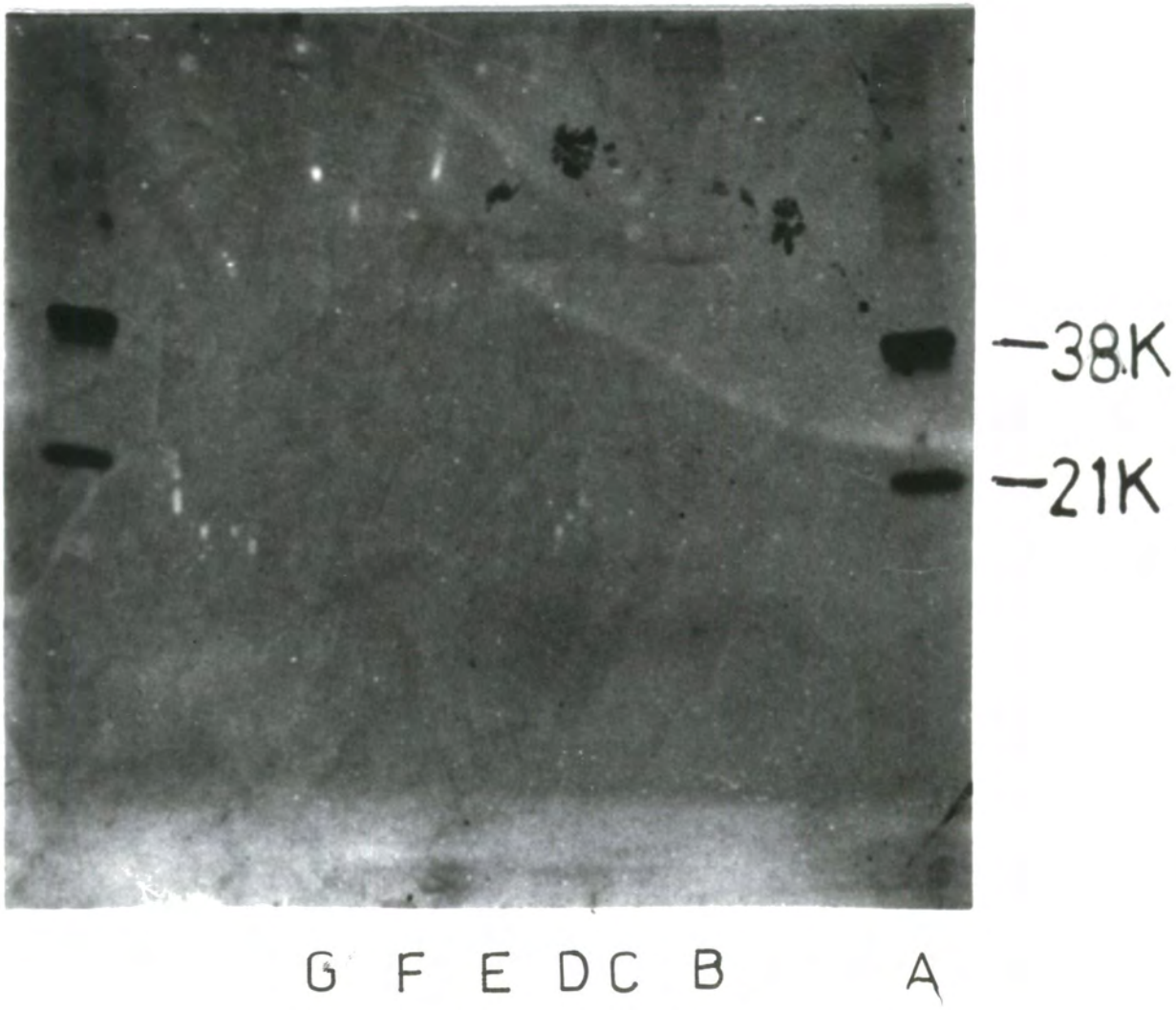


Figure 11 - Western blot photograph of PJY 28 yeast pellet protein at different stages of growth in enriched-medium.

A) 15 µg legumin standard

B) 50 µl yeast pellet protein extracted at optical density 0.89

C) 50 µl yeast pellet protein extracted at optical density 1.15

D) 50 µl yeast pellet protein extracted at optical density 1.42

E) 50 µl yeast pellet protein extracted at optical density 1.65

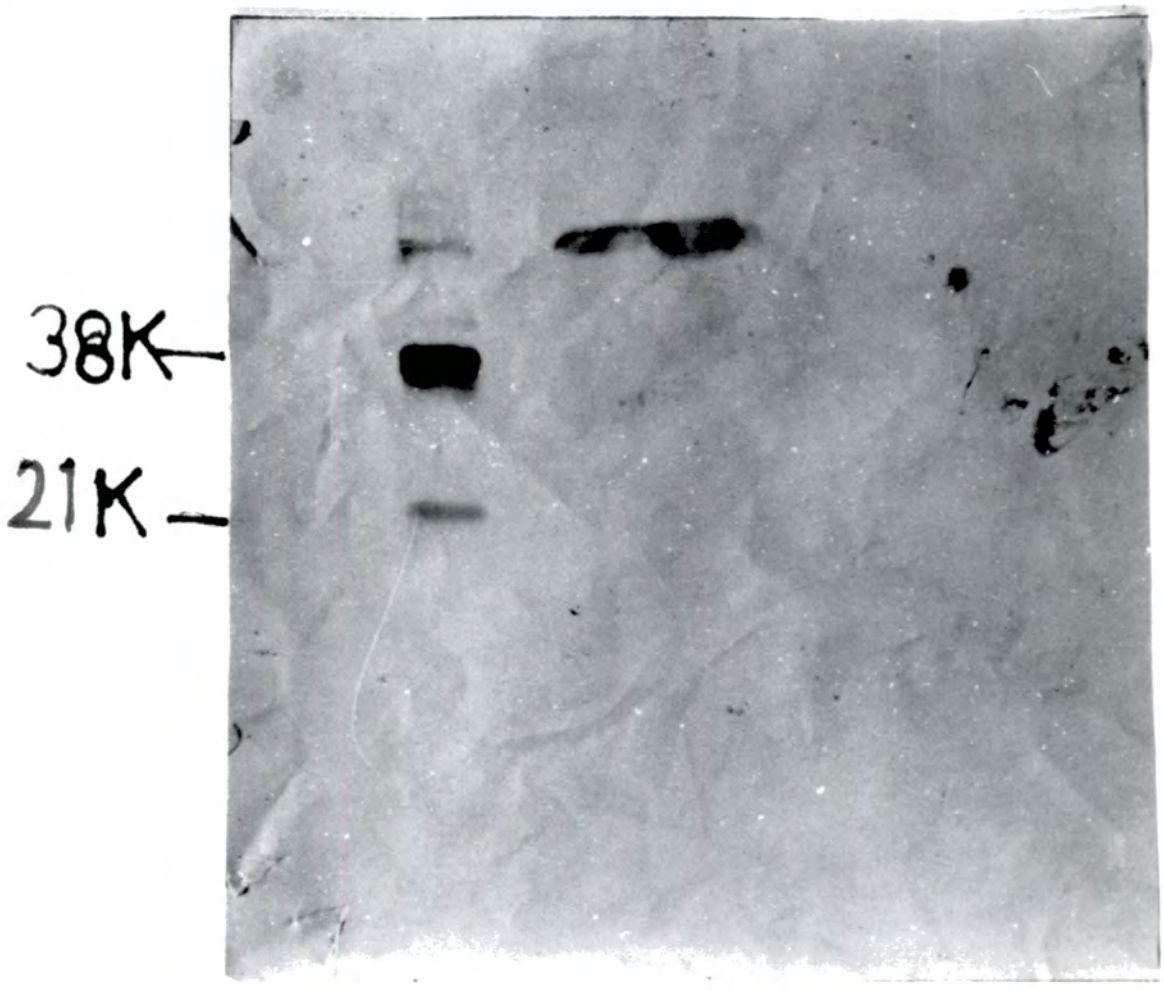
F) 50 µl yeast pellet protein extracted at optical density 1.71

G) 50 µl yeast pellet protein extracted at optical density 1.84

H) 50 µl yeast pellet protein extracted at optical density 1.90

I) 50 µl yeast pellet protein extracted at optical density 1.99

Fig: 11 Pellet fraction



3.4.3 Comparison of the level of legumin expression at same period of growth and same optical density in minimal and enriched-media

Results are shown in Figure 12. They indicate that when grown at identical optical densities, in minimal and enriched media, the level of expression was in the latter medium, suggesting that there were higher levels of expression, when growth occurred, in a selective medium in which active translation from the plasmid was necessary to provide requisite leucine.

Figure 12 - Western-blot photograph showing the level of expression at same preiod of growth and same optical density.

A) 15 µg legumin standard

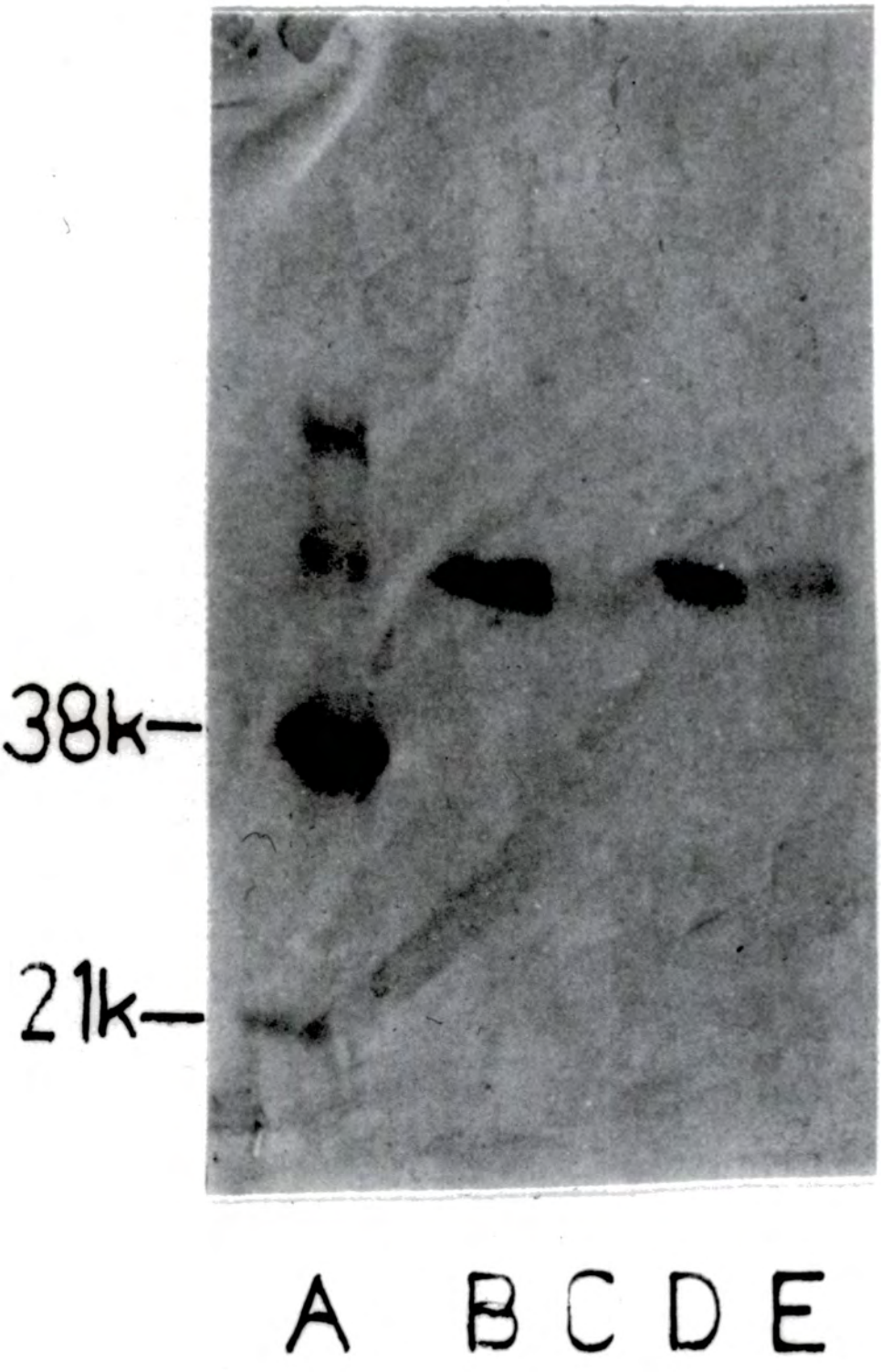
B) 50 µl pellet protein in minimal medium at O.D. 1.28

C) 50 µl pellet protein in enriched-medium at O.D. 1.28

D) 50 µl pellet protein in minimal medium at 48 hours (O.D. 1.34)

E) 50 µl pellet protein in enriched-medium at 48 hours (O.D. 1.99)

Fig : 9



3.4.5 Growth curve of PJY 28 and Mc 16 control yeast

1 ml of medium culture was removed at time intervals of 2 - 6 hours. Optical density at 590 nm was measured and time of growth was noted.

3.4.5.1 Growth curve of PJY 28 in minimal and enriched media

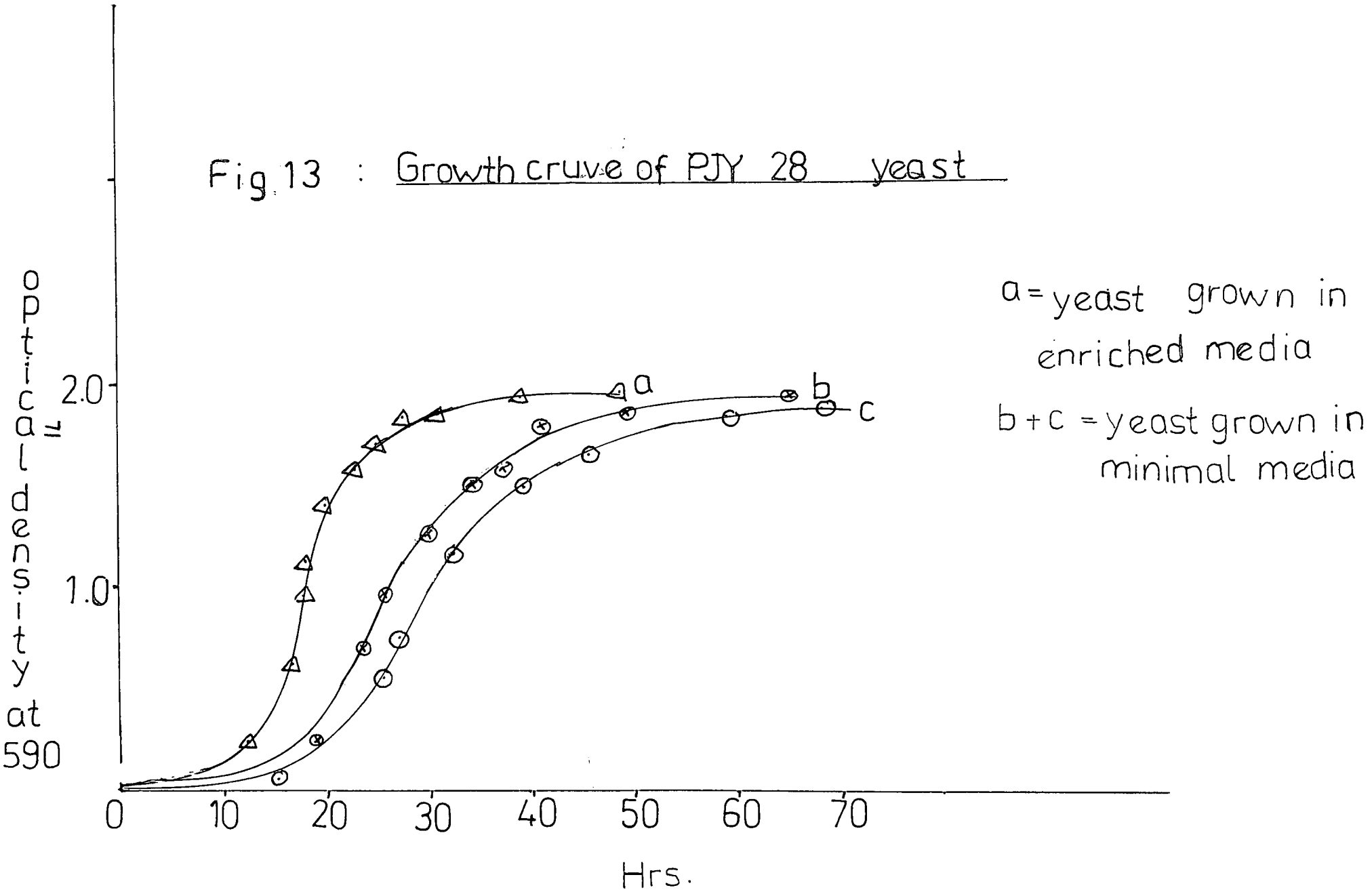
The results were plotted with optical density against time of growth; shown in Figure 13.

3.4.5.2 Growth curve of Mc16 control yeast in minimal and enriched media

The results are plotted with optical density against time of growth.

The results from Figure 13 and 14 show that there was a large increased in cell density when the yeast grown in enriched-medium. The growth curve of PJY 28 and Mc 16 yeast have a similar pattern, showing that legumin expression did not significantly interfere with the normal growth of yeast.

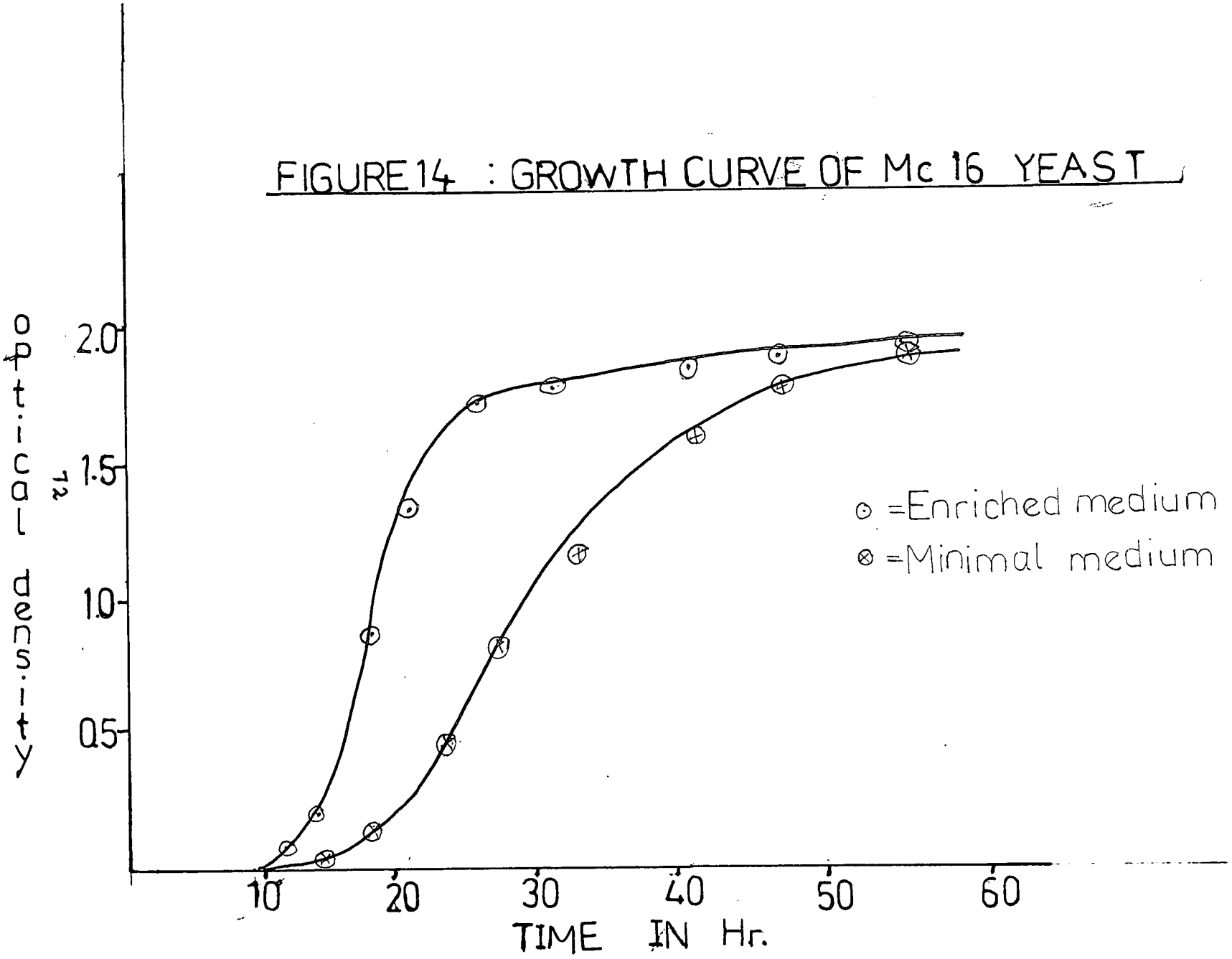
Fig 13 : Growth cruve of PJY 28 yeast



a = yeast grown in enriched media

b + c = yeast grown in minimal media

FIGURE 14 : GROWTH CURVE OF Mc 16 YEAST



3.6 Quantitative analysis

3.6.1 Determination of total yeast protein by Microbiuret

BSA was used to set up a standard curve (as described in the methods) to estimate the total protein in yeast extract. The standard curve is shown in figure 15 with optical density plotted against BSA (mg/ml). 25 µl yeast protein was placed in an Eppendorf tube and made up to 250 µl with distilled water. 750 µl 1M sodium hydroxide and 50 µl Benedict's solution was added. The absorbance at 330 nm was measured after 15 minutes (as described in the methods). From the standard curve, the level of yeast protein could be estimated.

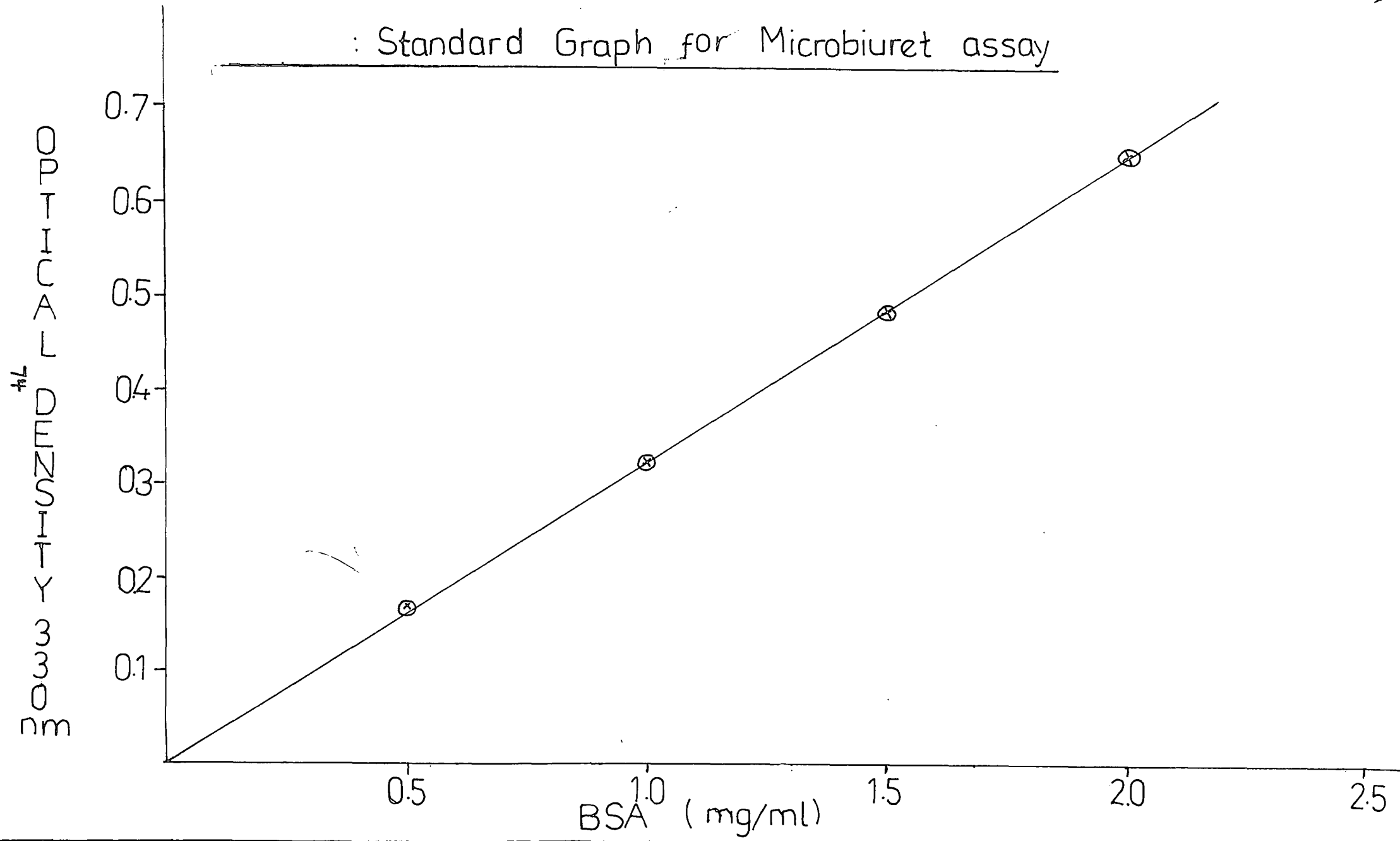
Results are shown in Table 3.

Table 3

Yeast extract	PJY 28 Supernatant protein in Tricine buffer	PJY28 Pellet protein in 1 x SDS
Dilution	25x	25x
O.D. (330 nm)	0.236	0.39
Protein (mg)	20.75	30.5

Total protein yeast protein = 51.25 mg/ml

: Standard Graph for Microbiuret assay



3.6.2 Estimation of yeast legumin by quantitative western blotting

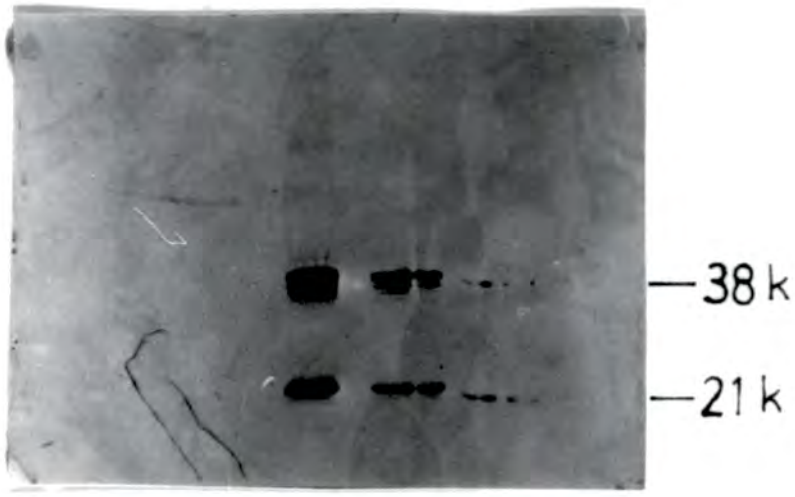
Yeast supernatant protein was extracted under non-denaturing condition and pellet protein was extracted under denaturing condition. Protein were fractionated by 17% SDS-PAGE and electroblotted and detected with anti-legumin IgG as described in the methods. The amount of legumin synthesized was estimated by comparison with the staining obtained with known amounts of purified legumin standard.

3.6.2.1 Quantitative western blotting of PJY 28 supernatant protein

Results are shown in Figure 16. The band of 50 µl supernatant protein gives a similar intensity as the 2.5 µg legumin standard. Therefore legumin present in supernatant protein is 50 µg/ml.

Fig: 16.

Quantitative western blot of legA in PMA 91 in soluble fraction by Tricine/NaCl



A B C D E F G H I

A=10µl yeast Supernatant fraction
B=25µl yeast Supernatant fraction
C=50µl yeast Supernatant fraction
D=50µg Legumin standard
E=30µg Legumin standard
F=20µg Legumin standard
G=10µg Legumin standard
H=2·5µg Legumin standard
I=1·0µg Legumin standard

3.6.2.2 Quantitative western-blottong of PJY 28 pellet protein

Results are shown in Figure 17. The band of 10 µl of pellet fraction gives a similar intensity as the 10 µg of legumin standard. Therefore legumin is in the pellet fraction of PJY 28 yeast in 1 mg/ml.

Level of expression in PJY 28 yeast

$$\frac{\text{Total PJY 28 yeast protein}}{\text{Total yeast protein (from the microbiuret estimation in section 3.6.1)}} \times 100$$

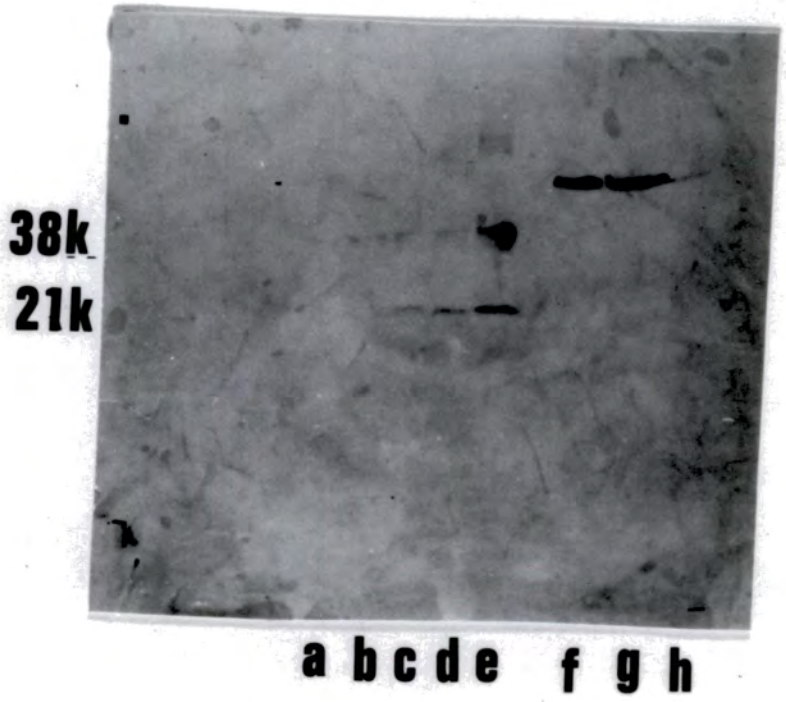
$$= 1 / 51.25 \times 100$$

$$= 1.95\%$$

Fig: 17

Quantitative western blot of leg A in PMA 91 by Tricine/Nacl

Pellet fraction



A = 5µg Legumin standard

B = 10 µg "

C = 20µg

D = 30µg

E = 50µg

F = 50µl Yeast pellet

G = 25µl "

H = 10µl "

3.7 Estimatiom of legumin in PJY 28 yeast by ELISA

3.7.1 Preliminary Antibody binding curve

Three different anti-sera of dilution 0 - 10^{-6} were used in the ELISA assay.

1) Preimmune serum = serum obtained before immunisation.
2) $R_{rb}B_{rb}$ serum = serum from immunised rabbit after red blood cells removed.
3) $R_{rb}B_{rb}$ IgG antibody = Affinity purified anti-legumin.

100 µl of diluted (1:1,000) PJY 28 total protein and Mc 16 control total protein was added to each well in duplicates. The three different types of serum at different dilutions were used as primary antibodies. Goat anti-rabbit IgG horseradish cojugate and DAB were used as the detection system. The results were plotted in a graph, with absorbance at 414 nm against the dilution factor (Figures 18 and 19).

For Mc 16 control yeast (figure 18), at dilution 1:100, it gives the highest binding. 1:1,000 (and below) dilutions show low non-specific binding to all anti-sera.

For PJY 28 yeast protein at 1:1,000 dilution, there is significant binding at both $R_{rb}B_{rb}$ serum and $R_{rb}B_{rb}$ IgG but fall to low level at 1;10,000 dilution.

At 1:5,000 dilution, the binding of $R_{22}B_{22}$ IgG still remains at a reasonable high level and Mc 16 control yeast gives a low binding at the same dilution. Therefore $R_{22}B_{22}$ IgG antibody at dilution 1:5,000 is the best acceptable primary antiboby which gives a high colour product.

Fig. 18:- Antibody binding curve of PJY 28 yeast protein

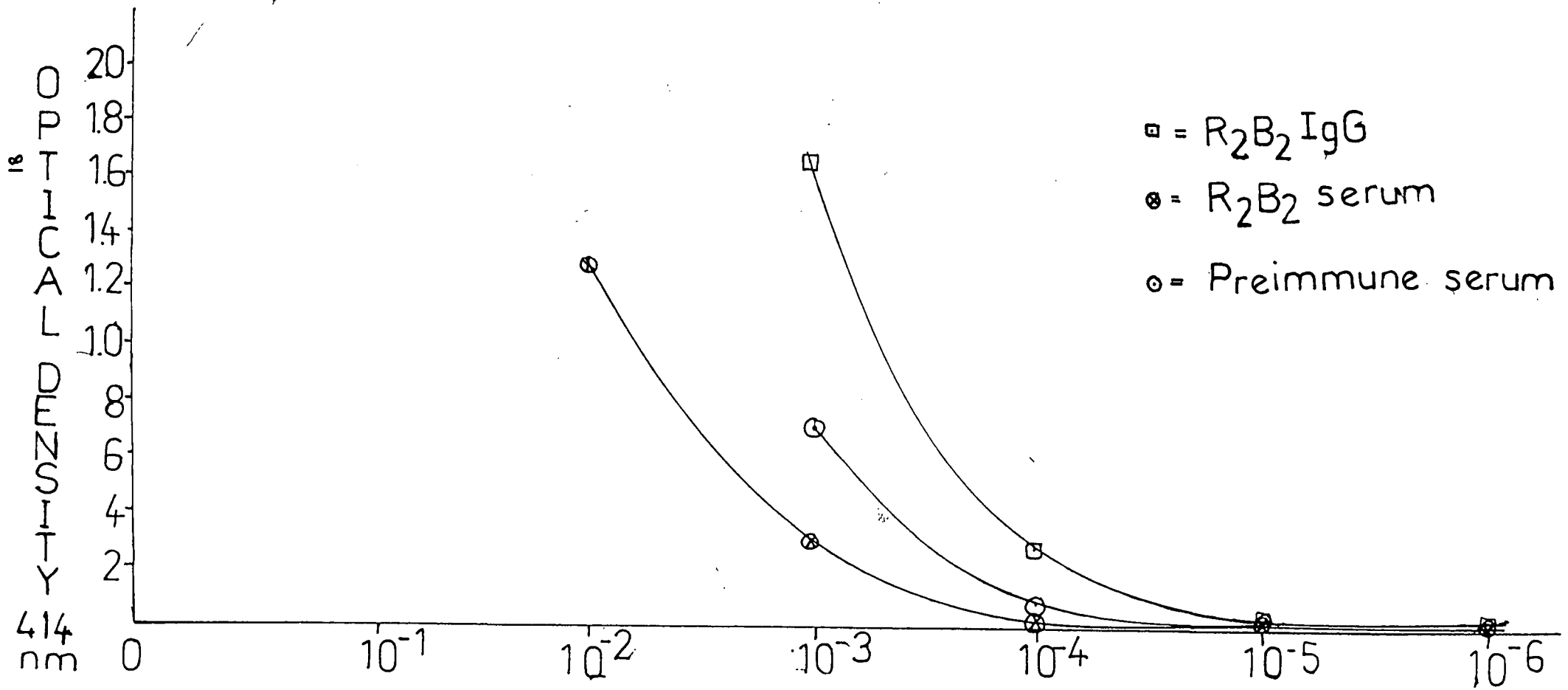
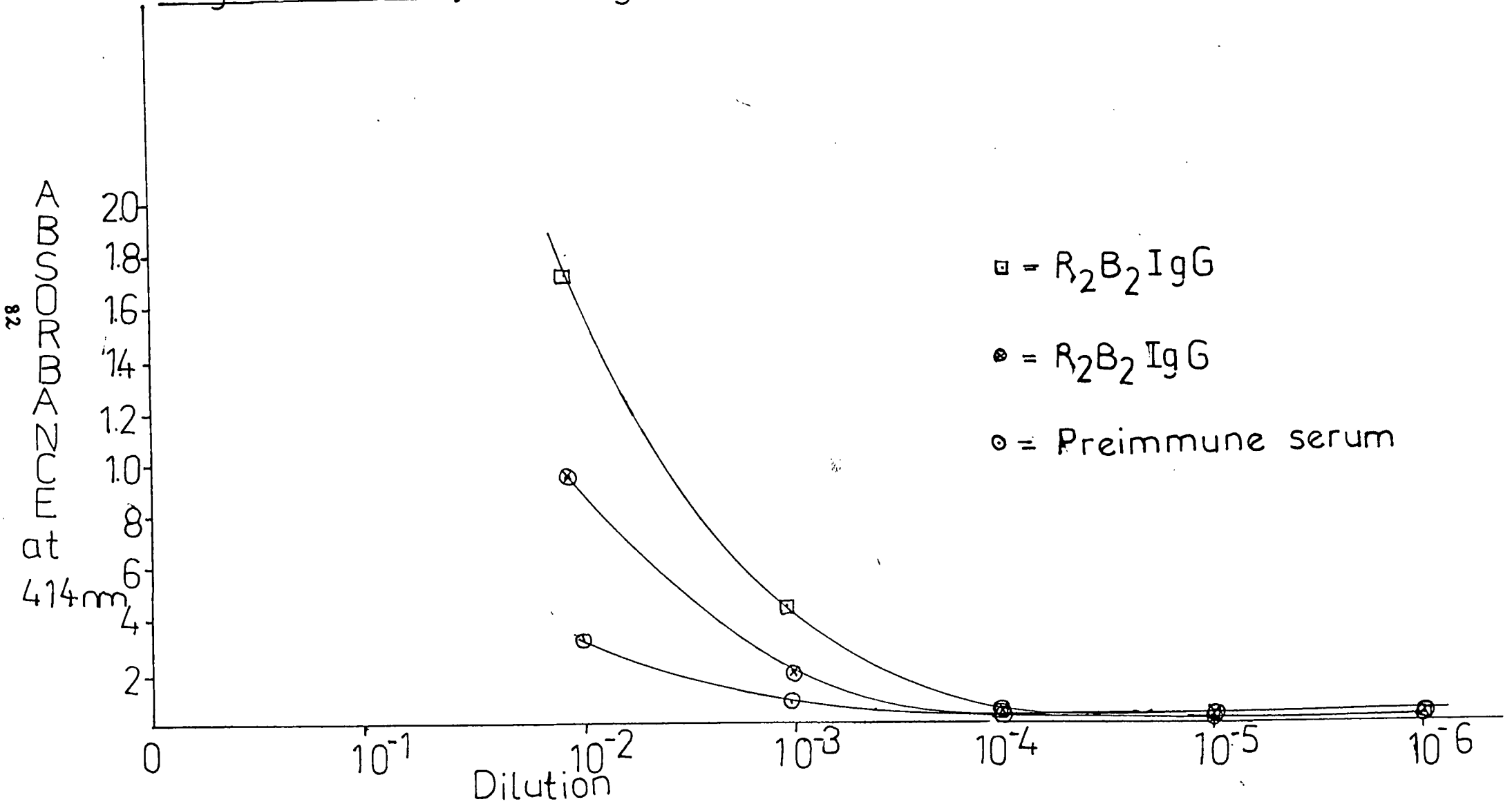


Fig. 19: Antibody-binding curve of Mc16 control Yeast Protein

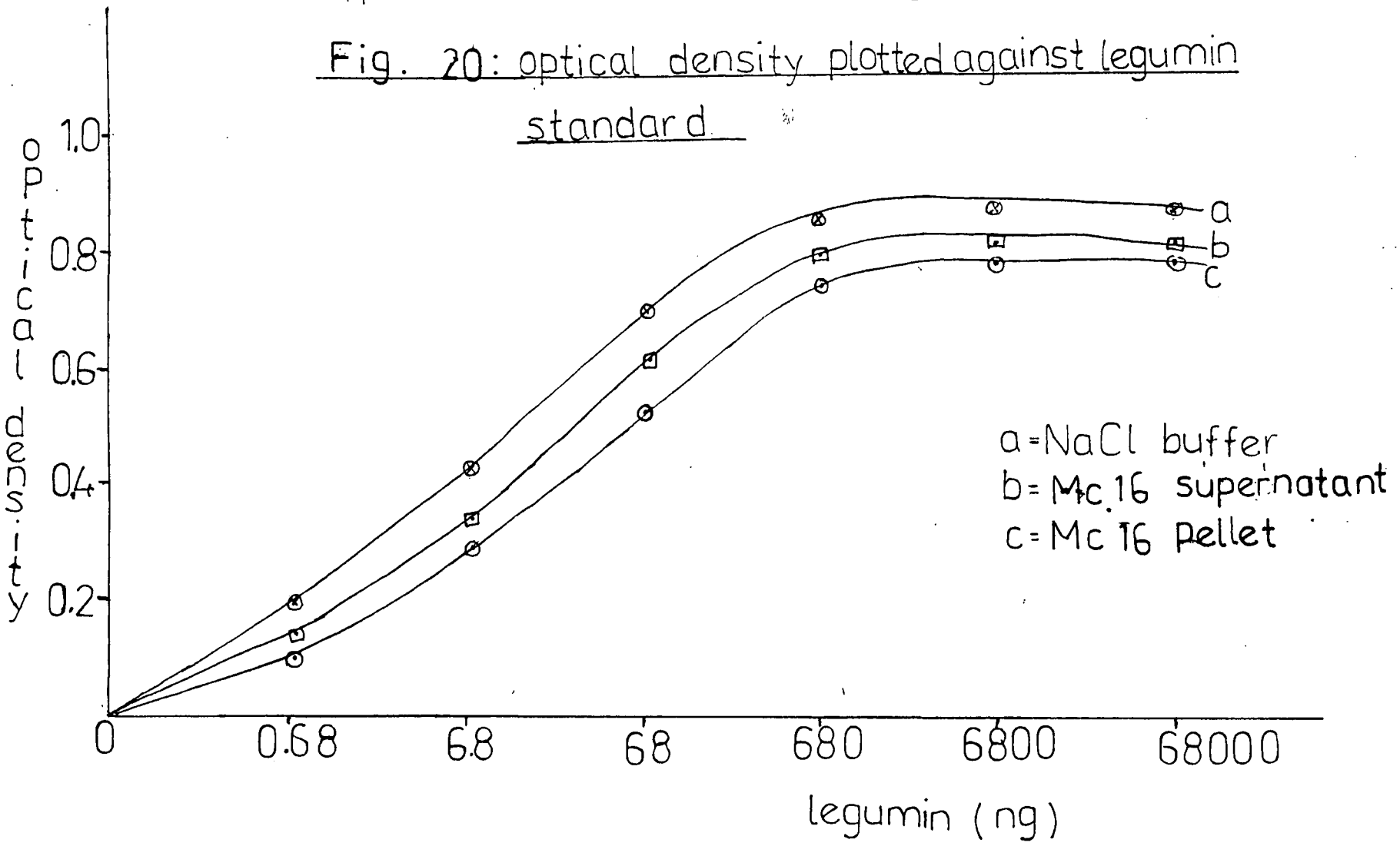


3.7.2 To investigate the effects of yeast protein on legumin standard curve

Pure legumin standard was diluted with 0.2 M NaCl / 0.3 M Tris buffer, pH 7.4, into a series of dilutions of legumin range of 0.68 - 68,000 ng. Similar series of dilutions of legumin standard were made with supernatant and pellet protein of Mc 16 control yeast. Both supernatant and pellet protein of Mc 16 yeast were initially diluted (1:1,000) with NaCl / Tris buffer. 100 µl of each diluted legumin was placed in each well in the microtiter plate in duplicates. ELISA was performed as described in methods. Results were plotted on a graph of optical density 414 nm against legumin standard as shown in Figure 20. The results showed that a linear relation was found between 0.68 - 368 ng.

Effects of yeast protein on legumin standard curve

Fig. 20: optical density plotted against legumin standard



3.7.3 Estimation of yeast legumin by ELISA

Standard graph of 17.2 - 82.5 ng of diluted supernatant and pellet protein of Mc 16 were set up.

From the quantitative western blots results dilution factor for supernatant protein is 1:500 and pellet protein is 1:8,000 so that the optical density produced will fall in the linear part of the standard curve. But preliminary ELISA estimation gave a high reading in PJY 28 supernatant protein which is off the scale of the standard graph. PJY 28 pellet protein gave a very reading instead. Therefore a dilution of 1:5,000 for supernatant protein and 1:1,000 for pellet protein protein were used. The following volume of diluted protein (as shown in table 4) was placed in each well and made up to 100 ul using NaCl / Tris buffer. Duplicates were set up for each and were subjected to ELISA. An average optical density was obtained from the results obtained from the duplicate wells were average out . The amount of legumin in ng was found from the standard curve and the amount of mg/ml were then calculated as shown in table 4.

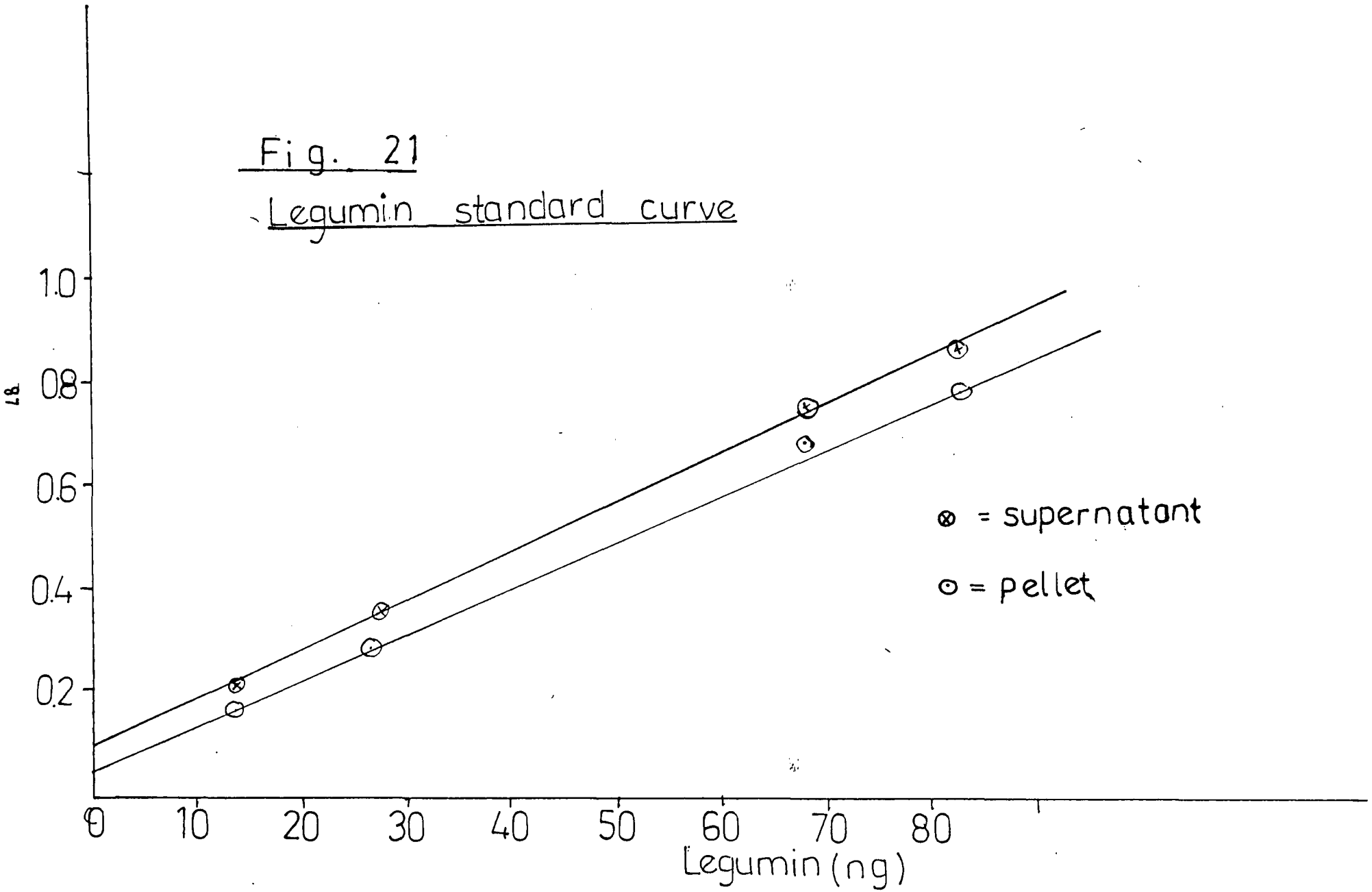
Table 4

Yeast fraction	Yeast supernatant protein extracted in non-reducing conditions	PJY 28 pellet extracted in denaturing conditions
O.D. 410nm		
40 µl yeast	0.101	0.310
60 µl yeast	0.135	0.365
80 µl yeast	0.152	0.445
100 µl yeast	0.205	0.523
Average legumin (mg/ml)	1.04	0.463

The results in Table 4 showed that the supernatant fraction contains more than twice the amount of legimin than the pellet fraction. The ELISA result contracdicted the by quantitive western blotting which showed that the pellet fraction contains 20x amount of legumin compared to supernatant.

Fig. 21

Legumin standard curve



3.8 To determine the processing of yeast legumin by comparing the mobility of legumin polypeptide with pea legumin using 12.5% SDS-PAGE and Western blotting

3.8.1 12.5% SDS-Page

Mc 16 control yeast protein, PJY 28 yeast protein and pea meal extracts from 13-17 DAF were separated on 12.5% SDS-PAGE and Fractionated proteins were visualised by 0.2% kenacid blue stain as described in method. The result is shown in figure 22. The distances, travelled by known molecular markers were measured. A graph of distances travelled by proteins was plotted against log M_r. shown in figure 23. Using this graph, the pea M_r. 59,000 precusor band was located. Knowing the approximate distance travelled by the M_r. 59,000 polypeptide of pea, by comparing the protein band of Mc 16 control yeast with PJY 28 yeast at that range. The yeast legumin polypeptide can be located as this band does not appear in control yeast. Results in Figure 23 showed that the distances travelled by both yeast and pea legumin in SDS-PAGE gel was 38 mm.

Figure 22 - Photograph of 12.5% SDS-PAGE gel comparing the mobility of pea and yeast leguminprecusor polypeptide.

A) 15 µg convicilin + vicilin standard

B) 2 µg BSA

C) 50 µl PJY 28 pellet protein

D) 50 µl PJY 28 pellet protein

E) 5 µl pea protein extract at 17 DAF

F) 5 µl pea protein extract at 16 DAF

G) 5 µl pea protein extract at 15 DAF

H) 5 µl pea protein extract at 14 DAF

I) 5 µl pea protein extract at 13 DAF

J) 5 µl pea protein extract at 12 DAF

K) 5 µl pea protein extract at 9 DAF

L) 15 µg legumin

Fig 22

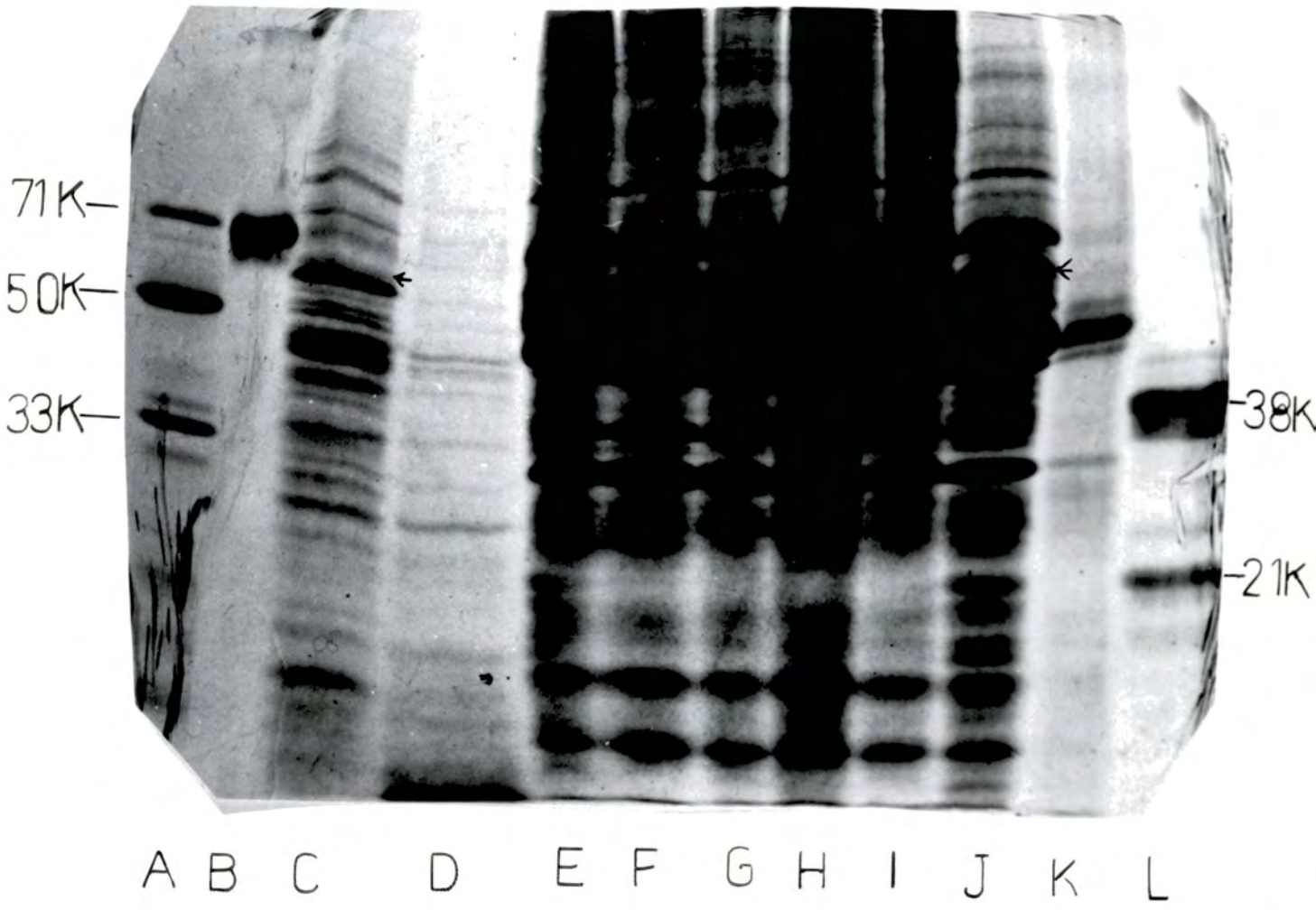
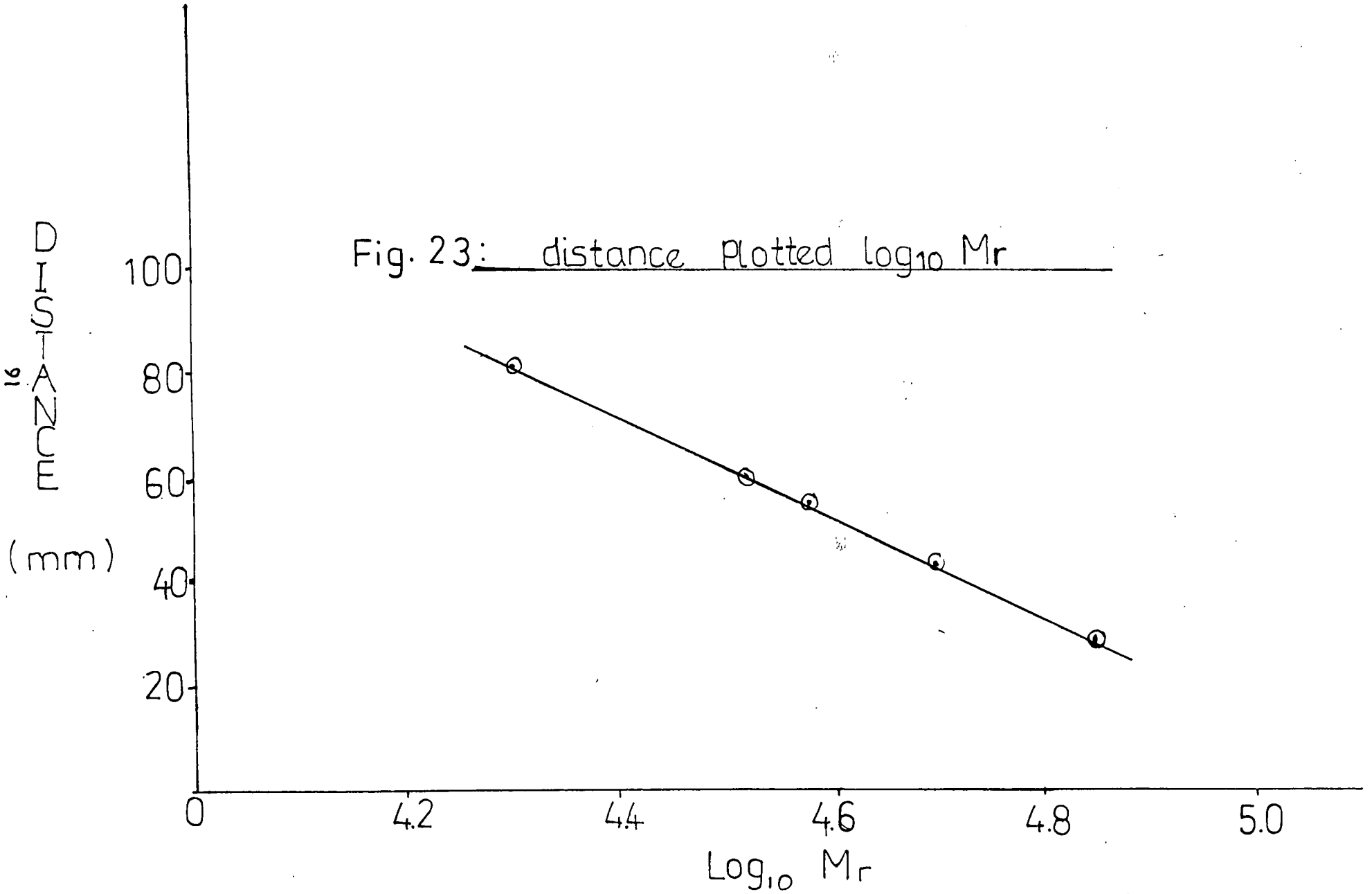


Fig. 23: distance plotted $\log_{10}$ Mr



3.8.2 Western - blotting

Yeast protein and pea protein extract were separated in 12.5% SDS-PAGE and electroblotted and detected by anti-legumin IgG and goat anti-rabbit IgG horseradish peroxidase as described in method. The result was shown in Figure 24. The distance travelled by the M.W. 59,000 legumin polypeptides of pea and yeast was found to be 34 mm (Figure 25).

Fig.: 24 Distance plotted against $Log_{10}Mr$

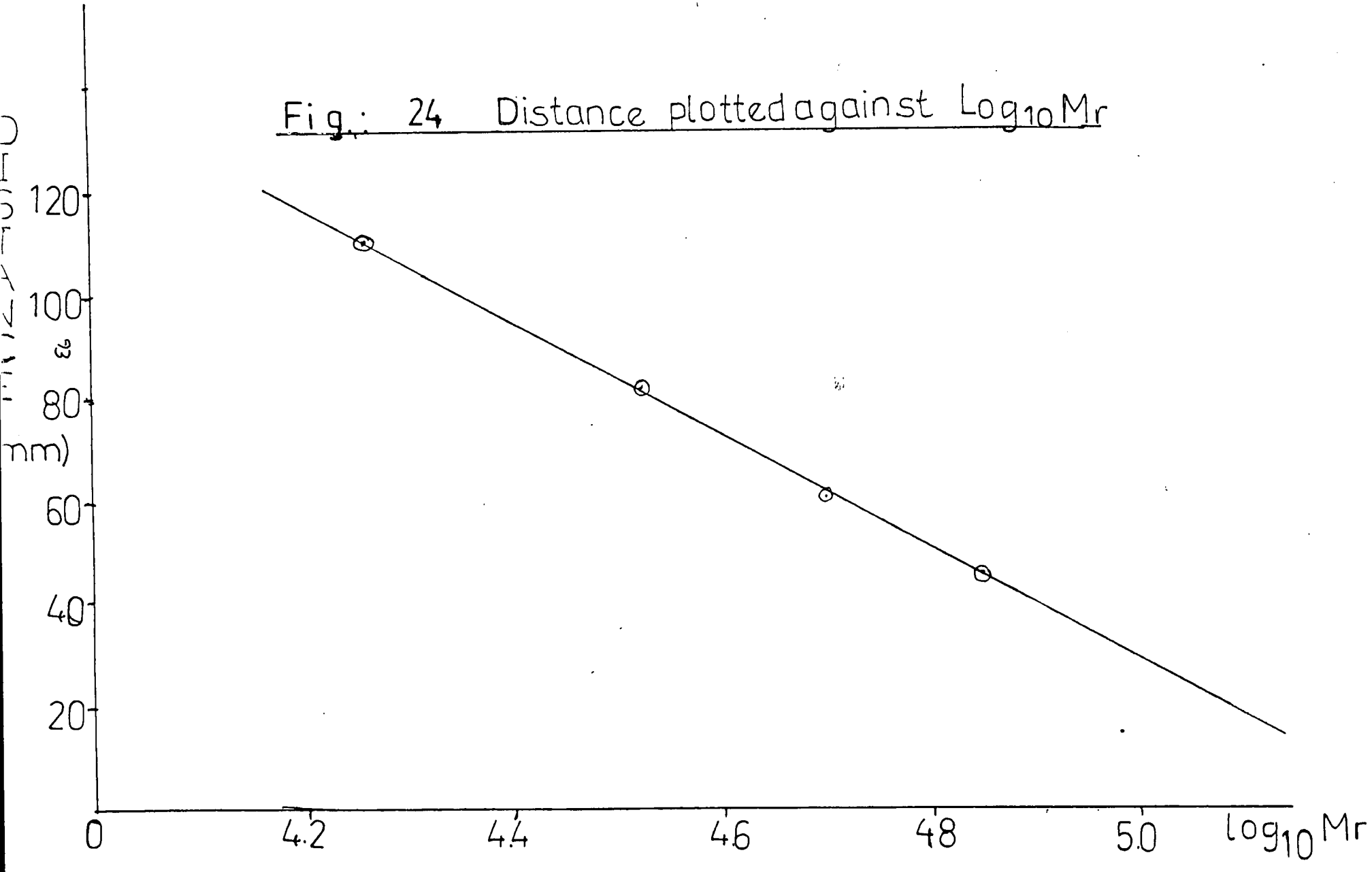


Figure 25 - Photograph of Western blotting of PJY 28 yeast and pea protein extract.

A) 50 µl PJY 28 yeast supernatant protein

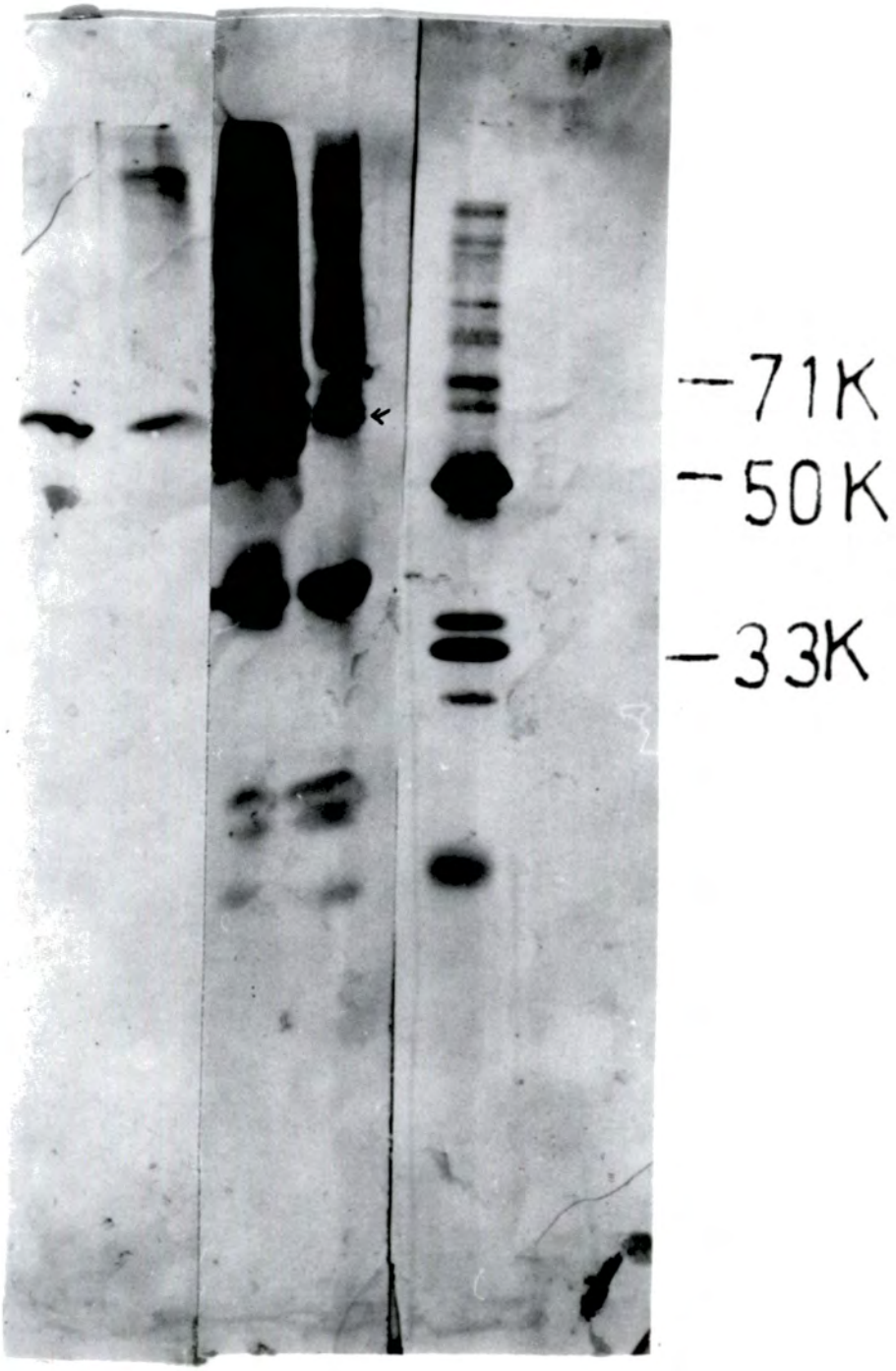
B) 50 µl PJY 28 yeast supernatant protein

C) 5 µl pea protein extract at 15 DAF

D) 5 µl pea protein extract at 14 DAF

E) 15 µg convicilin + vicilin standard

Fig 25



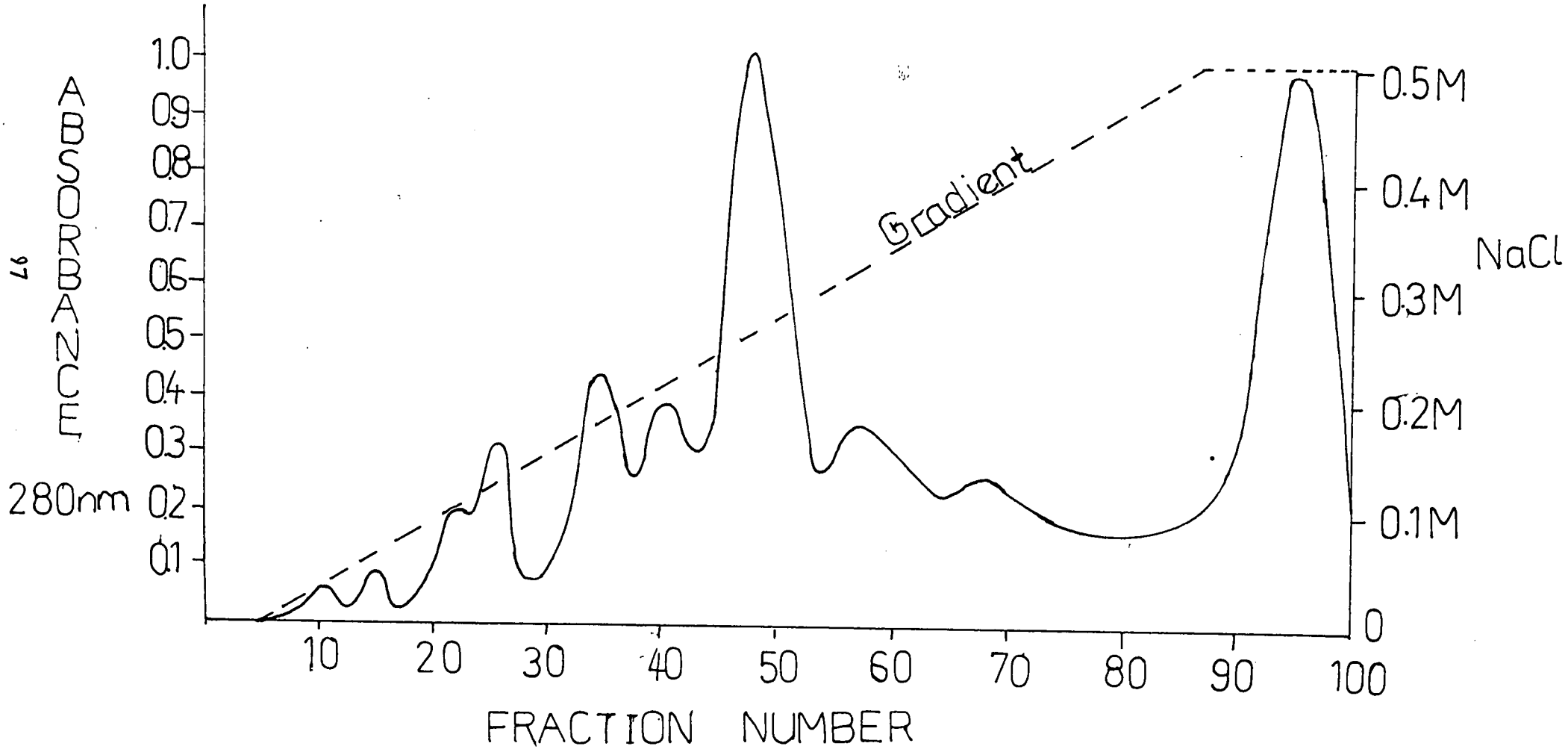
3.9 Purification of yeast legumin M_r. 59,000 polypeptide

3.9.1 DEAE-sepharose column

3 ml of yeast supernatant protein was extracted using 6 M urea in Tricine / NaCl buffer. Yeast legumin was purified by running on a DEAE-sepharose column, with a gradient of 0 - 0.5 M NaCl. Eluted fractions were collected and the absorbance at 280 nm were measured.

The results were plotted in a graph with absorbance at 280 nm against fractions. The results are presented in figure 26; there are 10 protein peaks.

Fig. 26: Elution profile of yeast proteins on DEAE-sepharose column.



3.9.1.1 17% SDS PAGE

50 µl of each peak fraction was loaded into each well, with legumin and vicilin standard. The separated proteins were visualised by 0.2% Kenacid blue. Results are shown in Figure 27.

The known distances of the known molecular weights were measured and a graph of distance plotted against log of M$_r$. (Figure 28). From the graph, the distance travelled by the 59,000 legumin polypeptide is 39 cm, which only corresponds to the second major band of fraction 34 (Figure 27).

Figure 27 - Photograph of SDS-PAGE gel of sepharose purified yeast extract.

A) 50 µl of sepharose purified protein fraction 22.

B) 50 µl of sepharose purified protein fraction 25.

C) 50 µl of sepharose purified protein fraction 34.

D) 50 µl of sepharose purified protein fraction 41.

E) 50 µl of sepharose purified protein fraction 47.

F) 50 µl of sepharose purified protein fraction 57.

G) 50 µl of sepharose purified protein fraction 68.

H) 50 µl of sepharose purified protein fraction 96.

I) 15 µl of vicilin + convicilin standard.

J) 15 µl of legumin standard.

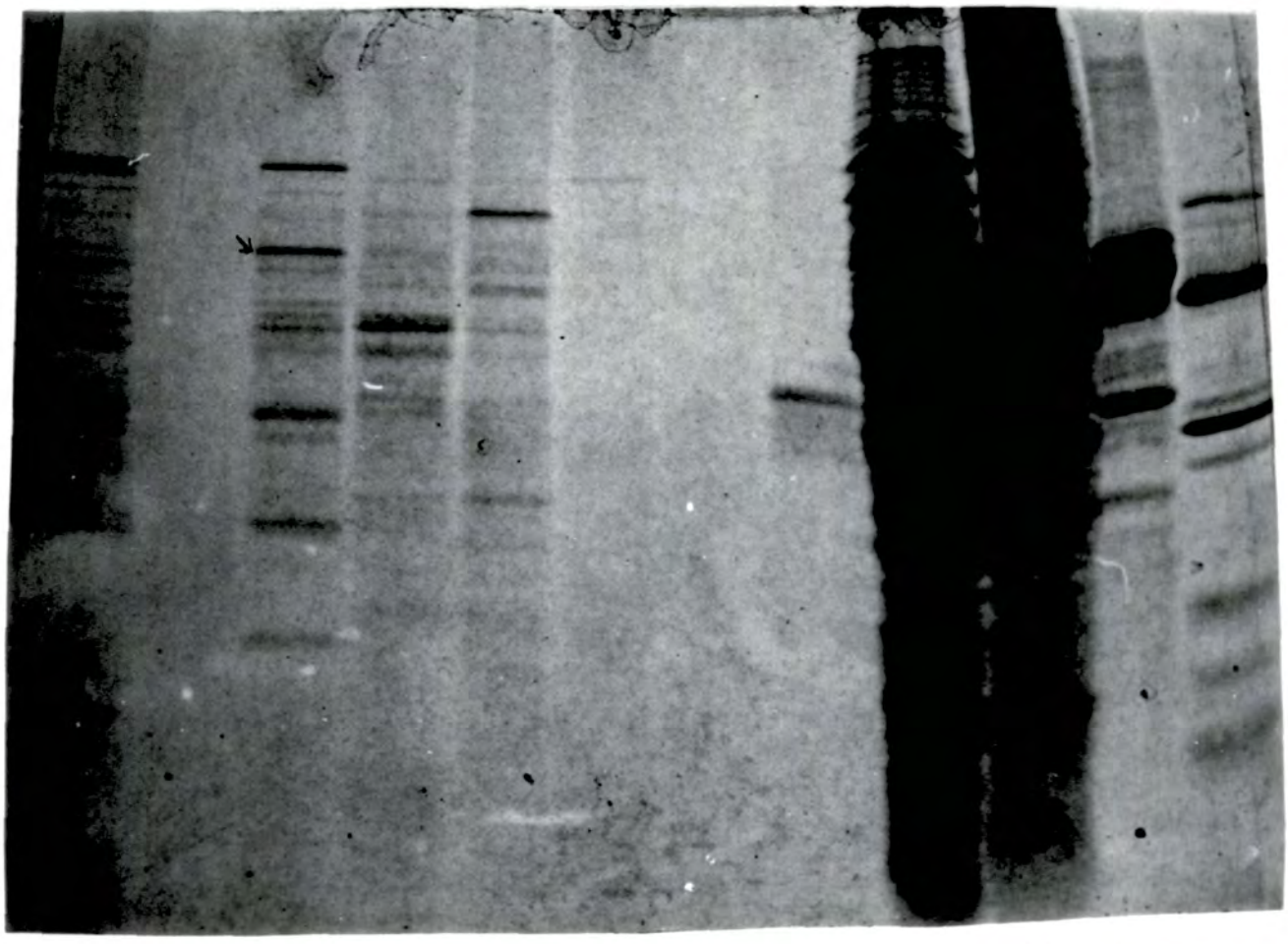
K) 50 µl of yeast supernatant extract.

L) 15 µl of yeast supernatant extract.

Figure: 27

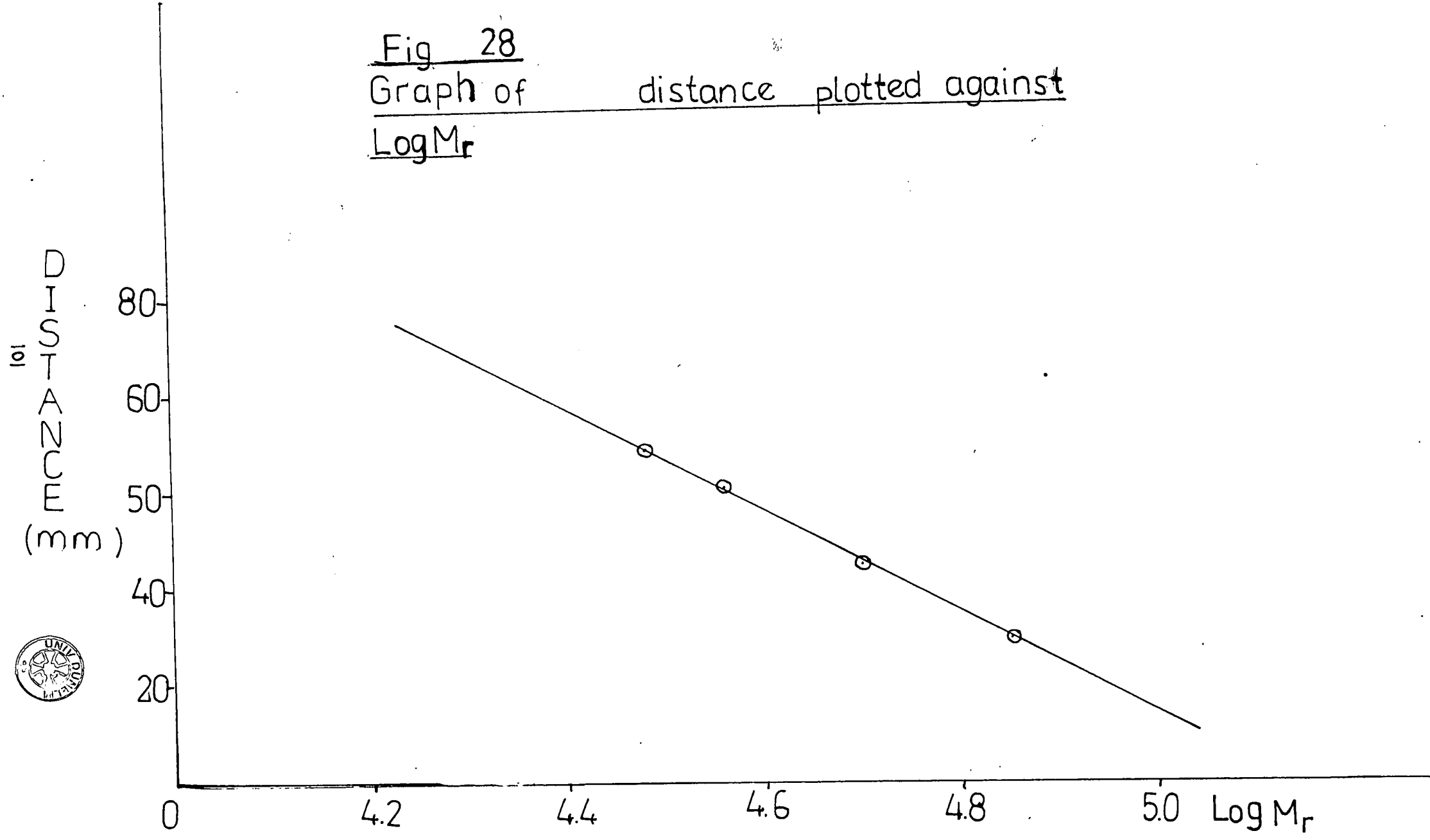
SDS-PAGE - OF SEPHAROSE

PURIFIED YEAST FRACTIONS



A B C D E F G H I J K L

Fig 28
Graph of distance plotted against Log M_r



3.9.1.2 Identification of DEAE-sepharose purified yeast legumin by 17% SDS-PAGE and western blotting

200 µl of each peak fraction was dialysed overnight, freezed-dried, dissolved in 100 µl of 1 x SDS sample buffer and boiled in 2 minutes. Only fraction 34 (track F) reacted with anti-legumin IgG to produce a band of legumin precursor polypeptide of M.. 59,000.

Figure 29 - Photograph of the Western blot of sepharose purified fractions.

A) 50 µl of sepharose purified protein, fraction 96.

B) 50 µl of sepharose purified protein, fraction 68.

C) 50 µl of sepharose purified protein, fraction 57.

D) 50 µl of sepharose purified protein, fraction 47.

E) 50 µl of sepharose purified protein, fraction 41.

F) 50 µl of sepharose purified protein, fraction 34.

G) 50 µl of sepharose purified protein, fraction 25.

H) 15 µl of legumin standard.

I) 15 µl of convicilin and vicilin standard.

J) 50 µl of sepharose purified protein, fraction 22.

K) 50 µl of sepharose purified protein, fraction 15.

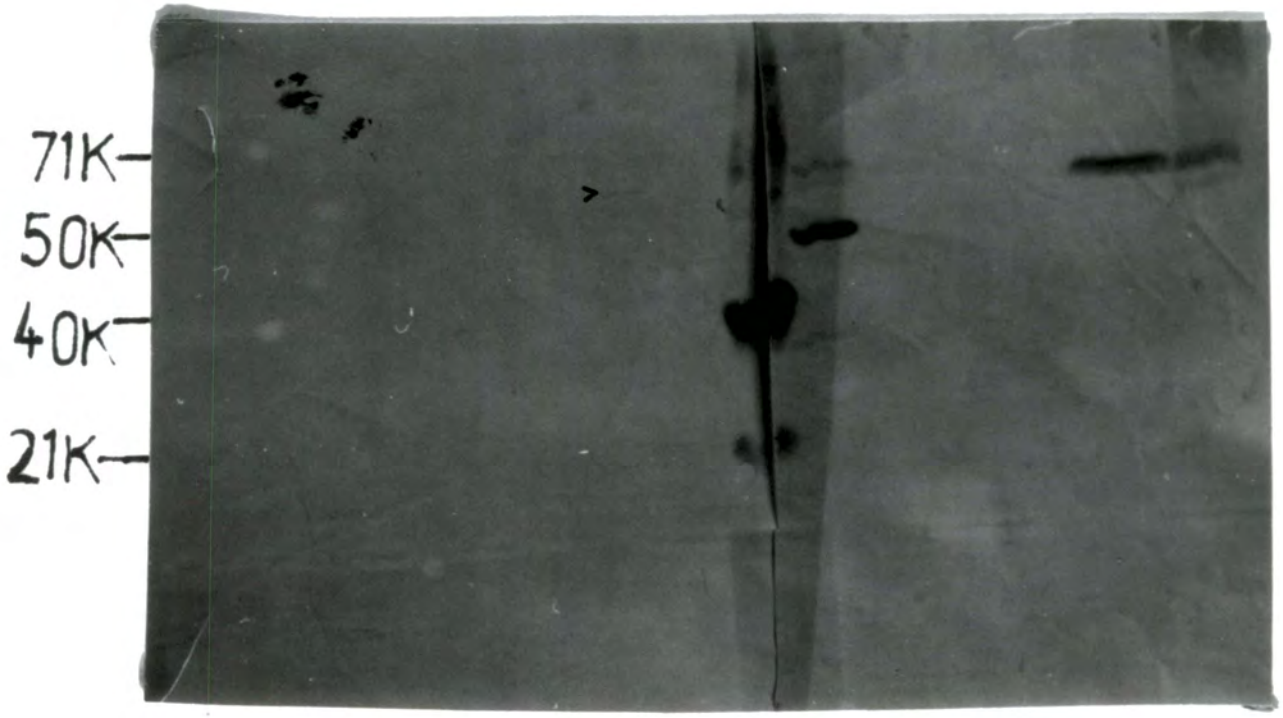
L) 50 µl of sepharose purified protein, fraction 10.

M) 50 µl of PJY 28 yeast supernatant protein.

N) 50 µl of PJY 28 yeast supernatant protein.

Fig: 29

western-blot of DEAE-sepharose purified yeast fraction



3.9.2 Quantitative estimations of Sepharose purified Legumin

A standard graph of 0 - 125 µg was set up using pure legumin standard, as shown in Figure 30. From the 2 ml fraction of 34, 200 µl was removed and dialysed , freeze-dried and dissolved in 100 ul of NaCl buffer. 50 ul was removed and made up to 1 ml with NaCl / Tris buffer. Triplicates of 100 ml samples were assayed, using ELISA.

Fraction number	Dilution	O.D.	Legumin (ng)
34	10,000	3.12	112

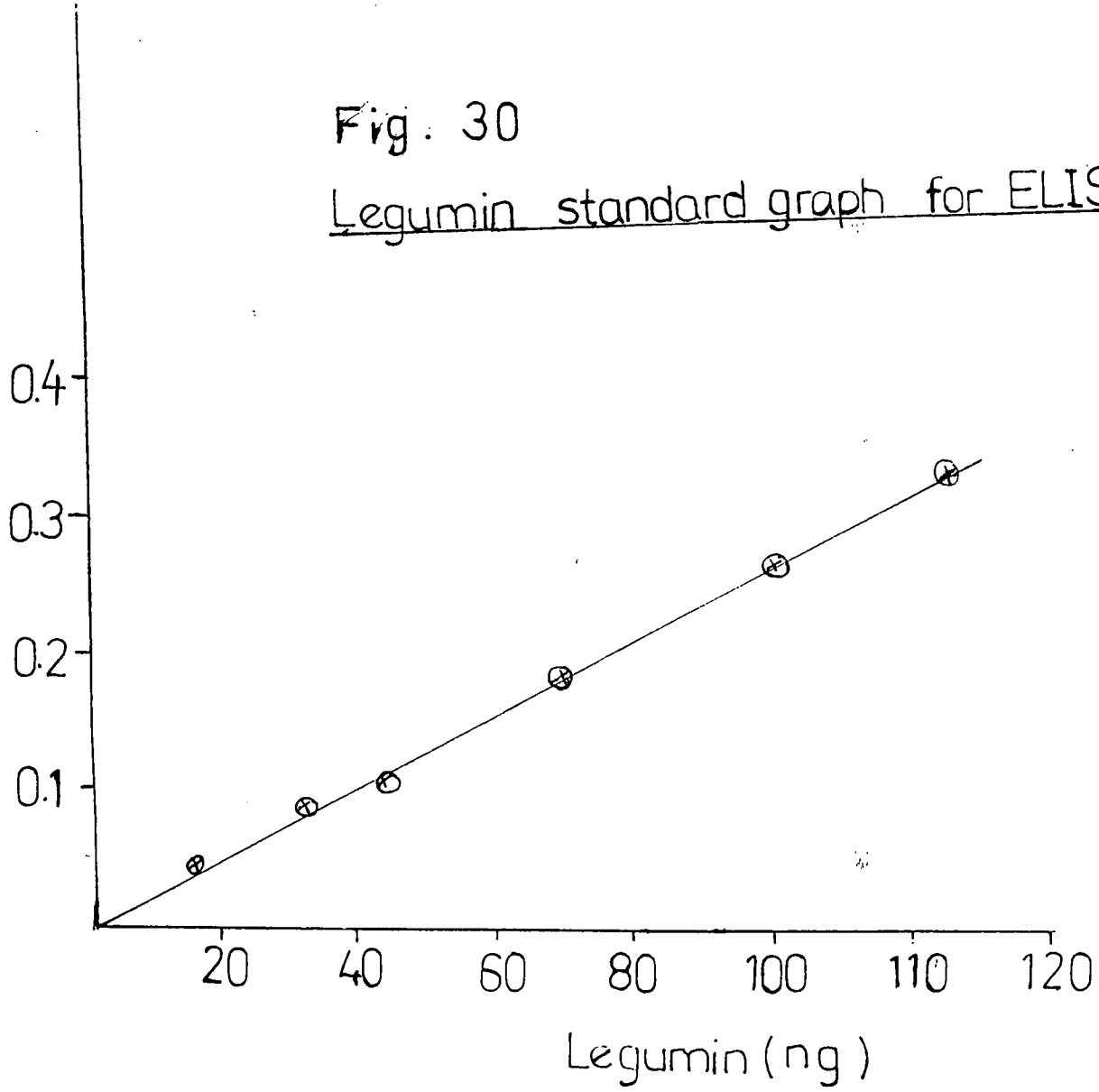
amount of legumin = $\frac{112 \times 10,000}{10^{-6}}$ = 1.12 mg/ml

level of expression = amount of legumin / total yeast protein

= 1.12 / 50.25 x 100 = 2.24%

Fig. 30

Legumin standard graph for ELISA



3.9.3 Further purification of yeast legumin in Fraction 34 by Reverse Phase HPLC

The fractions which fell below the peak of fraction 34 were collected and placed in a dialysis tubing and dialysed for 48 hours and the freeze-dried under a vacuum pump as mentioned in the methods. The solid sample was dissolved in 6 M of Guandine hydrochloride and 0.1% Trifluoro-acetic-acid and subjected to reverse phase HPLC (profile of protein diluted is shown in Appendix V).

The six major peaks observed on the chart recorder represent the separated proteins. These samples were freeze-dried and subjected to N-terminal sequencing.

3.9.4 N-Terminal Sequencing by the Edman Method

A manual double coupling Edman method, as described in the methods was used.

The first peptide from the first dehydration cycle were analysed by thin layer chromatography using both polyamide sheet and silica gel plates. After exposure to hydrochloride vapour, no spots were observed.

CHAPTER 4

4 Discussion

4.1 Comparison of the level of legumin expression with expression of other heterologous proteins in yeast

It was demonstrated that pea legumin was successfully expressed in yeast tranformed with the PJY 28 construct. The levels of expressions estimated by quantitative western blots and ELISA were comparable ; approximately 1.95% and 2.24% of total cell protein respectively. Numerous investigators have demonstated the expression of heterologous eukaryotic gene sequences in *S. cerevisiae* under the control of various yeast gene 'promoter' regions. The yields of a heterologous product in yeast have, however been only 1-5% of total cell protein, even 2 um plasmid based vectros have been used. In comparison, the parent plasmid pMA 27 which carries the complete active PGK gene produces a level of expression of PGK of 50-80% of the total cell protein (23). The possible explanations for the low expression of heterologous protein will discussed later.

Mellor *et al* (15) expressing chymosin cDNA species in vectors containing the yeast PGK promotor showed that the level of expression was dependent upon the length of to cDNA insert. Levels of 1-2% of total

yeast protein were obtained when the cDNA contained an insert 'leader' sequence but the removal of leader sequence caused the level of chymosin to drop tp 0.5% of the total yeast protein. A partial cDNA lacking both leader sequence and approximately 120 bp of coding sequence gave a very low level of expression. These differences in levels of expression were shown not to be due to low plasmid copy number, or to low level of RNA transcription, codon bias, or to ATG initiation enviroment. It was suggested that the low levels of expression may have resulted from high levels of protein turnover. However, in the case of an intereron alpha-2 cDNA construct different limits to expression were identified. Interferon alpha-2 was expressed a chimeric protein in a PGK fusion vector (pMA 230-1) and as a single protein in a closely related PGK vector (pMA 301-1)(25,31) pMA 203-1 contains 1.5 kb of 5'-flanking region of the PGK gene and 37 bp of coding sequence. This vector is used to produce PGK fusion proteins in yeast, and in this construct the correct 'signal' for transcription and translation of yeast gene have been preserved. pMA 301-1 contains all the 5' flanking region of PGK gene up to one nucleotide before the ATG initiation codon. Both pMA 301-1 and pMA 230-1 gave similar expression levels of interferon alpha (1-3% of total cell protein), but the expression levels were considerably

less than those found in the homogenous situation in which a plasmid based PGK structural gene was under its own promoter. This difference was attributed priminary to 5-10 fold reduction in levels of interferon alpha-2 mRNA compared to PGK mRNA resulting from inefficient transcription in the heterologous situation. A difference in steady state protein level was also present. A number of consensus sequences for transcription and initiation in highly expressed yeast genes have been described (34). These genes contain no G residue in the 15-20 bases proceeding the initiation codon, an A residue at -3, and a purine at +6 (12)A number of consensus requirements for yeast termination have also been discussed. These are provided by the 3' region of PGK sequence present in pMA 91, but in addition the legumin cDNA has a termination codon and a polyadenylation site. However whether transcription terminates within yeast or legumin sequences has not been investigate

Recently, a few plant storage proteins have been expressed in yeast. The expression of the major storage protein of french bean, *phaseolin*, under control of natural plant DNA sequences yields a level of 0.01-0.05% of total cellular protein (7). Under the yeast phosphatase promoter in a multicopy expression plasmid, the expression level of bean phaseolin increased to 3% of total cell protein (8). When the

high efficiency promoter of PGK was used to express the pea storage protein, vicilin, the level of expression was approximately 5% of soluble protein (32). The cDNA sequence encoding vicilin used in this experiment lacks a leader sequence, resulting in the encoding protein being located in the cytosol. Estimation of the levels of expression in legumin depend upon visual comparison of the intensity staining on western blots. However the standard legumin under the denaturing conditions employed is dissociated into and B subunits, while the unprocessed expression product remains as a M- 59,000 precusor molecule. Unpublished data consistently demonstrated that the subunit is considerably more antigenic than the subunit. Staining obtained by subunit is always exceeds that of B sub-unit. However comparison of intensity by staining to estimate the level of expression of legumin might be underestimated since the comparison is not between identical polypeptides. Further experiments in progress use monoclonal antibodies to compare the amounts of types of legumin.

4.2 Post-translational proteolysis of legumin

Legumin polypeptides are synthesized in vivo as precusor of M-. 59,000 from which a 21 amino acid leader sequence is removed, followed by cleavage of a single Asn-Gly peptide bond (24) to yield the and b

subunits. The latter process probably involves a single enzyme located in the protein bodies of seed cotyledons. The presence in yeast of a membrane and vascular system sugests that such post-translational modifications may be carried out by yeast and indeed several proteins expressed in yeast have been shown to undergo varying degrees of post-translational modification (7,8,14).

The western blots consistently demonstrate a M$_r$. 59,000 polypeptide, suggesting the failure of yeast to carry out post-translational cleavage of legumin precusor to generate the mature, disulphide-linked and b polypeptides (M$_r$. 38,000 and 21,000).

This could be due to several causes. Since the legumin polypeptide can only extracted under denaturing conditions this suggests legumin polypeptide is enclosed in a membrane system. Electron microscopy localisation studies of legumin support this view (33). Legumin was located using gold labelled goat anti-rabbit IgG and rabbit anti-legumin IgG Legumin polypeptides were synthesized at the endoplasmic recticulum and are sequestered within golgi apparatus, in a mannner similar to that occuring within pea cotyledonary tissue. It is likely that the newly synthesized legumin may be sequestered into an intracellular compartment in yeast where it is inaccessible to the yeast protease.

It is also possible that the susceptible bond for the cleavage may not be avaliable. The legumin synthesized in yeast may be associated within the endoplasmic recticulum into an insoluble oligomeric form (6). Result shown in figure 4 and 5 show the presence of partially assembled molecules. A similar observation was made for vicilin assembly when cDNA was expressed in the same host (32).

A third possibility is that yeast lacks the neccessary enzyme to carry out cleavage. The endoproteolytic enzyme which carries out the processing in the plant is highly specific. The post-translational cleavage of the legumin precusor occurs at a specific single site, C-terminal to the asparagine residue in the sequence -R-R-Q-G-D-N/ G-L-E-E-T- (16). The commonly described yeast protease have no such specificities. Indeed, when limited proteolysis was allowed to occur the legumin synthesized in yeast generated a heterologous pattern of bands representing polypeptides of M_r. less than 59,000 (Figure 4 and 5). The bands vary from preparation to preparation indicating that this represents non-specific proteolysis by the abundant yeast protease.

The particular asparaginyl residues susceptible for cleavage are situated in accessible region of the protein (typically B-turn conformation) in the

hydrophilic region of the protein (Appendix 6.6 shows secondary structure prediction and hydrophilicity index profile for amino acid sequence of legumin). It may be that this geometrically well-defined domain which faciiates the intraction with the processing enzymes was altered and distorted in yeast, when legumin was extracted under denaturing condition.

Recent investigation suggested that some specific features of plant signal sequences were required for post-translational modifications to occur (8). Bean phaseolin under the yeast phosphatase promoter with no signal peptide showed no post-translational modification in yeast. Partial or complete substitution of phaseolin signal peptide with that from acid phosphatase dramatically inhibited both processing and glycosylation, suggesting that some specific feature of phaseolin signal peptide was required for this modification to occur. But it is not clear whether the failure of acid phosphatase signal peptide to direct post-traslational processing in the partial hybrid protein examined here was a function of a phosphatase sequence itself, yeast signal sequence as a whole, or some special feature of the phaseolin polypeptide. When a large portion of the mature and phosphatase protein was present as a hybrid protein with phaseolin mature polypeptide, this hybrid protein was glycosylated in yeast cells and underwent a number

of proteolytic processing steps. In addition, some feature of hybrid protein appeared to allow proteolytic cleavage at numerous sites in the acid phosphatase amino sequence that were not used in the wild type form of the enzyme. From the analysis of the average hydrophobicity of amino acid of acid phosphatase and phaseolin. It was shown that the phaseolin signal peptide has a short hydrophilic sequence whereas the N-terminal of acid phosphatase signal peptide is hydrophobic in nature. It is not clear how this difference may effects on the processing of the expressed heterologous protein therefore it is necessary to develop more understanding of the requirements for the modification of the hybrid protein as a whole.

4.3 Co-translational processing of legumin in PJY 28 yeast

The legumin construct PJY 28 directs the the synthesis of legumin precusor polypeptide complete with signal peptide. Transport of newly synthesized legumin into the lumen of the endoplasmic recticulum would be expected to involve removal of signal peptide. Comparing the mobilities of yeast legumin and pea legumin o 12.5% SDS-PAGE and western blotting (Figure 23 and 24) indicated that the 21 amino acid signal peptide of the precusor legumin polypeptide had been removed. This strongly suggests a recognition of the

plant signal sequence by yeast. These results indicate that processing af signal peptides is accomplished by a universal mechanism. Therefore, it is assumed that removal of the signal sequence from legumin precusor by yeast occurs at a site very near to the cleavage site used in plant. Several plant proteins have been shown to undergo signal peptide cleavage in S. cerevisiae for example bean phaseolin (7 and 8), plant thaumatin (14), -amylase (28), eventhough the amylase gene has the signal sequence located internally, not at the N-terminal.

However, in view of somewhat limited resolution of SDS-PAGE, and the relatively small difference in M_r. expected between the processed and non processed polypeptide, it is difficult to demonstrate conclusively that the leader peptide has been removed.

The first amino acid on the N-terminal is methionine. With the removal of signal peptide, the first amino acid on the N-terminal would be leucine (Appendix 5.5 shows the N-terminal sequence of legumin). Analysis of the amino acid sequence in the N-terminal of purified yeast legumin precusor could therefore confirm that processing does occur. However,the attempt at amino acid sequencing of the N-terminal was not successful. This could be due to small amount of purified sample present. Another reason might be the N-terminal was blocked. The lack

of time made it impossible to resolve this.

4.4 Possible conditions and strategy to improve yield of legumin

By exploring other growth media it might be possible to increase further the level of legumin produced from the existing construct. When expressing a vicilin cDNA in the same host there, is an increase of 75% in cell density when yeast is growth in complete enriched medium compared with cells grown in minimal medium.This results in a significant increase in yield of vicilin.

However, a preliminary attempt of growing PJY 28 in complete medium resulted in a decrease or cessation in legumin expression when compared with cell grown in minimal medium. This may be due to the turnover of the expressed legumin. Alternatively, in the minimal medium transcription and translation from the plasmid must be occuring at a high level, in order to supply the leucine necessary for growth of the Leu- yeast in a leucine free medium. This is accompanied by high levels of expression from the legumin cDNA encoded by the plasmid.

Due to insolubility of yeast legumin, the polypeptide can only be extracted by denaturants. The presence of denaturant causes many problems. It interferes with many protein assays and also supressed the antigenity of legumin. The ELISA method depends on the surface antigenity of the antigen (legumin) binding

onto the surface of the microtiter wells, and subsequent binding of the primary antibody to the antigen followed by binding of secondary antibody to the primary antibody. As the antigenity of the antigen is supressed less legumin will be antigenically available for the binding to the surface of the wells. This explain the low detection of legumin in the unpurified PJY 28 pellet protein, which was extracted under denaturing condition. The low amount of extracted under non-denaturing condition showed much higher antigenity. Even though the estimation of yeast legumin by quantitative western blot and ELISA of purified yeast protein were comparable, the level of expression of legumin detected by these methods might be underestimated. It would be more suitable if the encoding protein could be directed to the cytosol where it can be harvested under non-denaturing coditions. Vicilin cDNA expressed in yeast without a leader sequence resulted in vicilin polypeptide being located in the cytosol (32). An analogous legumin construct is in progress.

Another possibility to produce soluble protein is to use a signal peptide to secrete the encoded protein into the growth medium. Hitzeman *et al* (18) constructed a series of plasmids in which either mature interferon or pre-interferon genes were placed downstream from a PGK promoter such that native, as

opposed to fused, protein were produced. Yeast cells carrying such plasmids synthesized interferon but only those encoding pre-interferon produced active interferone secreted in the growth medium. Expression of other secretory proteins such as -amylase (28) and prochymosin (29), both under the control of PGK promoter with the presence of a secretory signal peptide resulted in secretion of encoded protein into the growth medium. The secreted chymosin is soluble and active. The -amylase was processed and secreted as an active form.

Heterologous plant gene and animal gene sequences have been successful expressed in yeast. The encoded proteins are processed, modified and secreted, as previously described. This clearly shows that yeast systems are able to recognise foreign gene signal peptides and to process them correctly. The successful processing and export of plant and animal proteins indicates the general similariity of all eukaryotic secretory signal and processing apparatus. The yeast system therefore provides an alternative system in which valuable and rare proteins can be produced. The ability to express plant genes in *S. cerevisiae* faciliates not only the production of large quantities of homogenous protein, but is also a vehicle for the production of novel proteins by in vitro mutagenesis, in which predictable changes in amino acid sequence can

be made, and their effects upon functional and physio-chemical properties can be determined. This will allow the basis of various properties of proteins to be determined.

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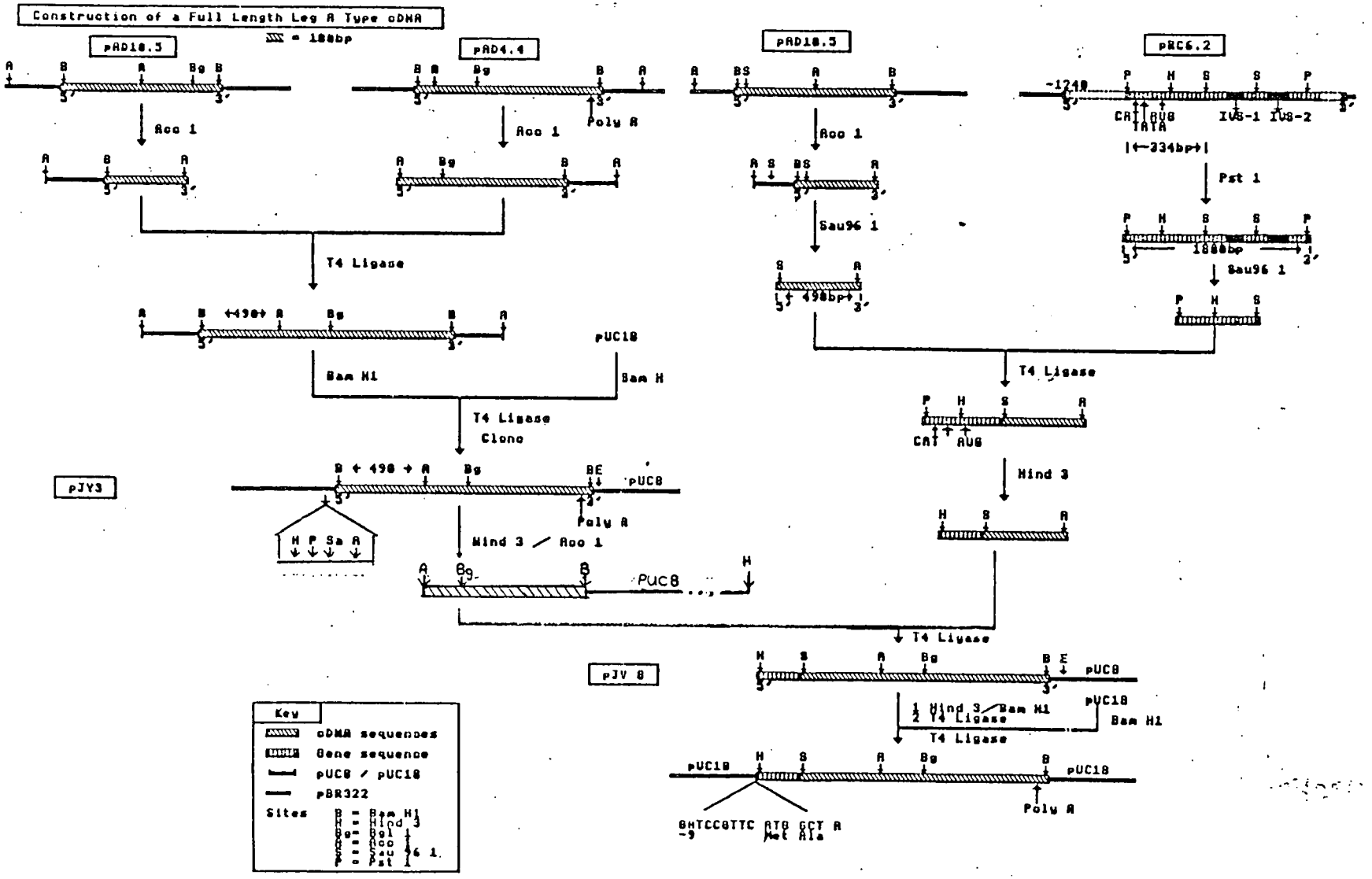
APPENDIX 6:1


Construction of a Full Length Leg A Type cDNA
= 188bp
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pAD4.4
pAD18.5
pRC6.2
Acc 1
Poly A
Acc 1
Acc 1
CAT
TATA
AUG
IVS-1
IVS-2
-1248
-234bp
Pst 1
Sau96 1
1888bp
Sau96 1
T4 Ligase
498bp
T4 Ligase
pUC18
Bam H1
Bam H
T4 Ligase
Clone
pJY3
Poly A
pUC8
Hind 3 / Acc 1
T4 Ligase
pJV 8
pUC8
pUC18
Bam H1
T4 Ligase
pUC18
pUC18
Poly A
Key
cDNA sequences
Gene sequence
pUC8 / pUC18
pBR322
Sites
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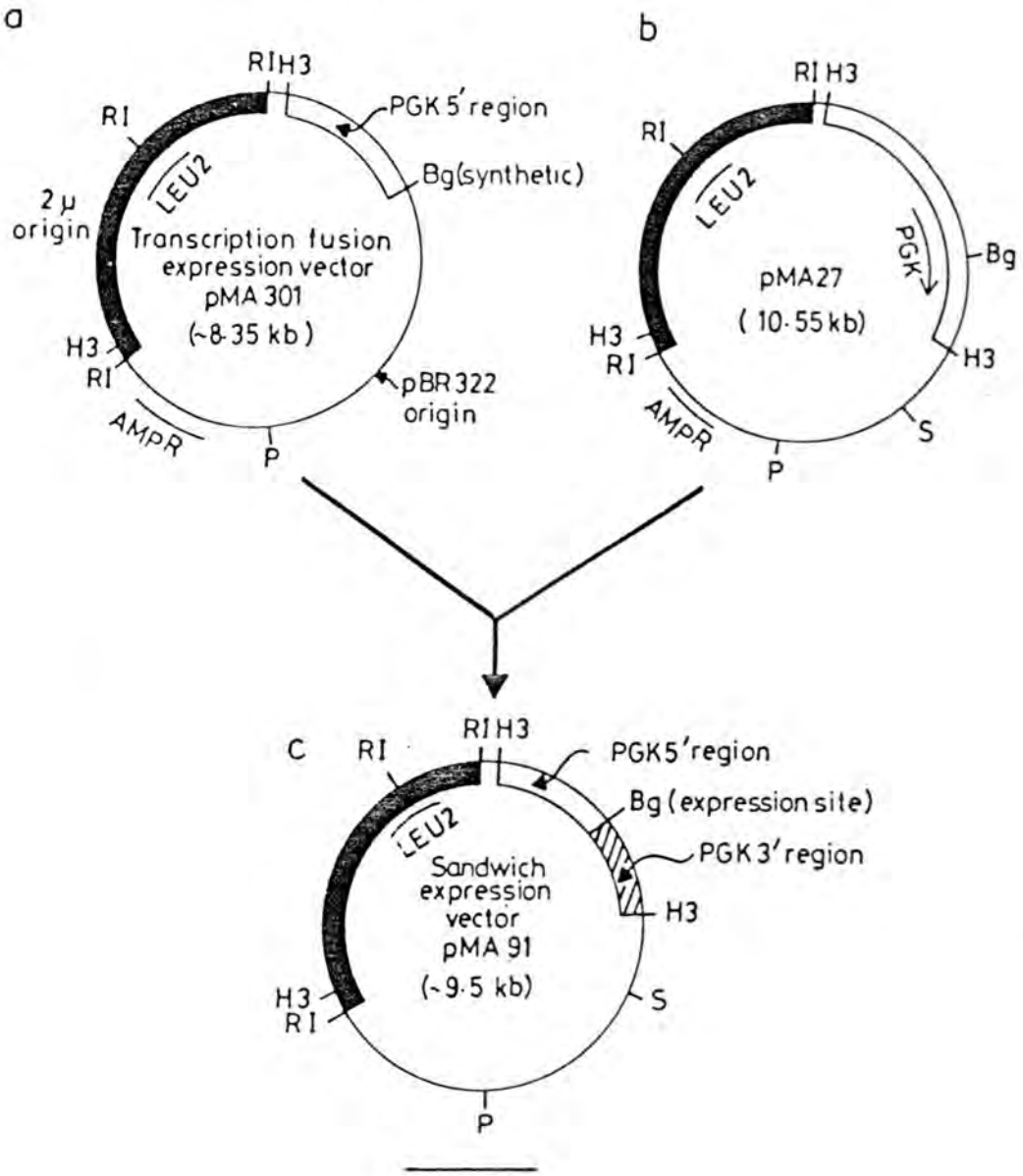
H = Hind 3
Bg= Bgl 1
A = Acc 1
S = Sau 96 1
P = Pst 1

APPENDIX 6:2



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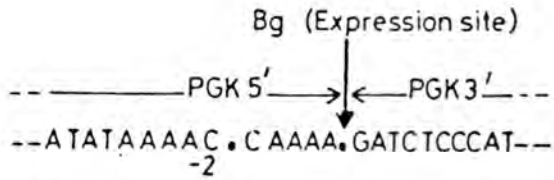
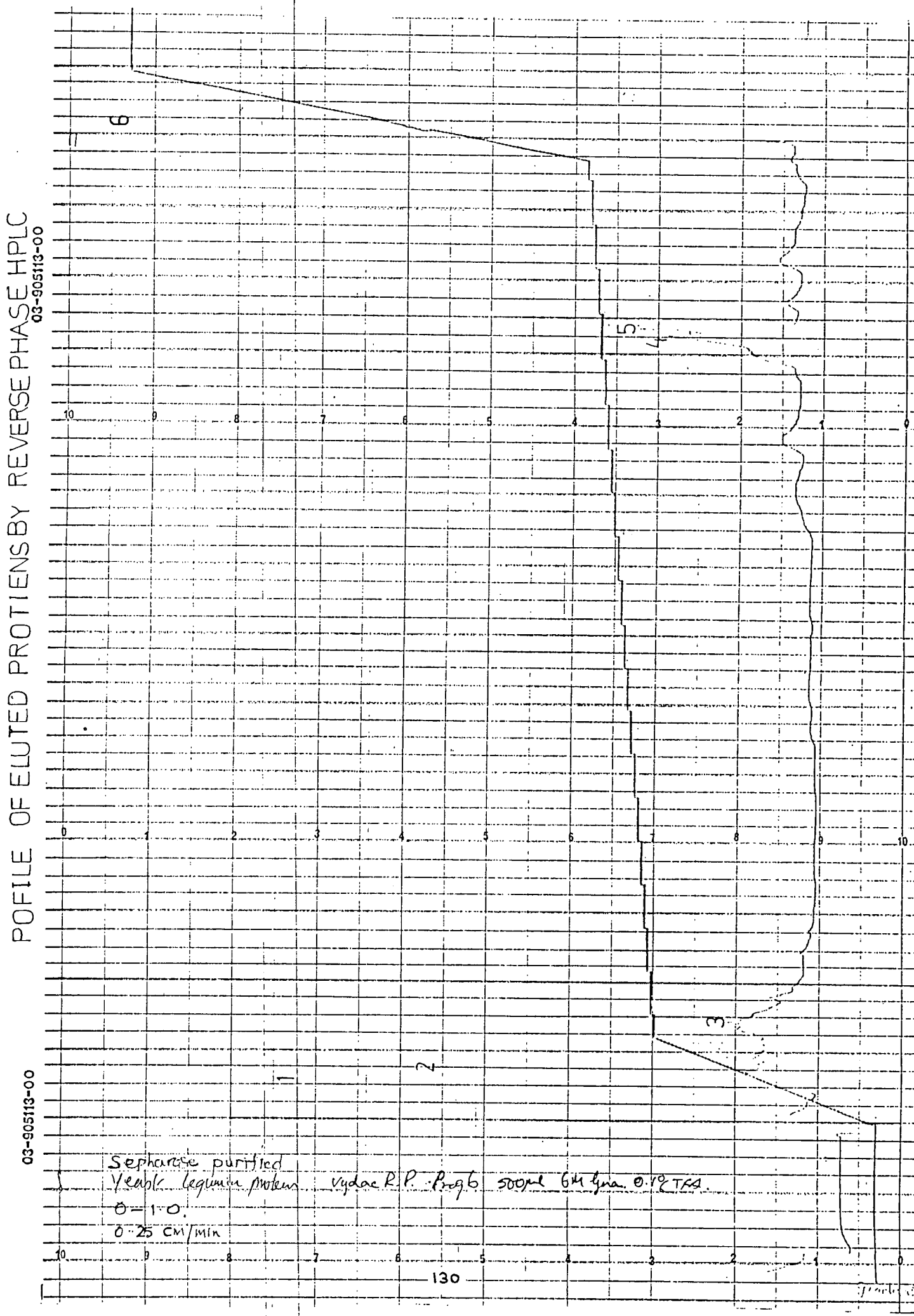
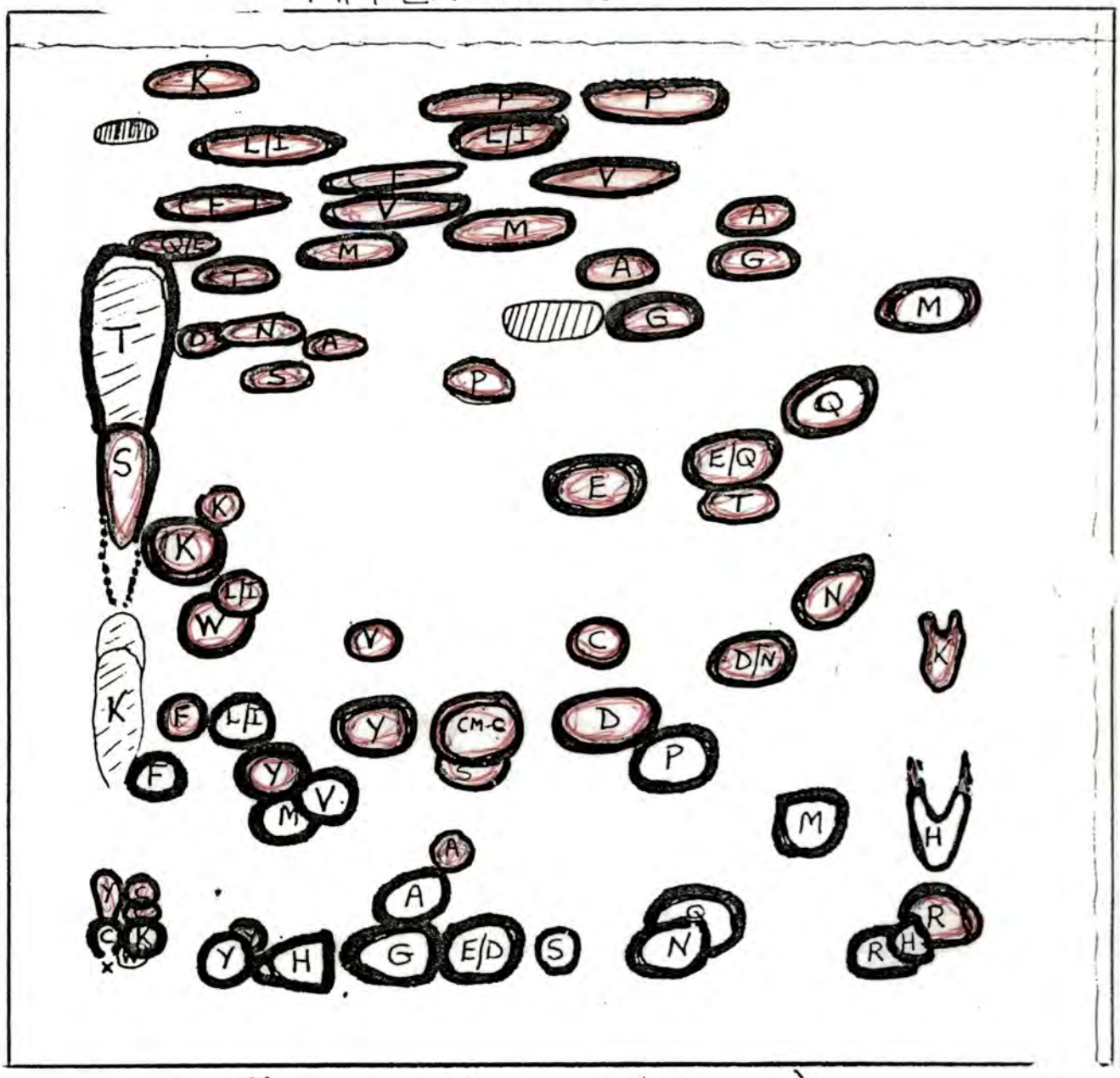


Construction of a sandwich expression vector. (a) Transcription fusion vector pMA301. Thin line, pBR322 sequences; Nar box, yeast DNA region containing the 2μ origin of replication and the *LEU2* gene; open box, 5′ region of the yeast *PGK* gene up t nucleotide −2 (Dobson et al., 1982). (b) Plasmid pMA27. Thin line and dark box as for (a), open box, 2.95-kb *Hin*dIII fragme containing the entire *PGK* coding sequence and some 5′ and 3′ non-coding sequences. The arrow marks the direction of transcriptio (c) Sandwich expression vector pMA91. Thin line, dark box and open box as in (a); hatched area is the carboxy terminus of the *PG* gene and some 3′ non-coding sequence. (d) Immediate nucleotide sequence around the *Bgl*II expression site in pMA91. The sequenc between the dots is the remainder of the synthetic oligonucleotide; sequences to the left and right are derived from *PGK* 5′ and regions, respectively. Bg = *Bgl*II; H3 = *Hin*dIII; P = *Pst*I; S = *Sal*I; RI = *Eco*RI.

APPENDIX 6:3


POFILE OF ELUTED PROTIENS BY REVERSE PHASE HPLC
03-905113-00
03-905113-00
6
5
4
3
2
1
10 9 8 7 6 5 4 3 2 1 0
0 1 2 3 4 5 6 7 8 9 10
Sephacryl purified
Yeast legumin protein vydac R.P. Prog 6 500µl 6M Gua. 0.1% TFA.
0=1.0.
0.25 cm/min
10 9 8 7 6 5 4 3 2 1 0

APPENDIX 6:4



STANDARD, DABITC-DIETHYLAMINE

MAJOR DABTH AMINO ACIDS

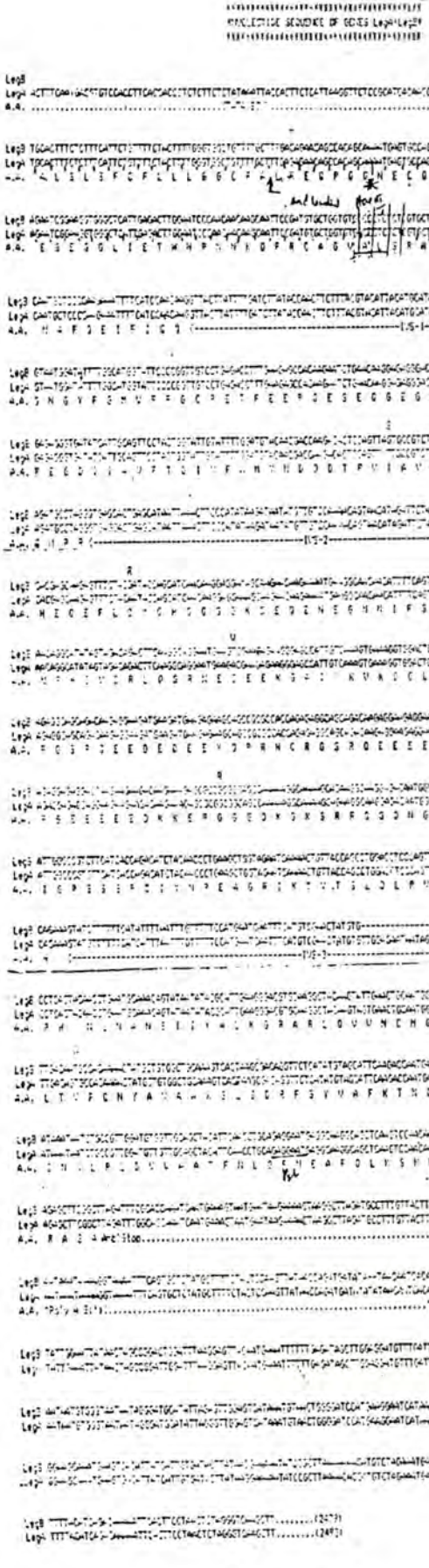
MINOR DERIVATIVES (UNKNOWN)

DABTC BYPRODUCTS (INCOMPLETE CONVERSIONS)

A = ALANINE
C = CM-CYSTEINE
D = ASPARTIC ACID
E = GLUTAMIC ACID
F = PHENYLALANINE
G = GLYCINE
H = HISTIDINE
I = ISOLEUCINE
K = LYSINE
L = LEUCINE
M = METHIONINE
N = ASPARAGINE
P = PROLINE
Q = GLUTAMINE
R = ARGININE
S = SERINE
T = THREONINE
V = VALINE
W = TRYPTOPHAN
Y = TYROSINE

Method used is essentially that of CHANG et al (1978) FEBS Lett. 93, 205

APPENDIX 6:5



APPENDIX 6.6