The intracellular sorting of vacuolar proteins in the yeast saccharomyces

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The Intracellular Sorting of Vacuolar Proteins in the Yeast *Saccharomyces cerevisiae*

by

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To My Wife and Children

DECLARATION

I declare that all the experiments involved in this thesis submitted by me for the degree of Doctor of Philosophy are my own original work and have never been submitted for a degree at this or any other university.

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April 1989

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ABSTRACT

The mechanism of protein sorting to the vacuole in yeast was studied both *in vitro* and *in vivo*. A series of experiments were performed to reconstitute transport of carboxypeptidase Y (CPY) from Golgi vesicles to vacuoles. In order to investigate this process, microsomes were purified from *sec, pep4-3* mutant strains that accumulate inactive proCPY in the Golgi when incubated at the nonpermissive temperature. These were mixed with purified vacuoles isolated from a mutant lacking CPY activity, but containing active proteinases A and B. Transported proCPY is maturated by these proteinases to active form. Experiments indicate that maturation of CPY is due to the correct transport of proCPY from microsomes to vacuoles because:- Firstly, the reaction is temperature sensitive, requires ATP and is stimulated by the addition of soluble factors (S100). Secondly, the addition of proteinase A and B inhibitors to the reaction mixtures has a negligible effect on the maturation process. Thirdly, disrupting the membranes by the addition of TritonX-100 before addition of the proteinase inhibitors, inhibited the maturation of proCPY. Fourthly, the majority of CPY activity was observed in the sedimented fraction of the reaction mixtures rather than supernatant fractions. Lastly, analysis with western blot shows a clear band of mature CPY only in the sedimented fraction of the reaction mixtures with ATP. This *in vitro* system will be invaluable in investigating the molecular events of vacuolar biogenesis.

For *in vivo* sorting of proteins to the vacuole, a series of experiments were performed that involved the genetic fusion of the CPY promoter and prepro-sequence of CPY to the bacterial Gus (β-glucuronidase) reporter gene. The Gus gene was expressed in yeast with high efficiency and the results of sub-cellular fractionation indicated that the Gus product was distributed in all cell components. Using a centromeric vector gave similar results but with a lower efficiency of Gus expression. Removal of 90bp from Gus, including Gus initiation codon does not completely inhibit Gus expression either in bacteria or in yeast. Fusion of the shortened Gus with the CPY prepro-fragment and expression in yeast led to the correct sorting of the CPY-Gus hybrid protein to the vacuole. This CPY-Gus fusion is potentially useful in the genetic analysis of mutations defective in vacuolar protein sorting.

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ABBREVIATIONS

Amp = Ampicillin

A_{260} = Absorbance at 260nm

A_{280} = Absorbance at 280nm.

bp = Base pair

BSA = Bovine serium albumin

BTPNA = N-benzoyl-L-tyrosine-p-nitroanilide

Cbz-Gly-Leu = Benzyloxycarbonyl-glycyl-leucine

Cbz-Phe-Leu = Benzyloxycarbonyl-phenylalanyl-leucine

CPS = Carboxypeptidase S

CPY = Carboxypeptidase Y

DAB = 3,3'-Diaminobenzidine tetrahydrochloride dihydrate

DMF = Dimethyl formamide

dNTP = Deoxyribonucleoside triphosphate

ddNTP = Dideoxyribonucleoside triphosphate

DTT = Dithiothreitol

EDTA = Ethylenediaminetetra-acetic acid

EtBr = Ethidium bromide

Exo III = Exonuclease III

IPTG = Isopropylthiogalactoside

Kan = Kanamycin

Kb = Kilobase

kD = Kilodalton

LMP = Low melting point

MUG = 4-Methylumbelliferyl glucuronide

ABBREVIATIONS (Cont.)

NEM = N-ethylmaleimide

PAGE = Polyacrylamide gel electrophoresis

PEG = Polyethylene glycol

PMSF = Phenylmethylsulphonylfluoride

RER = Rough endoplasmic reticulum

RNAase = Ribonuclease

SDS =Sodium dodecyl sulphate

Tet = Tetracycline

Tris = Tris(hydroxymehtyl)methylamine[2-amino-2-(hydroxymethyl)propane-1,3-diol, (tris)]

W/o = Without

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CHAPTER ONE

1. INTRODUCTION

1.1 Vacuolar Proteinases

The yeast lysosomal-like vacuole is considered to be the digestive compartment of the yeast cell (Matile and Wiemken, 1967; Wiemken, *et al.,* 1979), which at least in part, is functionally related to the lysosome or vacuole of higher eukaryotic organisms. The work of Hata, *et al.,* (1967a,b), Lenney and Dalbec (1967), Lenney, *et al.,* (1974) and Wiemken, *et al.,* (1979) led to the characterization and separation of three vacuolar enzymes, namely proteinase A, proteinase B, and proteinase C. This last enzyme was later termed carboxypeptidase Y (Hayashi, *et al.,* 1970, 1972, 1973a). These enzymes for a long time were the only well known yeast proteinases (Wolf, 1980; Wolf and Holzer, 1980).

Proteinase A is a soluble carboxylic endopeptidase (Hata, *et al.,* 1967a; Lenney and Dalbec, 1967; Meussdoerffer *et al.,* 1980; Magni *et al.,* 1982). The enzyme consists of a single polypeptide chain and has a molecular weight of 42kD of which about 8.5% is carbohydrate (Meussdoerffer, *et al.,* 1980; Mechler *et al.,* 1982b). The structural gene for proteinase A is *PRA1.* The enzyme can be assayed using acid denatured haemoglobin or casein (Hata, *et al.,* 1967b; Meussdoerffer, *et al.,* 1980) at a pH optimum of 3 and 6 respectively. The enzyme can also be measured using the peptide succinyl-arg-pro-phe-his-leu-leu-val-tyr-7-amino-4-methyl-coumarin as a substrate at pH5. Hydrolysis occurs at the leu-val bond (Yokosawa, *et al.,* 1983) and the product can be assayed fluorometrically. Proteinase A is inhibited by pepstatin (Meussdoerffer, *et al.,* 1980; Wolf, 1986; Mechler, *et al.,* 1987) and its activity is

inhibited in cell extracts (Saheki, *et al.*, 1974) due to a specific cytoplasmic proteinase A inhibitor of 7.7kD molecular weight (Saheki, *et al.*, 1974; Matern, *et al.*, 1974a; Lenney, 1975). Incubation of cell extracts at low pH releases proteinase A activity due to the digestion of proteinase A inhibitor by proteinase B (Matern, *et al.*, 1974; Lenney, 1975; Beck, *et al.*, 1980).

Proteinase B is a soluble serine, sulphhydryl endopeptidase with optimum activity at neutral pH (Hata, *et al.*, 1967a,b; Lenney and Dalbec, 1967; Ulane and Cabib, 1976; Kominami, *et al.*, 1981). It has a molecular weight of about 33kD, consisting of a single polypeptide (Kominami, *et al.*, 1981; Mechler, *et al.*, 1982b). 10% of the molecular weight is carbohydrate (Kominami, *et al.*, 1981). The structural gene for proteinase B is *PRB1* (Zubenko, *et al.*, 1979). The enzyme cleaves acid denaturated haemoglobin and casein (Hata, *et al.*, 1967a) and Azacoll (Cabib and Ulane, 1973; Saheki and Holzer, 1974; Ulane and Cabib, 1976). Proteinase B exhibits some activity towards ester substrates such as N-benzoyl-L-arginine ethyl ester and N-acetyl-DL-phenylalanine-β-naphthyl ester (Ulane and Cabib, 1976; Wolf and Ehmann, 1978b; Wolf, 1980; Kominami, *et al.*, 1981). The enzyme activity is strongly inhibited by phenylmethylsulphonylfluoride (PMSF) and diisopropylfluorophosphate (Hata, *et al.*, 1967a; Lenney and Dalbec, 1967) and by chymostatin (Wolf, 1986: Mechler, *et al.*, 1987). Mercury compounds are also strong inhibitors of proteinase B (Hata, *et al.*, 1967a; Ulane and Cabib, 1976; Kominami, *et al.*, 1981). The activity of the enzyme is inhibited in yeast crude extracts due to the action of a cytoplasmic proteinase B inhibitor of about 8.5kD molecular weight (Lenney and Dalbec, 1969; Lenney, 1975; Dunning and Holzer, 1977; Maier, *et al.*, 1979). Incubation of yeast cell extracts at low pH releases proteinase B activity from the inhibition due to digestion of the inhibitor by proteinase A (Saheki, *et al.*, 1974).

Carboxypeptidase Y. This enzyme was originally termed proteinase C (Hata, *et al.,* 1967a,b) but now designated carboxypeptidase Y (CPY) to distinguish it from similar enzymes from other sources and because of its broad substrate specificity (Hayashi, *et al.,* 1970; Aibara, *et al.,* 1971; Hayashi, 1976). It is the most studied peptidase in *Saccharomyces cerevisiae* (Hayashi, *et al.,* 1968, 1975a,b,c; Kuhn, *et al.,* 1974, 1976; Bai, *et al.,* 1975a,b; Bai and Hayashi, 1979; Johansen, *et al.,* 1976). CPY is a serine sulphydryl carboxypeptidase (Doi, *et al.,* 1967; Hata, *et al.,* 1967a,b; Hayashi, *et al.,* 1973a; Kuhn, *et al.,* 1974; 1976) consisting of one polypeptide chain of 61kD molecular weight (Aibara, *et al.,* 1971; Kuhn, *et al.,* 1974; Hayashi, 1976; Hasilik and Tanner, 1978a,b). Mature CPY carries four asparagine-linked oligosaccharides, three of which are phosphorylated (Mazon and Hemmings, 1979; Hashimoto, *et al.,* 1981; Trimble *et al.,* 1983).

The structural gene for CPY is *PRC1* (Wolf and Fink, 1975; Wolf and Weiser, 1977; Hemmings, *et al.,* 1981). The enzyme exhibits a broad specificity against peptides, esters and amides as well as against the chromogenic substrate benzoyl-tyrosine-4-nitroanilide (Hayashi, 1976) and also against proteins (Doi, *et al.,* 1967; Hayashi, 1976). The enzyme is strongly inhibited by PMSF and diisopropylphosphate (Wolf and Fink, 1975; Wolf and Weiser, 1977). Mercurial compounds also inhibit the peptidase activity of the enzyme by binding to the single SH-group (Hayashi, 1976: Wolf, 1980). A cytoplasmic inhibitor of 25kD molecular weight inhibits CPY activity (Matern, *et al.,* 1974a,b; Lenney, 1975). Incubation of yeast cell extracts at pH 5.0 for 20hrs at room temperature or for longer at 4°C leads to reactivation of CPY activity (Hayashi, *et al.,* 1968,1972; Saheki, *et al.,* 1974) due to inactivation of its specific inhibitor by proteinase A (Wolf and Fink, 1975; Wolf and Holzer, 1980; Wolf, 1980).

Synthesis of Vacuolar Proteinases

According to Hasilik and Tanner (1978b) and Mechler *et al.,* (1982a) the soluble vacuolar proteinases (A, B and CPY) are synthesized as a higher molecular weight precursors with molecular weights of 52, 42, and 67kD respectively. These high molecular weight precursor forms are reported to be inactive (Hasilik and Tanner, 1978b; Hemmings, *et al.,* 1981; Jones, *et al.,* 1982). ProCPY can be activated *in vitro* by proteinase B alone (Hasilik and Tanner, 1978b), however, mutants lacking proteinase B activity still exhibit normal CPY and proteinase A activity (Wolf and Ehmann, 1978b, 1979; Zubenko, *et al.,* 1979). ProCPY is also activated by proteinase A which is believed to be self-activating.

pep4-3 mutant yeast cells lead to a deficiency of a variety of vacuolar hydrolases (Jones, 1977, 1984; Jones, *et al.,* 1981, 1982; Zubenko, *et al.,* 1982). *pep4-3* cells accumulate inactive precursors for proteinase B (Mechler, *et al.,* 1982b), CPY (Hemmings, *et al.,* 1981) and proteinase A (Zubenko, *et al.,* 1983). This finding strongly suggests that the *PEP4* gene product is required for the processing and activation of the vacuolar enzymes (Jones, 1984), but not for localization, since these precursors are found as normal in the vacuole (Stevens, *et al.,* 1982, 1986b)

Accumulated proproteinase B and proCPY could be immunologically detected in a *pep4-3* mutant but no immunoreactive proteinase A could be found (Mechler, *et al.,* 1982b). Recently, Ammerer, *et al.,* (1986), Woolford, *et al.,* (1986) and Mechler, *et al.,* (1987) found that *PEP4* is the structural gene for proteinase A, identical with the gene previously identified as *PRA1.*

Mechler, *et al.,* (1987) have also described that proCPY can be processed *in vitro* to active mature CPY by the action of proteinase B alone. Purified proteinase

A, from a mutant lacking proteinase B activity, can activate proCPY *in vitro* to an active form of somewhat higher molecular weight as compared to the authentic mature CPY. This intermediate processed form of proCPY can be further processed to the authentic mature CPY by the addition of proteinase B.

Carboxypeptidase S. In a *Saccharomyces cerevisiae* mutant lacking CPY activity a second carboxypeptidase called carboxypeptidase S (CPS) could be detected (Wolf and Fink, 1975; Wolf and Weiser, 1977). This enzyme cleaves the dipeptide Cbz-Gly-Leu with high efficiency and its activity is strongly inhibited by EDTA (Wolf and Weiser, 1977; Wolf and Ehmann, 1978a; Jones, 1984). Reactivation of CPS can be achieved by Co^{2+}, Mn^{2+} and Zn^{2+} ions (Wolf and Weiser, 1977; Wolf, 1982). The structural gene for CPS is *CPS1* (Achstetter and Wolf, 1985).

Other Vacuolar Proteases. Three other soluble metallo exopeptidase enzymes can be detected in the vacuole, namely aminopeptidase I, aminopeptidase Co and dipeptidylaminopeptidase V. The molecular weight for aminopeptidase I is 640kD (Frey and Rohm, 1978, 1979), for aminopeptidase Co 100kD (Achstetter *et al.*, 1981; Emter and Wolf, 1984) and for dipeptidylaminopeptidase V 40kD (Achstetter and Wolf, 1985). Aminopeptidase I is active at a slightly basic pH (Frey and Rohm, 1978) as is aminopeptidase Co but only in the presence of Co^{2+} ions (Achstetter, *et al.*, 1981). Dipeptidylaminopeptidase V is active at neutral pH (Achstetter and Wolf, 1985).

1.2 Protein Processing and Transport in Eukaryotes

A property of all eukaryotic cells is their ability to direct the efficient localization of defined subsets of proteins from their cytoplasmic site of synthesis to their various

non-cytoplasmic destinations. The localization of newly synthesized polypeptides into their respective sub-cellular compartments is thought to be mediated by signals within proteins which are recognized by specific receptors contained within the organelle membranes (Horwich, *et al.,* 1985).

The majority of mitochondria and chloroplast proteins are encoded by nuclear genes and are synthesized on free cytoplasmic ribosomes as high molecular weight precursors bearing a charged, hydrophilic, N-terminal transit peptide (Coleman and Robinson, 1986; Rothman and Kornfeld, 1986). The precursors move to the relevant organelles and seem to unfold (Eilers and Schatz, 1986) before being translocated into their specific organelles. In the case of the chloroplast this was shown to require energy from ATP (Grossman, *et al.,* 1980; Schatz and Butow, 1983), whereas, mitochondria require both ATP (Pfanner and Neupert, 1986; Eilers, *et al.,* 1987) and the transmembrane potential of the inner mitochondrial membrane (Grossman, *et al.,* 1980; Schatz and Butow, 1983). Proteolytic cleavage of the transit peptide occurs during or shortly after import, by the action of a specific peptidase (Maccecchini, *et al.,* 1979).

In contrast, the precursors of secretory proteins (Blobel and Dobberstein, 1975), proteins of intracellular organelles such as the lysosome (Erickson, *et al.,* 1981) and proteins of the plasma membrane (Anderson, *et al.,* 1983), are synthesized by membrane bound ribosomes on the rough endoplasmic reticulum (RER). They are co-translationally translocated across the ER membrane under the control of a generally hydrophobic signal peptide (Blobel and Dobberstein, 1975). This is aided by two protein complexes, the signal recognition particle (SRP) (Walter and Blobel, 1981) and the SRP-receptor or docking protein (Meyer, *et al.,* 1982). The process is energy dependent in the form of ATP. During translocation most signal peptides are removed

by signal peptidase (Blobel and Dobberstein, 1975) and the core asparagine-linked carbohydrate units are added (Kiely, *et al.,* 1976; Rothman and Lodish, 1977)

The newly synthesized membrane and secretory proteins are transported through the ER, where they fold into their correct 3-dimensional conformation. They are then transported to the Golgi stack at different distinct rates (Fitting and Kabat, 1982; Lodish, *et al.,* 1983), where they may be further glycosylated, before delivery to the plasma membrane, secretory vesicles, or lysosome.

Immunoelectron microscopy experiments of viral glycoproteins have shown that they enter the Golgi face adjacent to the ER (*cis* side) and exit at the opposite *trans* face (Bergmann and Singer, 1983; Saraste and Hedman, 1983). Elucidation of the biochemical steps involved in the processing of the asparagine-linked oligosaccharides and immunocytochemical and biochemical localization of the enzymes involved in these reactions, have shown that the Golgi stack is composed of at least three functionally distinct compartments (Dunphy and Rothman, 1985; Farquhar, 1985).

The *cis* Golgi is the site where lysosomal hydrolases are recognised and modified. This compartment is the location of N-acetylglucosaminylphosphotransferase (GlcNAc-p) (Goldberg and Kornfeld, 1983; Pohlmann, *et al.,* 1982). The lysosomal hydrolases are selectively recognized by GlcNAc-p and modified at α-1,2-linked mannose residues present in N-linked oligosaccharides by the addition of GlcNAc phosphate to the six carbon atom of the mannose residues (Reitman and Kornfeld, 1981; Lang, *et al.,* 1984). After modification, 1-phosphodiester N-acetylglucosaminidase releases GlcNAc leaving mannose-6-phosphate (man-6-p) residues which act as the molecular signal for the sorting of lysosomal hydrolases. In the *trans* Golgi the man-6-p modified lysosomal hydrolases bind to one of the two man-6-p receptors in the

membrane before lysosomal delivery (Geuze, *et al.*, 1984; von Figura and Hasilik, 1986). Mutant cells that fail to phosphorylate mannose aberrantly secrete lysosomal proteins.

In the *medial* Golgi N-linked oligosaccharides are modified by the action of two mannosidases which trim off five mannose residues, and two GlcNAc transferases which add GlcNAc (Dunphy, *et al.*, 1985). In the *trans* Golgi addition of galactose and sialic acid residues occurs (Roth and Berger, 1982; Roth, *et al.*, 1985). Therefore, the newly synthesized proteins are transported from the ER to the *cis*, then *medial* and then the *trans* Golgi, before being distributed from the *trans* Golgi en route to their correct destination. Protein transfer from one Golgi compartment to another is in a unindirectional fashion (Rothman, *et al.*, 1984a,b) via vesicles which are the agents of protein transfer (Pfeffer and Rothman, 1987).

The formation of secretory storage vesicles has been found to occur at the Golgi *trans* face (Palade, 1975). The components are transported from the *trans* Golgi and then self-aggregate in a manner that seems to be a pH dependent process (Pfeffer and Rothman, 1987). Most secretory storage vesicles have an acidic environment (Mellman, *et al.*, 1986) and it has been shown that the *trans* Golgi is also a slightly acidic compartment (Anderson and Pathak, 1985), reflecting the first appearance of condensed secretory products.

1.3 Protein Processing and Transport in Yeast

Yeast is a model eukaryotic organism and has all of the organelles associated with a typical eukaryotic cell. Yeast is currectly used to investigate the secretory pathway, targeting to mitochondria, the nucleus and peroxisomes.

1.3.1 Transport into the Endoplasmic Reticulum

Proteins that are transported to the vacuole, the plasma membrane, that are secreted into the medium and the periplasmic space and those that make up the Golgi apparatus, all originate in the ER. Two methods have been used to demonstrate initial accumulation of proteins in the ER. First, translation of mRNA in the presence of dog pancreas microsomal vesicles and second, the influence of pleiotropic secretion mutants that block translocation into or sorting from the ER.

The signal peptide (Blobel and Dobberstein, 1975) provides a satisfactory explanation of the targeting and translocation of protein molecules across the membrane of the ER in eukaryotic cells. It is usually a 20-24 amino acid sequence of the N-terminal end of the protein. The nascent signal peptide binds to the signal recognition particle leading to the formation of a complex that interacts with an integral membrane protein of the ER known as the docking protein or signal recognition particle receptor (Gilmore, *et al.*, 1982; Meyer, *et al.*, 1982). Cotranslational translocation of the preprotein into the ER lumen then occurs (Walter and Blobel, 1981; Walter, *et al.*, 1984; Meyer, 1985). However, Ainger and Meyer, (1986), Perara, *et al.*, (1986) and Mueckler and Lodish, (1986b) report that translocation may occur posttranslationally, but Waters and Blobel, (1986) state that this is only true for the α-mating-factor preprotein.

Translocation of proteins does not usually occur in the absence of a signal peptide (Gething and Sambrook, 1982; Carlson and Botstein, 1982; Chao, *et al.*, 1987). There are exceptions to this, for example CPY is successfully translocated in the absence of a signal peptide but at a much slower rate (Blachly-Dyson and Stevens, 1987).

Signal peptides from both prokaryotic and eukaryotic secretory proteins have the same general organization (von Heijne, 1981; Pearlman and Halvorson, 1983). A typical signal sequence appears to consist of three regions, a positively charged amino terminal region, a central hydrophobic region and a polar carboxy terminal region, all without acidic residues (von Heijne, 1985).

Signal peptides from one organism can function in another because of their structural similarities. The bacterial β-lactamase signal sequence is functional both *in vivo* and *in vitro* in vertebrate systems (Muller, *et al.*, 1982; Wiedmann, *et al.*, 1984). That of rat preproinsulin functions in bacteria (Talmadge, *et al.*, 1980), the signal peptide of both human interferon (Hitzeman, *et al.*, 1983) and influenza virus haemagglutinin (Jabbar, *et al.*, 1985) function in yeast.

Translocation *in vitro* into microsomal vesicles can be detected by cleavage of the signal peptide, protein glycosylation and/or protection from proteinase digestion. These methods have been developed for investigating translocation of several proteins such as invertase (Perlman and Halvorson, 1981), CPY (Muller and Muller, 1981; Blachly-Dyson and Stevens, 1987), α-factor precursor (Julius *et al.*, 1984a) and killer toxin precursor (Bostian, *et al.*, 1983).

During translocation or immediately upon completion, two events may occur. Firstly, the signal peptide is removed in the lumen of the ER by signal peptidase. Secondly, the protein may be glycosylated.

In yeast two form of glycosylation occur within the ER. One form of glycosylation takes place by the formation of O-linked saccharides. The structure of O-linked saccharides, the pathway of their synthesis and cellular localization in yeast differ from that found in mammalian cells.

In yeast synthesis of O-linked saccharides is achieved by the involvement of the donor dolichol monophosphate mannose (Dol-p-man) representing the first mannosyl residue that is attached to the protein (Babscziniski and Tanner, 1973; Sharma, *et al.*, 1974; Parodi, 1979). The attachment takes place on a serine or threonine residue and stimulated by a proline residue on the N-terminal side (Lehle and Bause, 1984) in the ER (Haselbeck and Tanner, 1983). The extension of the O-linked mannose residue to di or oligosaccharides most likely takes place in the Golgi apparatus (Haselbeck and Tanner, 1983), since the *sec18* mutation that blocks protein transport between the ER and Golgi (Novick, *et al.*, 1980) was shown to accumulate protein containing only one O-linked mannose per mannosylation side. In mammalian cells, Dol-p-man is not involved (Babscziniski, 1980; Beyer, *et al.*, 1981) and O-linked glycosylation takes place in Golgi cisternae (Niemann, *et al.*, 1982; Roth, 1984).

The majority of glycosylation that occurs in the ER is N-linked glycosylation. This is achieved in two stages, firstly core glycosylation in the ER and then outer-chain modification in the Golgi (Kukuruzinska, *et al.*, 1988). Core glycosylation of proteins may occur cotranslationally (Larriba, *et al.*, 1976). The path of core glycosylation takes place by transfer of GlcNAc from UDP-GlcNAc to Dol-p to form Dol-p-p-GlcNAc on the cytoplasmic surface of the ER. The further addition of a GlcNAc residue then the sequential addition of five mannose residues, derived from GDP-mannose, then occurs. The $Man_5GlcNAc_2$ unit is then translocated to the ER lumen, followed by the addition of extra mannose units donated from Dol-p-man and glucose units from Dol-p-glu (Lehle, 1980; Trimble, *et al.*, 1980). When the synthesis of the $Glu_3Man_9GlcNAc_2$ core oligosaccharide is completed it is transferred from the dolichol-p to an asparaginyl residue. This residue is part of a tripeptide acceptor sequence of Asn-X-Ser/Thr that is necessary and sufficient for glycosylation

of proteins (Struck and Lennarz, 1980). The efficiency of glycosylation improves with increasing peptide chain length (Lehle and Bause, 1984). It has also been found that the rate of the glycosylation reaction is stimulated by about 20 fold by the presence of the glucose residues on the $Man_9GlcNAc_2$ core (Lehle, 1980). Protein glycosylation is then terminated by the removal of three glucose molecules and one mannose unit (Byrd, *et al.,* 1982; Esmon, *et al.,* 1984) prior to transport from the ER to the Golgi apparatus.

Proteins that are destined to leave the ER must first be sorted from those that remain in the ER. This is likely to involve a certain signal or structure. Those proteins that remain in the lumen of the ER of mammalian cells have KDEL as a C-terminal end; in yeast it is HDEL (Munro and Pelham, 1987; Pelham, *et al.,* 1988).

1.3.2 Transport Through the Golgi Body

Convincing profiles of Golgi cisternae in wild type *Saccharomyces cerevisiae* are not common (Schekman and Novick, 1982), however, some have been puplished (Matile *et al.,* 1969). Evidence of the existence of this organelle has been shown from the investigation of *sec7* mutant strains (Schekman, 1982; Schekman and Novick, 1982). In *sec7* mutant cells large stacks of Golgi-like cisternae are observed when incubated at 37^{o}C in medium containing 0.2% glucose (Novick, *et al.,* 1981; Schekman, 1982).

As in all organisms further glycosylation of proteins occurs in the Golgi aparatus (Esmon, *et al.,* 1981) leading to the elongation of N-linked oligosaccharides to produce a structure referred to as the outer chain (Ballou, 1982). However, outer chain modification in yeast is not typical of that found in the other organisms. The vacuolar

hydrolases received only 3-7 mannosyl units per carbohydrate chain (Jones, 1984), whilst, more than 100 mannosyl units are added to some of the chains of secreted glycoproteins such as invertase. (Ballou, 1982; Tanner and Lehle, 1987).

1.3.3 Transport to the Vacuole

The yeast vacuole is different from a mammalian lysosome in containing hydrolytic glycoproteins (Wiemken, *et al.,* 1979), many of which are derived from proenzyme forms (Jones, *et al.,* 1981; Jones, 1984). Transport of several vacuolar proteins depends upon early stages of the secretory pathway (Novick, *et al.,* 1981). Carboxypeptidase Y (CPY) is one whose biosynthesis and transport through the secretory pathway has been well characterized (Hasilik, *et al.,* 1974; Hasilik and Tanner, 1978a; Stevens, *et al.,* 1982).

The proenzyme is synthesized as a larger, inactive precursor (Hasilik and Tanner, 1978a), which is directed into the ER lumen, where signal peptide cleavage occurs (Blachly-Dyson and Stevens, 1987; Johnson, *et al.,* 1987). Deletion of the signal peptide fails to fully inhibit the translocation of the CPY precursor (Blachly-Dyson and Stevens, 1987). It may be the signal sequence is redundant because of another signal located internally in the precursor. In the ER, proCPY receives four asparagine-linked oligosaccharide chains leading to the formation of a precursor form of molecular weight equivalent to 67kD and termed the P1 form (Trimble and Maley, 1977; Hasilik and Tanner, 1978b). From the ER, proCPY is transported to the Golgi apparatus, where a further 2kD of carbohydrate is added to yield the 69kD P2 form of proCPY for delivery to the vacuole (Stevens, *et al.,* 1982). Maturation of active CPY involves removal of 8kD of N-terminal propeptide (Hemmings, *et al.,* 1981) by both

proteinase A and proteinase B (Mechler, *et al.*, 1987). *pep4* mutant cells accumulate enzymatically inactive proCPY in the vacuole, which suggests the maturation occurs immediately after the delivery of the CPY precursor to the vacuole (Stevens, *et al.*, 1982; Distel, *et al.*, 1983; Zubenko, *et al.*, 1983). Processing and delivery of CPY to the vacuole occurs with a half-time of about six mins (Hasilik and Tanner, 1978b; Stevens, *et al.*, 1982) Maturation of and transport of CPY normally occurs in the absence of glycosylation (Hasilik and Tanner, 1978a; Onishi, *et al.*, 1979; Schwaiger, *et al.*, 1982).

Sec mutants that block the transport of secretory vesicles have no effect on CPY localization, suggesting that vacuolar and secretory proteins travel together through the ER to the Golgi apparatus, where sorting occurs.

To identify the signal that targets CPY to the vacuole, amino-terminal segments of CPY were fused to the secretory enzyme invertase (Johnson, *et al.*, 1987). They have shown that the N-terminal 50 amino acids of CPY are quite sufficient to direct delivery of a CPY-invertase hybrid protein to the vacuole. This observation suggests that 20 out of the 50 amino acids constitute the N-terminal signal peptide that directs translocation of CPY to the ER, and the remaining 30 amino acids contain the vacuolar targeting signal. Deletion of this vacuolar sorting signal from wild type CPY leads to missorting and eventual secretion of CPY (Johnson, *et al.*, 1987).

Valls, *et al.*, (1987) have observed that the N-terminal region of the CPY propeptide near amino acid 24 is required for targeting of CPY to the vacuole. Moreover, a deletion of *prc1* corresponding to amino acids 29-30 of the propeptide resulted in secretion of 40-90% of CPY. A deletion in *prc1* around amino acids 62-63 has no such effect.

The presence of the CPY structural gene (*PRC1*) on a multicopy plasmid causes yeast to overproduce CPY by 3 to 4 fold, resulting in the secretion of about 10% of the CPY to the cell surface (Stevens, *et al.*, 1986a,b). More than 50% of the total CPY was secreted when the acid phosphatase promoter was substituted for the CPY promoter, strongly suggesting that the sorting of CPY is saturable (Stevens, *et al.*, 1986a,b). Rothman, *et al.*, (1986) have found that proteinase A is also secreted to the cell surface when *PRA1* is present on a multicopy plasmid.

The secreted form of CPY which appears in the periplasm due to overexpression of *PRC1* is the 69kD precursor species suggesting that the same carbohydrate modification occurs to the vacuolar and secreted forms of CPY (Stevens, *et al.*, 1986b). This observation suggests that vacuolar proteins are not recognised or are blocked in formation of high mannose type glycoproteins. It is also confirms that the site of maturation of proCPY is the vacuole.

1.3.4 Transport to the Cell Surface

The intracellular transport and processing of two other glycoproteins, invertase and α-factor pheromone, have also been studied with a view to using their signal peptides to direct the processing and transport of heterologous proteins expressed in yeast (Emr, *et al.*, 1983; Brake, *et al.*, 1984). These proteins, like CPY, are synthesized as large precursor molecules on the RER, which are subsequently proteolytically processed to yield the mature species. Both are secreted into the periplasmic space, where invertase remains, whilst the α-factor diffuses into the medium.

The α-factor is a peptide pheromone produced by haploid cells of mating type α (Emr, *et al.*, 1983). The precursor molecule, prepro-α-factor, consists of 80 amino

acids. The 'prepro' region contains three N-linked glycosylation sites (Kurajan and Herskowitz, 1982; Julius, *et al.,* 1983). The precursor enters the secretory pathway and the signal peptide is removed during the final processing stage in α-factor maturation (Emr, *et al.,* 1983; Julius, *et al.,* 1984a). The cotranslational translocation of this polypeptide has been shown not to be obligatory, since the precursor can be *in vitro* posttranslationally translocated in yeast microsomes (Rothblat and Meyer, 1986b; Hansen, *et al.,* 1986; Waters and Blobel, 1986; Baker, *et al.,* 1988). No further glycosylation occurs to the prepro-fragment after being core glycosylated (Emr, *et al.,* 1983; Julius, *et al.,* 1984a). Inhibition of glycosylation does not alter the rate of α-factor secretion, proteolytic cleavage or transport (Julius, *et al.,* 1984a).

Maturation of the pheromone involves proteolytic cleavage by the *KEX2* gene product to yield α-factor with the spacer peptides still attached at the N-termal end (Julius, *et al.,* 1984b), whilst subsequent proteolysis to liberate the mature pheromone is mediated by membrane-bound dipeptidylaminopeptidase A (Julius, *et al.,* 1983). Maturation occurs in the *trans* Golgi or in secretory vesicles (Julius, *et al.,* 1984a; Fuller, *et al.,* 1988).

Two forms of invertase are synthesized in *Saccharomyces cerevisiae*, a glycosylated, secreted form and a non-glycosylated, cytoplasmic form which is produced constitutively (Pearlman and Halvorson, 1981; Carlson and Botstein, 1982). They are encoded by separate mRNA species which differ in size and in the nature of $5'$ terminal sequences, but are transcribed from a common gene (*SUC2*) (Pearlman and Halvorson, 1981). mRNA's encoding the precursors of secreted invertase specify a 19 amino acid signal peptide, involved in the cotranslational translocation of preinvertase into the ER, whereas those encoding cytoplasmic invertase have no signal peptide coding sequences.

1.3.5 Transport to Mitochondria

Assembly of the mitochondrion appears to be independent of the secretory process. The import of proteins into mitochondria in some cases appears to be similar to assembly of certain membrane proteins in *E. coli* (Neupert and Schatz, 1981). Nuclear encoded mitochondrial precursors are made on cytoplasmic ribosomes, and the completed protein then inserted at the surface of the outer membrane (Neupert and Schatz, 1981; Schatz and Butow, 1983).

Many different nuclear-encoded mitochondrial proteins have been synthesised *in vitro* in reticulocyte lysates, and the precursors are taken up by the mitochondria after polypeptide synthesis is completed. The results show that translation and import occur independently (Schekman, 1985). Butow, *et al.,* (1975) found that a thin section of yeast cells shows some enrichment of ribosomes at the mitochondrial surface. Furthermore, cytoplasmic ribosomes found in a purified mitochondrial fraction appear to be involved in the systhesis of certain proteins that are different from those made by soluble ribosomes (Ades and Butow, 1980).

As in the case for secretory proteins, it has been found that a mitochondrial specific transit peptide consisting of 16-25 amino acids is sufficient for transport and localization of mitochodrial proteins (Geller, *et al.,* 1983; Douglas, *et al.,* 1984; Hurt, *et al.,* 1984).

1.4 Sec Genes

The process of intracellular protein transport and secretion requires large num-

bers of gene products. In *Saccharomyces cerevisiae* a series of temperature-sensitive secretory mutants have been isolated and complementation analysis shows that there are more than 27 genes (*SEC*) involved in the yeast secretory pathway (Novick and Schekman, 1979; Novick, *et al.*, 1980; Ferro-Novick, *et al.*, 1984a; Deshaies and Schekman, 1987). At the restrictive temperature, these mutants accumulate organelles and protein precursors, which are usually secreted again upon shifting back to the permissive temperature (Novick and Schekman, 1979; Novick, *et al.*, 1980).

The *sec* mutants are of two types. Class A *sec* mutants are temperature sensitive for growth, secretion and plasma membrane assembly (Novick and Schekman, 1979; Novick, *et al.*, 1980). Biochemical and morphological analysis of class A *sec* mutants, has revealed that protein processing and transport through the secretory pathway can be divided into three stages (Novick, *et al.*, 1980; Ferro-Novick, *et al.*, 1984a; Schekman, 1985). Transport from the ER (*sec12, sec13, sec16, sec17, sec18, sec19, sec20, sec21, sec23*), accumulation at the Golgi vesicles (*sec7, sec14*) and accumulation of secretory vesicles (*sec 1, sec2, sec3, sec4, sec5, sec6, sec8, sec9, sec10, sec15*).

Class B *sec* mutants include *sec53* and *sec59* (Ferro-Novick, *et al.*, 1984a). This class is unlike the class A mutants in that both *sec53* and *sec59* cause plasma membrane, vacuolar and secretory proteins to remain associated with the ER membrane but not inserted into the lumen of the ER (Ferro-Novick, *et al.*, 1984a, 1984b). The *sec53* block is thermoreversible, returning of the blocked cells to the permisive temperature in the presence of cycloheximide, results in glycosylation and secretion of accumulated secretory precursors (Julius, *et al.*, 1984a; Feldman, *et al.*, 1987).

Yeast vacuolar protein targeting (*vpt*) mutant exhibits defects in the delivery of some vacuolar hydrolases, including CPY (Bankaitis, *et al.*, 1986; Rothman and

Stevens, 1986; Banta, *et al.*, 1988). This observation led to the identification of eight vacuolar protein localization (*vpl*) complementation groups (Rothman and Stevens, 1986) that cause aberrant secretion of up to 90% of CPY and about 60% of proteinase A. These mutants, which also have a mutation in the *pep4* locus and are leucine auxotrophs, are able to grow on Cbz-Phe-Leu as a source of leucine (Rothman and Stevens, 1986) due to *PEP4* independent periplasmic activation of proCPY (Stevens *et al.*, 1986b).

Electron microscopy revealed the presence of three classes of *vpt* mutants with respect to vacuolar morphology (Banta, *et al.*, 1988). Class A *vpt* mutants, consisting of 26 complementation groups, contain 1-3 large vacuoles similar to wild type, but some of them are sensitive to low pH that leads to missorting and secretion of CPY and proteinase A. Class B *vpt* mutants, three complementation groups, posess a different vacuole morphology. They contain about 35 very small vacuoles, but have the same vacuolar acidity as wild type strains. Class C *vpt* mutants, four complementation groups, exhibit extreme deficiencies in vacuolar biogenesis. This class of mutant lacks functional vacuoles, but accumulate other membrane-bound organelles including vesicles of 80nm in diameter that have a very different appearance from the vesicles of about 100nm in diameter that accumulate in the secretory mutant *sec1*. Class C mutants also accumulate large multi-lamellar membrane-bound structures which are electron transparent, suggesting that they do not contain large amounts of glycoprotein or sugar. Finally, in summary, there are at least 50 complementation groups, representing *VPT, VPL, SEC and PEP* genes involved in vacuolar biogenesis (Robinson, *et al.*, 1988).

1.5 In Vitro Protein Translocation and Transport

Different parts of the secretory pathway have been reconstituted *in vitro*. Cell-free reconstitution of protein translocation across the ER membrane has been developed in *Saccharomyces cerevisiae* (Waters and Blobel, 1986; Rothblatt and Meyer, 1986a) and *Neurospora crassa* (Addison, 1987). *In vitro* translocation of the yeast secretory protein prepro-α-factor into the lumen of the ER can occur posttranslationally (Hansen, *et al.*, 1986; Waters and Blobel, 1986; Rothblatt and Meyer, 1986b; Rothblatt, *et al.*, 1987). Translocation was shown to require membrane proteins of the microsomal vesicles (Hansen, *et al.*, 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986) and to be stimulated by soluble factors (Waters, *et al.*, 1986; Baker, *et al.*, 1988; Ruohola, *et al.*, 1988).

The requirements of prepro-α-factor translocation are similar to those found for bacterial secretory proteins, which can be translocated posttranslationally (Date and Wickner, 1981; Koshland and Botstein, 1982; Chen and Tai, 1985), as well as those described for posttranslational transport of mitochondrial protein precursors (Gasser, *et al.*, 1982; Pfanner and Neupert, 1985). Moreover, Fecycz and Blobel (1987) have demonstrated that the postribosomal supernatants isolated from bacteria and yeast can substitute for each other in promoting the posttranslational translocation of prepro-α-factor and invertase from the plasma membrane of *E. coli* or across the microsomal membrane of *Saccharomyces cerevisiae*.

In addition to prepro-α-factor, preinvertase and prepro-CPY synthesized in a yeast lysate are also successfully translocated and glycosylated by yeast microsomes (Rothblatt and Meyer, 1986a,b; Rothblatt, *et al.*, 1987).

Until recently, translocation across the ER was thought to be only cotranslational (Blobel and Dobberstein, 1975; Schmidt, *et al.*, 1981; Walter, *et al.*, 1984). However it has been reported that translocation into isolated canine pancreatic microsomes can also occur posttranslationally for a truncated form of the human glucose transporter (Mueckler and Lodish, 1986a,b) and for a fusion protein of lactamase and β-globin (Perara, *et al.*, 1986). Recently, Hansen and Walter, (1988) reported that prepro-CPY and a truncated form of preinvertase but not full length preinvertase, can also be translocated posttranslationally into ER vesicles *in vitro*.

In vitro reconstitution of intercompartmental transport between the cisternae of the Golgi stack has also been demonstrated (Pfeffer and Rothman, 1987). Since the oligosaccharide chains of glycoproteins undergo well characterized modifications in the successive compartments of the Golgi complex (Dunphy and Rothman, 1985), this has been used as a means of assaying transport.

The transport of vesicular stomatitis virus (VSV) encoded G protein from the *cis* compartment of a mutant Chinese Hamster ovary (CHO) cell line that is unable to incorporate GlcNAc into glycoprotein oligosaccharides, due to a deficiency in GlcNAc transferase I, to the *medial* compartment of a wild type CHO Golgi stack recipient was measured (Fries and Rothman, 1980; Balch, *et al.*, 1984; Dunphy, *et al.*, 1985). The movement was detected biochemically by the incorporation of ^{3}H-GlcNAc in a complementation assay when G protein is transported from the *cis* donor compartment to the *medial* recipient. Transport was absolutely dependent upon the presence of a crude cytosolic fraction, ATP and proteins from the surface of the Golgi membranes (Fries and Rothman, 1980; Balch, *et al.*, 1984; Balch and Rothman, 1985). *In vitro* intercompartmental transport of G protein occurs via a series of steps that reflect the stages in budding and fusion of the transport vesicles (Balch, *et al.*, 1984;

Wattenberg, *et al.*, 1986), which require both energy from ATP and cytosolic proteins. Moreover, studies of the coated vesicles containing G protein (Balch, *et al.*, 1984; Orci, *et al.*, 1986) which are thought to be responsible for transport between the cisternae of the Golgi stack, show that they are coated with a protein other than clathrin.

It has been reported that a cytosolic fraction isolated from yeast (Dunphy, *et al.*, 1986) and from plants (Paquet, *et al.*, 1986) can substitute for the animal cell cytosol in promoting *in vitro* transport between the cisternae of the Golgi stack. This shows that the mechanism of transport is likely to be similar in all eukaryotes.

Recently, Balch, *et al.*, (1987) have introduced a cell free system that reconstituted the transport of the VSV G protein from the ER to the Golgi of CHO cells. The digestion of high mannose oligosaccharides present on ER-derived G protein by the Golgi enzyme Mannosidase I was used to monitor this event. The transport was shown to require ATP and a cytosolic fraction.

Haselbeck and Schekman, (1986) described *in vitro* transport of core glycosylated invertase from the ER to Golgi vesicles using temperature sensitive strains that accumulate core-glycosylated invertase in the ER at the restrictive temperature. A double mutant strain was constructed also bearing a mutation in the Golgi α-1,3-mannosyltransferase. The transport of invertase from the donor membrane to the recipient membrane containing α-1,3-mannosyltransferase activity but lacking invertase was detected by monitoring the acquisition of outer chain α-1,3-linked mannose residues on invertase. The reaction was found to require ATP, GDP mannose and either Mg^{2+} or Mn^{2+} as a divalent cation. However, for intercompartmental transport Baker, *et al.*, (1988) have developed a promising powerful method for preparing yeast

ghosts. They used freeze-thaw lysis of sphaeroplasts that cause gentle breakage of the plasma membrane. This releases cytosolic proteins but leaves the organelles in an intact form. Utilizing such ghosts, they have shown the transport of prepro-α-factor from the cytosol through the ER to Golgi vesicles. The reaction was temperature dependent, required ATP and stimulated 6 fold by the addition of a cytosolic fraction.

Very recently, Ruohola, *et al.*, (1988), have developed another system that involves the *in vitro* reconstitution of translocation of prepro-α-factor from the ER to Golgi vesicles. This translocation system is based on the preparation of permeabilized cells. It uses the translocation of newly translated prepro-α-factor into the ER in the presence of an S3 fraction and energy. Translocation of this protein from the ER to Golgi complex occurs by the addition of the recipient compartments (Golgi vesicles) plus an S3 fraction and ATP. They also found that ER to Golgi transport is inhibited in the presence of GTPγS as previously described by Melancon, *et al.*, (1987). This implicates the involvement of a GTP-binding protein in inter-Golgi transport. It has recently been reported that mutations in the GTP-binding protein *YPT1* can affect ER to Golgi transport in yeast (Segev, *et al.*, 1988; Schmitt, *et al.*, 1988).

1.6 β-Glucuronidase and Gus Fusions

E. coli β-glucuronidase (Gus) has a molecular weight of about 68.2kD (Jefferson, *et al.*, 1986). The behaviour of the native enzyme on gel filtration columns indicates that it is probably a tetramer. The enzyme is most active in the presence of thiol-reducing agents such as β-mercaptoethanol or DTT. Gus has no co-factor nor any ionic requirement and it is inhibited in the presence of some divalent metal ions such as Zn^{2+} and Cu^{2+} (Stoeber, 1961). β-glucuronidase can be assayed at different

physiological pH, exhibiting an optimum pH between 5.0-8.0. It is still 50% active at pH4.3 (Jefferson, 1985; Jefferson, *et al.*, 1986). The enzyme is also resistant to high temperature and only loses half of its activity at 55°C (Jefferson, *et al.*, 1986). Gus is encoded by the *uidA* locus (Novel and Novel, 1973). It is an acid hydrolase that catalyzes the cleavage of a wide variety of β-glucuronides (Stoeber, 1961). Substrates of β-glucuronidase are generally water soluble (Paigen, 1979) and many are available, including those for spectrophotometric (p-nitrophenyl glucuronidase), for fluorometric (4-methyl umbelliferone and 4-methyl umbelliferyl glucuronide (MUG)) and for histochemical (5-bromo-4-chloro-3-indolyl-glucuronide) analysis.

The β-glucuronidase gene has been cloned and sequenced (Jefferson, *et al.*, 1986). The coding region of the gene is 1809bp long.

Gus was used initially as a gene fusion marker in *E. coli* and in *Caenorhabditis* (Jefferson, *et al.*, 1987a). There is no detectable β-glucuronidase activity in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, some strains of *Caenorhabditis elegans*, *Dictyostelium discoidum* and most higher plants (Jefferson, *et al.*, 1987a,b; Sebastiano, *et al.*, 1986). The enzyme can tolerate a large amino-terminal addition without loss of activity (Jefferson, *et al.*, 1987b; Jefferson, 1987).

1.7 Aim of work

The major aims of this work were to develop *in vitro* and *in vivo* systems for the study of protein transport from the Golgi apparatus to the vacuole in the yeast *Saccharomyces cerevisiae*. The transport of the enzyme carboxypeptidase Y was studied in detail.

To accomplish these aims necessitated the production of an anti-CPY antibody using CPY purified form dried Baker's yeast as part of this work.

The *in vitro* system required the construction of specific genetically marked yeast strains carrying both *sec* and *pep4-3* mutations. The *in vitro* transport system followed the transport of CPY using enzyme assays and western blots. The effects of energy and cytoplasmic proteins on transport were investigated.

As a compliment to these *in vitro* studies, an *in vivo* system was also developed. This uses the bacterial enzyme Gus as a reporter gene for the targeting of CPY to the vacuole. Current systems for the study of vacuolar targeting in yeast use invertase as a reporter gene. The use of Gus potentially has significant advantages over invertase because of its ease of assay and the range of synthetic chromogenic substrates available.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 MATERIALS

All chemicals and biological reagents were from the Sigma Chemical Company Ltd, Poole, Dorset, unless otherwise stated.

Restriction endonucleases, T4 DNA ligase, X-gal, Agarose, LMP Agarose were from Bethesda Research Labs (U.K.) Ltd, Cambridge, U.K.; Boeringer Corporation (London) Ltd, Lewes, East Sussex; or New England Biolabs, CP Labs, Bishops Stortford, Herts, U.K.

Klenow DNA polymerase I was from Northumbria Biological Ltd, Cramlington, Northumberland, U.K.

Caesium chloride, Sodium chloride and Potassium chloride were from Koch-light Ltd, Haverhill, Suffolk, U.K.

Bactoagar, Bactopeptone and Yeast nitrogen base without amino acids were from Difco Labs, Detroit, Michigan, U.S.A.

Yeast extract was from Sterlin Ltd, Teddington, U.K.

DEAE-Sephadex A-50, Bio-Gel P-200, Ficoll-400 and Glusulase were from Pharmacia Fine Chemicals, Uppsala, Sweden.

DEAE-Cellulose, Glass beads and Dried yeast cells were from BDH Chemicals Ltd, Poole, Dorset, U.K.

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

Nitrocellulose filters were from Schleicher and Schuell, Postfach-4, D-3354, Dassel,

High vacuum grease was from Dow Corning S.A., Senefte, Belgium.

Polaroid 667 film was from Polaroid (U.K.) Ltd, St. Albans, Hertfordshire.

BBL trypticase peptone was from Becton Dickinson and Co, Cockeysville, M.D., U.S.A.

2.1.1 Yeast Strains

All strains used throughout this work are *Saccharomyces cerevisiae.*

MC16 α *leu2-3, his4-712, ade2-1, lys2-1.*

MD40-4C α *ura2, trp1, leu2-3, leu2-112, his3-11, his3-15.*

MT302-1C a *ade1, his3-11, arg5-6, leu2-12, his3-15.*

BYS (232-31-42) α *his7,lys2, leu2 prb1-1, prc1-1, prs1-3.*

BYS (232-31-4) a *ade, ura, leu2 prb1-1, prc1-1, prs1-3.*

BJ1075 α *trp1, prc1-229.*

YHH19 α *leu, his, ura3Δ5.*

HMSF1 a *sec1-1.*

SF294-2B a *sec7-1.*

HMSF169 a *sec4-3.*

HMSF176 a *sec18-1.*

HMSF331 a *sec53-1.*

2.1.2 Bacterial Strains

All bacterial strains used during the course of this work are *Escherichia coli*

JM83 *ara*, Δ(*lac-pro*A,B), *rps*L, [ϕ80*lacZ*ΔM15]

GM119 *dcm-6, dam-3, metB1, thi-1, lacY1, lacZ4, galK2, galT22, mtl-2, tonA2, tonA31, tsx-78,* λ, *supE44.*

JM101 (Δ *lac-pro*A,B), *sup*E, *thi*, /F', *tra*D36, *pro*AB *lac*I^q,ZΔM15.

2.1.3 Plasmids

pTSY3: (Stevens, *et al.,* 1986a), Intact PRC-1 gene encoding CPY, Amp^R.

YCp50: (Cathering, *et al.,* 1985), Yeast centromeric plasmid, Amp^R, Tet^R.

pEMBLYe31: (Baldari and Cesareni, 1985) Yeast shuttle vector, Amp^R.

pUC19: (Vieira and Messing, 1982), Amp^R.

2.1.4 Glassware and Other Equipment

All solutions, glassware, plasticware and other equipment used in DNA manipulation, growth and maintenance of yeast and bacterial cultures were autoclaved at 15p.s.i for 15mins or filter sterilized through a 0.25μm filter before use.

Glass beads were prepared by soaking in 4% nitric acid for 1hour, washing extensively with sterile distilled water and then dried.

2.2 Growth Media

YPD Medium, (Complete Medium)

10g yeast extract

20g bacto-peptone

20g glucose

(2% Bacto-agar)

per 1 litre distilled water.

YMM, (Yeast Minimal Medium)

6.7g yeast nitrogen base without amino acids

20g glucose

20mg amino acid as required

(1.5% Bacto-agar)

per 1 litre distilled water.

Amino acids were dissolved in distilled water at a concentration of 2mg/ml and autoclaved.

L-Broth

20g tryptone

5g yeast extract

5g sodium chloride

(1.5% Bacto-agar)

per 1 litre distilled water.

2YT-Broth

16g tryptone

10g yeast extract

5g sodium chloride

per 1 litre distilled water.

Antibiotics

Antibiotic	Stock Solution mg/ml	Final cconcentration μg/ml
Kanamycin (kan)	12.5	25
Chloramphenicol (Cm)	12.5	25
Ampicillin (Amp)	25	50
Tetracyclin (Tc)	12.5	10

2.3 Benedicts Reagent

Solution 1: 173g trisodium citrate. $2H_2O$; 100g Na_2CO_3 per 500ml distilled water.

solution 2: 17.3g $CuSO_4$ per 100ml distilled water.

Solution 1 was dissolved in distilled water with warming then solution 2 was added to solution 1 and the volume was completed to 1 litre.

2.4 METHODS

2.4.1 Growth Conditions

Yeast strains were grown in YPD as a complete medium or supplemented yeast minimal medium as a minimal medium. Growth took place at 30^{o}C unless otherwise stated. Liquid cultures were agitated on rotary shakers to ensure good aeration.

2.4.2 Preparation of Crude Extracts

Large Scale Preparation:- The method of Jones (1977), was used for large scale preparations of crude extracts with the exception that NaCl was added to the extraction buffer. Overnight 500ml cultures of yeast cells were harvested by centrifugation at 6000rpm for 5mins, washed once with distilled water and then resuspended in 2ml extraction buffer (0.2M Tris.HCl pH7.6, 0.1M NaCl) for each gram of cells. The cells were then broken by sonication using a Soniprep MSE Sonicator (6 x 30sec) with glass beads (equal volume of glass beads and cell suspension), continually cooling on ice. The suspension was then centrifuged for 15mins at 15000rpm and the supernatant was retained for further analysis.

Small Scale Preparation:- 10ml of cells were harvested by centrifugation in a benchtop centrifuge for 10mins at 4000rpm. The supernatant was carefully drained off and the pellet kept on ice before being resuspended in 200ml sample buffer, (see SDS-PAGE). The suspension was then transferred to an eppendorf tube, pelleted lightly and an equal volume of glass beads was added to the cell pellet. The cells were then vortexed for 5 x 5sec, cooling on ice between vortexing. This was followed by centrifugation in a MSE microfuge for 3mins at 12000rpm. The supernatant was retained and kept on ice before further analysis.

Fig 2.4.3: Standard curve of bovine serum albumin.

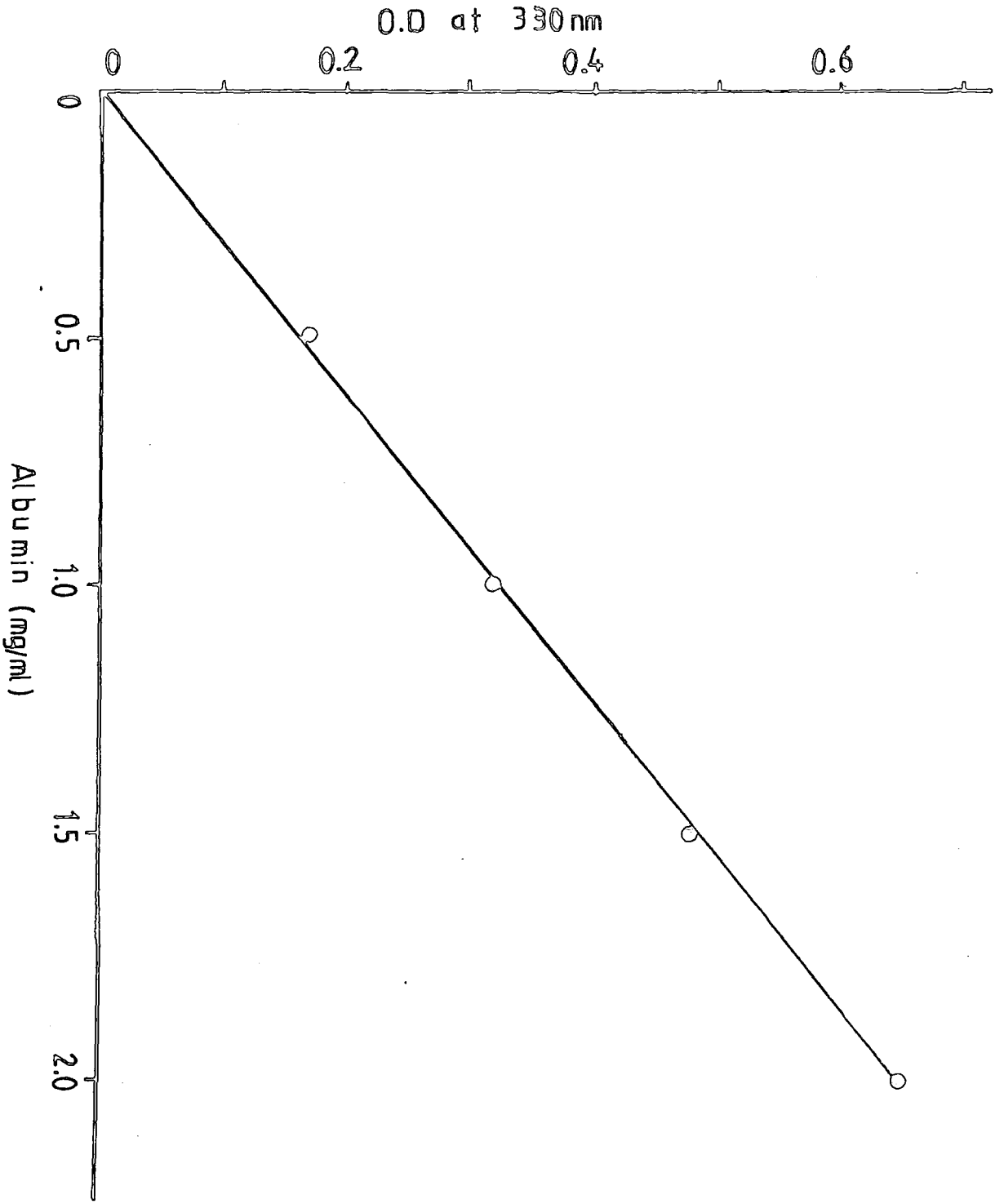
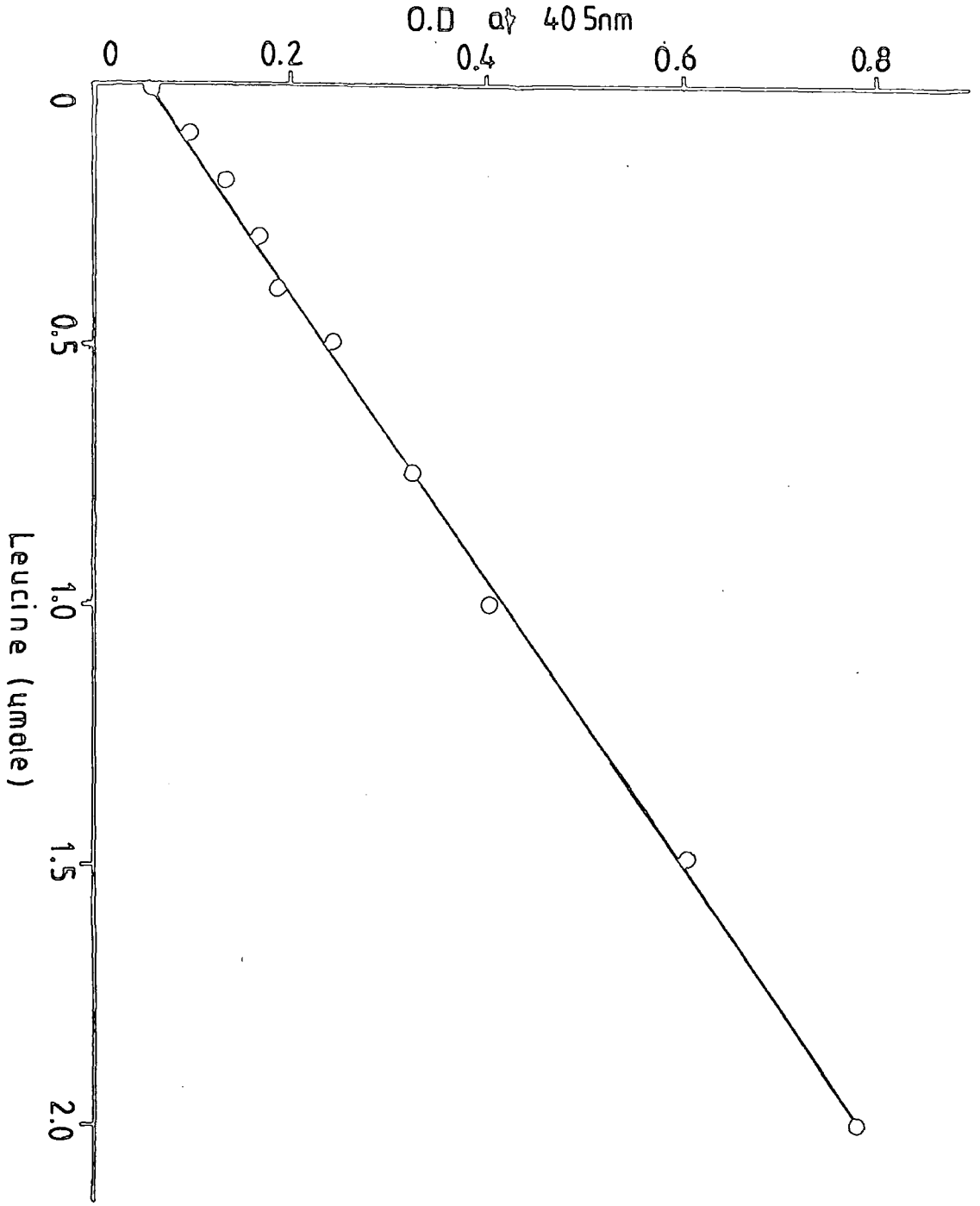

O.D at 330nm
0
0.2
0.4
0.6
0
0.5
1.0
1.5
2.0
Albumin (mg/ml)

Fig 2.4.4: Leucine standard curve.


O.D at 405nm
0
0.2
0.4
0.6
0.8
0
0.5
1.0
1.5
2.0
Leucine (umole)

2.4.3 Protein Standard Curve

A protein standard curve was made using the method of Legatt-Bailey, (1967). 0.2ml Benedict reagent (Section 2.3) and 3ml of 1M NaOH were added separately to 0.5, 1.0, 1.5, and 2.0mg/ml of bovine serum albumin. The mixture was then shaken immediately and left at room temperature for 15mins. The optical density of each sample was read at 330nm using a sample lacking protein as a blank (Fig 2.4.3)

2.4.4 Carboxypeptidase Y and S Determination

In order to study the total carboxypeptidase Y, (CPY) and carboxypeptidase S, (CPS) activities, the method as described by Wolf and Weiser (1977) was used. This assay is based on the liberation of leucine from the Cbz-Phe-Leu substrate. The free leucine is detected by a coupled assay of L-amino acid oxidase and horseradish peroxidase.

First a standard curve of leucine was constructed as follows: A 0.5ml test solution containing 0.25mg/ml L-amino acid oxidase, 0.4mg/ml horseradish peroxidase and 0.5mM $MnCl_2$ were mixed with 0.5ml of aliquots containing 0.01, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5, and 2.0μmole leucine respectively. (All solutions were in 0.2M potassium phosphate buffer, pH7.0). To this 0.05ml of o-dianisidine di-hydrochloride (2mg/ml) dissolved in sterile distilled water was added. Finally the assay mixture was completed to 1.1ml by adding sterile distilled water. After 90mins of incubation at 30^{o}C the change of absorbance at 405nm was determined using zero leucine as a blank (Fig 2.4.4)

Total CPY plus CPS activity in extracts was determined using 0.5ml of 10mM Cbz-Phe-Leu (20.5mg dissolved in 5mls of 0.2M potassium phosphate) in a coupled assay as detailed in the preparation of the leucine standard curve using 50μl of ex-

tracts. CPY specific activity was in nMole of liberated leucine/min/mg protein.

2.4.5 Determination of Carboxypeptidase Y in Colonies

The method described by Wolf and Fink, (1975) was used to detect the CPY activity in yeast colonies grown on solid medium. Yeast cells of wild type (MC16) and a mutant lacking CPY activity (MT302/1C) were grown on solid YPD medium for 24hrs using glass petridishes. The plates were placed in a fume-hood at room temperature and the surface of each was covered by 10ml of toluene. The toluene was evaporated completely after 2hrs. The cells were then stained with APNE-Agar solution (20mg N-acetyl-phenyl-β-naphthyl ester, 200mg fast garnet, 10ml DMF, 0.75% agar and 90ml sterile distilled water). 5ml of autoclaved APNE-Agar was added to each plate and left for 2hrs at 30°C. Colonies containing CPY activity showed a dark red color, whereas mutants lacking CPY activity showed only a slight reddish color during this incubation period.

2.4.6 Determination of Carboxypeptidase Y Activity with BTPNA

The described method of Aibara, *et al.*, (1971) was adapted to determine the anilidase activity of CPY. CPY was assayed with 300μM N-benzoyl-L-tyrosine *p*-nitroanilide (BTPNA) at pH7.0, in which 1ml of 100mM sodium phosphate buffer, pH7.0 was added to 0.2ml enzyme solution and the mixture was pre-incubated at 25°C for 5mins, then 0.2ml of 3mM BTPNA was added. After 1hr of incubation the reaction was terminated by the addition of 1ml of 1mM mercuric chloride. The color which developed from *p*-nitroaniline during the enzyme activity was then measured spectrophotometrically at 410nm.

2.4.7 Determination of Glucose-6-Phosphate Dehydrogenase Activity

The method of Kato, *et al.*, (1979) was employed for the determination of glucose-6-phosphate dehydrogenase activity. Enzyme activity was assayed kinetically at 340nm using a Pye Unicam SP8-150 UV/Visible Spectrophotometer equipped with a line-log converter and recorder. The assay mixture consisted of 3ml of 33mM glycyl-glycine buffer, pH8.0, 0.2mM $MgCl_2$ 4mM glucose-6-phosphate, 0.1mM NADP and a limiting amount of enzyme (Yeast crude extracts). The enzyme specific activity was measured in μmols of NADPH/min/mg protein.

2.4.8 Determination of NADPH-Cytochrome c Reductase

The method of Polakis, *et al.*, (1965) was developed for the measurement of NADPH-cytochrome c reductase activity. The activity of the microsomal enzyme was determined kinetically at 550nm using a Pye Unicam SP8-150 UV/Visible spectrophotometer provided with a line-log converter and recorder. The composition of the assay mixture was 50mM potassium phosphate buffer (pH6.5), 33mM EDTA, 1.7M potassium cyanide, 0.333mg/ml cytochrome c and limiting amounts of crude enzyme in total volume of 3ml. The specific activity of the enzyme was measured in μmols NADP/min/mg protein.

2.4.9 Determination of α-D-Mannosidase Activity

The reaction buffer (0.5ml) for the measurement of α-D-mannosidase activity contained 0.4mM p-nitrophenyl-α-D-mannosidase, 40mM sodium acetate (pH6.5) as described by Opheim, (1978). The reaction was initiated by the addition of enzyme. After 15mins incubation at 25°C, the reaction was stopped with 0.2ml 10% trichloroacetic acid (w/v). The reaction sample was then centrifuged for 10mins at

6000rpm, the supernatant was removed and added to 0.5ml of 1M glycine, (pH10.4). The absorbance was determined at 400nm and the amount of p-nitrophenyl released during the reaction was calculated. Specific activity was in μmols of p-nitrophenyl-α-D-mannopyranoside hydrolysed per min per mg protein.

2.4.10 Determination of β-Glucuronidase Activity

β-glucuronidase activity was assayed fluorometrically as described by Jefferson, *et al.*, (1987b). Yeast cells were extracted in assay buffer, (50mM sodium phosphate pH7.0, 10mm EDTA, 0.1% TritonX-100 and 10mM β-mercaptoethanol). The fluorogenic reaction was performed in assay buffer containing 1mM MUG (4-methyl umbelliferyl glucuronide) as a substrate, with a total reaction volume of 3ml. The mixture was incubated at 37°C, and 0.6ml of reaction mixture was removed at zero time and after each specified interval. The reactions were then terminated by the addition of 2.4ml of 0.2M sodium carbonate. Fluorescence was then measured with excitation at 365nm and emission at 455nm using a Bairo-Atomic spectrophotometer, with slit widths set at 10 or 30nm.

2.4.11 In Vitro Transport Assay

A standard incubation mixture contained 15μl of the microsomal preparation (10-15mg/ml protein) and 15μl of vacuoles (1.0-1.5mg/ml protein), 14.25μl of buffer B (80mM HEPES-KOH pH7.5, 600mM KOAc, 12mM $Mg(OAc)_2$, 12mM DTT), 3.95μl of an energy source (9.21mM ATP, 456.2mM Creatine phosphate), 1.82μl of 8mg/ml creatine kinase. The final volume was brought to 72μl with sterile distilled water. The mixtures were then incubated at 24°C for the times shown in the figures. The control mixtures contained 15μl of microsomes or 15μl of vacuoles only. After incubation for the desired time interval 50μl of the reaction mixtures was taken for determination

of CPY specific activity as described in section 2.4.4.

2.4.12 Purification of Carboxypeptidase Y

CPY was purified by combining the published protocols of Hayashi *et al.*, (1973a) and Kuhn *et al.*, (1974) as follows:

Step 1: Autolysis Step. 100g of fresh dried bakers yeast was crumbled and mixed with 200ml of chloroform. The mixture was occasionally kneaded with a spatula until the yeast had liquefied (30-60mins). After liquefacation the mixture was stirred for 30mins, then 200ml of distilled water was added. The pH of the mixture was adjusted to pH7.0 with 1N NaOH and the mixture left at room temperature for an additional 20hrs.

Step 2: Removal of Debris. The autolyzed mixture was centrifuged at 10000rpm for 10mins at 4^{o}C and the residue was discarded.

Step 3: Fractionation with Ammonium Sulphate. Solid ammonium sulphate was added gradually to the supernatant with stirring to reach 90% saturation, while the pH was maintained at pH7.0 with 1N NaOH at 25^{o}C. The precipitate was collected by centrifugation at 15000rpm for 30mins.

Step 4: Activation. The precipitate was dissolved in a minimal amount of 0.05M sodium acetate buffer, pH5.0 and the pH was readjusted to pH5.0 with 1N acetic acid. After that, the solution was left overnight. The precipitate produced was removed by centrifugation and the supernatant solution was readjusted to pH5.0 with 1N acetic acid.

Step 5: Chromatography on DEAE-Cellulose. The activated enzyme solution was brought to pH7.0 with 1N NaOH and dialysed overnight at 4^{o}C against three changes of sodium phosphate buffer pH7.0, containing 0.1M NaCl. The dialysed solution was then applied to a DEAE-cellulose column (2 x 12cm) which was previ-

ously equilibrated with 0.01M sodium phosphate buffer pH7.0 containing 0.1M NaCl, and then developed by applying a linear gradient of NaCl (0.15-0.45M) in the same buffer. Active fractions were pooled and the protein was precipitated by the addition of sufficient solid ammonium sulphate to reach at least 90% saturation at 25°C. The precipitate was collected by centrifugation for one hour at 15000rpm and then dissolved a in minimal amount of 0.01M sodium phosphate buffer, pH7.0 containing 0.1M NaCl and dialysed overnight against the same buffer at 4°C with three changes.

Step 6: Chromatography on DEAE-Sephadex A-50. The dialysed solution was applied to a column (0.8 x 20cm) of DEAE-Sephadex A-50 which was previously equilibrated with 0.01M sodium phosphate buffer, pH7.0 containing 0.1M NaCl. The column was developed by applying a linear gradient of NaCl (0.1-0.45M) in the same buffer. Active fractions were pooled and the protein was then precipitated with ammonium sulphate to 90% saturation at 25°C. The precipitate was dissolved in 1ml of 0.07M sodium phosphate buffer pH7.0 containing 0.15M NaCl.

Step 7: Gel Filtration on Bio-Gel P-200. The enzyme solution was applied to a column (1.5 x 100cm) of Bio-Gel P-200 which was previously equilibrated with 0.07M sodium phosphate buffer pH7.0 containing 0.15M NaCl. Active fractions were pooled and tested for CPY activity.

The homogeneity of the purified enzyme was demonstrated by SDS-gel electrophoresis and the protein bands detected by silver staining.

2.5 SDS Polyacrylamide Gel Electrophoresis, (Laemmli, 1970)

2.5.1 Acrylamide Stock

30g acrylamide

0.8g N′-N′-methylene-bisacrylamide

per 100ml sterile distilled water.

2.5.2 Standard Acrylamide Gel (10%)

22.5ml 1M Tris.HCl, pH8.8

23.8ml 30% acrylamide stock

11.2ml Sterile distilled water

degassed

Then just before pouring add

20μl TEMED and 1.5ml of 15mg/ml ammonium persulfate

2.5.3 Stacking Gel

2.5ml 1M Tris.HCl, pH6.8

2.0ml 30% acrylamide stock

14.8ml sterile distilled

Degassed

Then just before pouring add

20μl TEMED and 0.5ml of 20% ammonium persulfate.

2.5.4 Polyacrylamide Gel Electrophoresis Buffer (PAGE)

141g glycine

30g Tris

10g SDS

per 1 litre distilled water.

2.5.5 Sample buffer

0.2M Tris. HCl, pH6.8

0.2% SDS

10% sucrose

2.5.6 PAGE Staining Solution

1 litre methanol

140ml acetic acid

1.9g kenacid blue R250

Distilled water to 2 litre.

2.5.7 PAGE Destaining Solution

50% methanol

7% acetic acid

43% distilled water.

2.5.8 Preparation of Protein Samples for SDS-PAGE

Up to 100μl of protein sample could be loaded per track. This was prepared by mixing equal volumes of protein extract and sample buffer. The sample was then boiled for 5-10mins, added to a well and then 1μl of 2-mercaptoethanol added to the top of the well. The gel was electrophoresed at 8 mAmp overnight.

2.5.9 Staining and Destaining

After electrophoresis the gel was removed from the plates and placed in a tray of staining solution overnight. The gel was removed and placed in another tray containing destain solution and left until the protein bands were visible. The gel was then dried under a vacuum gel drier.

2.5.10 Silver Staining of SDS-Polyacrylamide Gels

The method of Wray, *et al.*, (1981) was followed. The gel was fixed for 30mins in 50% methanol and 10% acetic acid, then transferred to another tray containing 5% methanol and 10% acetic acid for 30mins. Next, the gel was rinsed with several changes of distilled water for about 2hrs. It was then soaked in 5μg/ml DTT for 30mins and the solution decanted without rinsing. 100ml of 0.1% silver nitrate was added and incubated at room temperature for 30mins before being washed again with a small amount of distilled water and twice rapidly with 10ml of developer (50μl of 30% formaldehyde per 100ml of 3% sodium carbonate). The gel was then soaked in the developer until the protein bands were visible and then stopped with 5ml of 2.3M citric acid. Finally, the stained gel was incubated for 30mins in distilled water and then for 10mins in 0.03% sodium carbonate before being dried under a vacuum gel drier.

2.6 Western Blots

The western blots were routinely carried out using the Bio-Rad Semi-Dry electrophoresis method as follows:-

The graphite plates were washed with distilled water and two layers of 16 x 16cm filter paper soaked in anode buffer no.1 (0.3M Tris.HCl pH9.4, 20% methanol, 0.1% SDS) were placed on the anodic plate. On top of these was placed another 16 x 16cm filter paper soaked in anode buffer no.2 (25mM Tris.HCl pH10.4, 20% methanol, 0.1% SDS) and then finally a piece of 16 x 16cm nitrocellulose membrane soaked in sterile distilled water. The gel was placed on top of the membrane and then a 16 x 16cm filter paper soaked in cathode buffer, (25mM Tris. HCl pH9.4, 40mM 6-amino-n-hexanoic acid, 20% methanol, 0.1% SDS) placed on top of that. A piece of dialysis membrane presoaked in distilled water was placed on top, followed by two layers of 16 x 16cm

filter paper soaked in cathode buffer. The cathode lid plate was then attached and the power supply connected and electrophoresed for 1hr at 0.8mA per cm^2 of gel. After that, the nitrocellulose membrane was removed for immunochemical detection of immobilized proteins.

The method of Johnson *et al.,* (1984) was used as follows: The nitrocellulose membrane was incubated for 1hr at 40°C with slight agitation in a sufficient amount of blotting buffer, (5% w/v non-fat dried milk dissolved in 1x Tris. Salt buffer (20mM Tris.HCl, 0.9% NaCl pH7.2)) to cover the surface of the membrane. The membrane was then washed three time for 10mins in blotting buffer. Then the membrane was incubated in blotting buffer containing 25μl of primary antibody (anti-CPY antibody) with agitation for 3hrs at 40°C. The membrane was then washed three times with blotting buffer as before, and then incubated at 40°C for 2hrs in the same buffer containing 20μl of secondary antibody. The membrane was then washed three more times in buffer and once quickly with 1x Tris-salt.

The membrane was stained using the method in the Amersham manual as follows: 25-50mg of DAB (3,3-Diaminobenzidine tetra-hydrochloride dihydrate) was dissolved in 100ml of 1x Tris-salt by heating to 65°C. The solution was then filtered and 3ml of 1% cobalt chloride added, followed by the addition of 50-100μl hydrogen peroxide, before pouring the solution into a tray containing the nitrocellulose membrane. After about 2-3mins the bands were visible.

2.7 Immunodiffusion (Ouchterlony, 1958)

2.7.1 Preparation of Agarose gel Plate, (Ouchterlony Plate)

A 0.3 x 3 x 8cm slab of 0.75% agarose dissolved in 1x TEB buffer (6.05g Tris., 0.46g Boric acid, 0.6g EDTA per 100ml distilled water) was prepared, and directly

runout onto a glass plate using a 10ml pipette. When the gel was set, the spacer was placed on the top of the gel and six wells were cut in a hexagonal array and one more well was cut in the centre of the array using a stainless steel bevelled-edge cutter.

2.7.2 Immunodiffusion and Precipitation

A 0.28mg/ml solution of CPY in TEB buffer was serially diluted two-fold five times. 50μl of each antigen dilution was placed in the outer wells. 50μl of antiserum was placed in the centre well. After loading, the gel was left at 4°C overnight, in order to allow diffusion to take place. The gel was then washed carefully with distilled water before being transferred to a clean plate, previously covered with a thin layer of 0.5% agarose containing 50% ethanol. The gel was then pressed with a sheet of filter paper and disposable nappies for about 10-15mins. The gel was then soaked in TEB buffer for 15mins before pressing again for 15mins. The gel was then dried slowly and stained with 5% staining solution, (Section 2.5.6) for 30-60mins. After staining the gel was rinsed with destaining solution, (Section 2.5.7), until the immunoprecipitated bands were visible.

2.7.3 The Detection of Anti-CPY Antibody by Enzyme-Linked Immunosorbent Assay (ELISA) (Engvall, 1980).

Aliquots of CPY (1μg/ml) in 0.1ml of Tris-HCl 0.2M NaCl 0.3M, pH9.5 were incubated on a microtitration plate (Nunc Immunoplate 1; obtained from Gibco Europe) for 18hrs at 4°C. The plates were washed with PBST buffer (NaCl 8.9g, KCl 0.2g, $Na_2HPO_4.12H_2O$ 2.9g, NaH_2PO_4 0.2g, Tween 20 0.5ml, Thimerosal 0.1g, Bovine serum albumin 1.0g per 1 litre distilled water), pH7.4, three times and with water three times also. Post-coating was carried out by incubating 0.3ml/well of a 1% solution of bovine serum albumin in PBS (W/o Tween 20) for 2hrs at ambient temperature

and washing as before. Rabbit anti-CPY IgG was added to the wells (Dilution series in PBST 0.1ml/well), incubated at ambient temperature for 2hrs more and washed as before prior to the addition of the second antibody (Goat anti-rabbit IgG coupled to horse radish peroxidase) (1/1000, by volume, in PBST, 0.1%PBS) (0.1ml/well). After a further incubation of 1hr at 35°C, plates were washed again and 0.1ml of substrate (PBST 55ml, H_2O_2 0.012%) in phosphate citrate buffer (0.2M NaH_2PO_4 25.7ml, 0.1M citric acid 24.3ml and 50ml deionised water), pH5.0 was added to each well. After approximately 30mins the peroxidase activity was inhibited by the addition of 0.05ml of 1M NaF and the well optical densities at 414nm determined on a Titertek Multiscan MCC plate reader (Flow Laboratories).

2.8 Preparation of Anti-CPY Antibody

For immunisation, the antigen (CPY) was emulsified with incomplete adjuvant and two rabbits were immunised subcutaneously three times at three week intervals with 130μg antigen. Two weeks after the last injection the antigen was emulsified with complete adjuvant and the two rabbits were boosted with 70μg of antigen.

Recovery of Blood Serum

70ml of blood was collected from both rabbits, three weeks after the first injection and two weeks after after the third and last injection. The tubes of blood were left in a water bath for two hours at 37°C and then kept overnight at 4°C to allow the red blood cells to clot. The blood serum was then transferred to centrifuge tubes and centrifuged at 3000rpm for 15-20mins. The supernatant was then dialysed against 50mM borate buffer saline (143mg sodium tetraborate, 126.5mg boric acid, 20mg sodium azide per 100ml distilled water), overnight with three changes. The crude anti-CPY antiserum was pooled and stored at -20°C.

2.8.1 Preparation of IgG Fraction from Crude Antiserum

Ammonium sulphate to 50% saturation was added slowly to the antiserum with continuous stirring and then left for 2hrs. The precipitated protein was recovered by centrifugation at 5500rpm for 10mins. The supernatant was discarded and the precipitate resuspended in 50mM borate saline buffer at about 1/2-1/5 of the original volume. This IgG fraction was dialysed against the same buffer with two changes overnight and the protein clarified by centrifugation. The purified IgG was then stored as before.

2.8.2 Removal of Non-Specific Anti-Yeast Antibodies

0.25ml of overnight YPD culture of a CPY deficient strain (BJ1075) was inoculated into 5ml fresh YPD and allowed to grow for 6hrs at 30°C with aeration. The cells were then pelleted by centrifugation at 3500rpm for 7mins, washed once with 10ml of Tween-20 precoat buffer (10mM $NaPO_4$ pH7.5, 140mM NaCl, 0.05% Tween-20) and then resuspended in 1ml of the same buffer. The cell suspension was transferred to a weighed eppendorf tube and centrifuged for 3mins at 12000rpm. The supernatant was discarded and the wet weight of cells was determined. The cells were resuspended at a concentration of 100μl buffer/mg cells and then transferred to two eppendorf tubes each receiving 0.2ml of cell suspension. The cells were pelleted again and the supernatants discarded. One portion of cells was resuspended in 0.4ml of anti-CPY antibody and kept at room temprature for 30mins with gentle agitation. The cell suspension was then centrifuged, the supernatant was removed and used to resuspend the second portion of cells, followed by incubation as above. The cells were removed by centrifugation and the supernant (purified anti-CPY antibody) transferred to a new eppendorf tube and 4μl of 1M sodium azide added as a preservative.

2.9 Genetic Crosses

Genetic crosses were performed essentially as described by Fink, (1970). The strains to be mated were grown on the surface of a YPD plate and incubated at 28°C overnight. The strains were then crossed by mixing them with a sterile toothpick on the surface of the YPD plate. The plate was then incubated for 4-8hrs, during which time the cells mate, first forming conjugating pairs and very soon thereafter, diploids.

In order to separate the diploid cells completely from haploid cells, they were streaked onto selective minimal agar plates and grown at 28°C for 2 days. The diploid cells were then transferred to 10ml of presporulation medium (10% glucose, 1% yeast extract, 3% tryptone) and incubated for 2 days at 25°C without shaking. They were then harvested by centrifugation at 3500rpm for 7mins, washed twice with distilled water and then resuspended in 0.5ml sterile distilled water. 0.1ml of cells was then transferred to sporulation slant medium (0.5% sodium acetate, 1% potassium chloride, 1.5% bacto-agar), followed by incubation at the above temperature for 4-5 days. The diploid cells undergo meiosis during this period, producing four haploid ascospores. The ascospores will not grow or germinate on sporulation medium, and can be stored in the refrigerator for at least 2 weeks. The separation of ascospores was achieved by enzymic digestion of the ascus wall with glusulase, (Fink, 1970) as follows: The asci and the unsporulated cells were washed carefully from sporulation slope medium. They were washed twice with distilled water and resuspended in 5ml of sterile distilled water. Glusulase was added to the suspension at the final concentration of 0.25%, followed by incubation at 25°C for 2hrs. The asci were then sonicated at low power to separate the adhesive sister ascospores from each other. The mixture was then centrifuged at 3500rpm for 7mins, washed twice with distilled water, resuspended in 0.5ml of sterile distilled water, vortexed gently and finally the suspension was diluted

to 5ml with sterile distilled water. The ascospores were freed from unsporulated cells by adding an equal volume of paraffin, and vortexing vigorously for 5mins, (Bevan and Woods, 1963). The ascospores which are hydrophobic remain in the paraffin layer whereas, the hydrophilic diploid cells do not. The paraffin layer was separated from the aqeous layer by centrifugation for 5mins at 4000rpm. The aqeous layer was discarded and the paraffin layer was washed four times more with sterile disilled water. 0.1ml of the purified spore suspension in paraffin was plated onto YPD medium and incubated either at 25°C for *sec* strains or at 30°C for other strains for 2-3 days.

In order to determine the mating type of the haploid colonies, they were mated separately on yeast minimal plates by cross-streaking with a and α mating types of an *ade1* strain.

2.10 Sub-Cellular Fractionation

2.10.1 Preparation of Vacuoles

To isolate yeast vacuoles, sphaeroplasts were prepared by the following method: 10ml of YPD medium was inoculated from a single yeast colony and grown overnight at 30°C. 2.5ml of this culture was used to start a fresh 250ml YPD culture. The cells were incubated with shaking to late exponential phase and the optical density at 600nm determined. Yeast cells were harvested by centrifugation at 8000rpm for 5mins, washed once in distilled water then with 100mM Tris.SO_4 (pH9.4), before being resuspended in the same buffer containing 10mM DTT at a density of 0.2g cells/ml. The cells were gently shaken for 30mins at 30°C. Following preincubation the cells were sedimented, washed twice with 1.2M sorbitol and thereafter, resuspended in 1.2M sorbitol (0.4g cells/ml), containing lyticase (25units/O.D_{600} unit). Sphaeroplasts formed during a 60mins incubation at 30°C were pelleted for 15mins

at 3500rpm and then washed twice with 1.2M sorbitol. Two methods were employed for the lysis of sphaeroplasts and the purification of vacuoles.

Method One.

In this method the procedure of Emter and Wolf, (1984) was followed. Sphaeroplasts were lysed by osmotic shock in 10mM Mes-Tris buffer pH6.8 containing 12% Ficoll-400 (1g cells/10ml). Lysis was then facilitated by gently pressing the mixture two times through a 50ml syringe. The lysed sphaeroplasts were then transferred to centrifuge tubes and overlaid with the same volume of 7% Ficoll in 10mM Mes-Tris (pH6.8) before centrifuging for 35mins at 12000rpm in a swingout rotor. Next, The float containing the vacuoles was removed. The crude vacuoles were further purified by adjusting the float with 13% Ficoll in the above buffer, to a final concentration of 10% Ficoll. An equal volume of 7% Ficoll buffer was carefully layered on top. Centrifugation was performed for 20mins at 12000rpm instead of 4500rpm (Kakinuma, *et al.*, 1981). The white float containing purified vacuoles was carefully collected and used for analysis.

Method Two.

The sphaeroplasts were resuspended in lysis buffer (20mM Heps-KOH pH7.5, 0.2M sorbitol) containing 15% Ficoll-400 at a density of 8ml/g cells. Lysis of sphaeroplasts was induced by the addition of DEAE-dextran to 30μg/1000 O.D$_{600}$ units of cells, and the vacuoles purified as described by Bankaitis, *et al.*, (1986). The lysed sphaeroplasts (8ml) were transferred to a nitrocellulose tube and overlaid with 6ml of 8% Ficoll in Hepes-KOH, buffer pH7.5 containing 0.2M sorbitol. A further 4ml layer of 4% Ficoll in the above buffer was carefully placed on the top. Lysis buffer (4ml) was then layered above the 4% Ficoll solution and the gradient centrifuged for 1.5hours at 33000rpm (100.000g) in a swinging bucket rotor. Purified vacuoles were then collected at the 4%/0% Ficoll interface.

2.10.2 Isolation of Microsomes "The Golgi fraction"

Microsomes from DUBY35 (*a sec14-3, pep4-3*) or DUBY29 (*a sec7-1, pep4-3*) were isolated by a modification to the procedure of Rothblatt and Meyer, (1986a) as follows: Yeast cells were grown in YPD medium containing either 0.2% or 2.0% glucose at 24°C to an $O.D_{600}$ of 1.5. The cells were then incubated at the restrictive temperature (37°C) for 2hrs to allow for accumulation of proCPY in the Golgi vesicles. Cells were then washed once with distilled water and resuspended in 100mM $Tris.SO_4$ (pH9.4), 10mM DTT at a density of 0.2g cells/ml. They were then incubated for 30mins at 33°C with shaking. Cells were then washed twice with 1.2M sorbitol and then resuspended in YPD medium with the appropriate concentration of glucose containing 1M sorbitol, 20mM potassium phosphate (pH7.2) and 25 units lyticase/$O.D_{600}$ at a concentration of 0.4g cells/ml. Sphaeroplast formation was allowed to proceed for 60-90mins at 33°C with gentle agitation. The sphaeroplasts were pelleted, washed twice with 1.2M sorbitol before being resuspended in buffer A (20mM HEPES-KOH pH7.5, 150mM KOAc, 3mM $Mg(OAc)_2$, 2mM DTT). The sphaeroplasts were homogenized with 40 strokes in a glass Dounce homogenizer on ice. Next, the homogenate was layered over a cushion of 1M sucrose, 2mM EDTA, 20mM HEPES-KOH pH7.5, and then centrifuged in a Sorval centrifuge using a sorval HB-4 rotor at 7000rpm for 10mins at 4°C. The upper layer which contained the microsomes was pooled and centrifuged in a Beckman JA-20 rotor for 25mins at 20000rpm. The supernatant was discarded and the lower microsomal fraction was resuspended carefully in buffer A containing 7% Ficoll-400. This preparation was used directly as a source of microsomes in the transport assay.

2.10.3 Isolation of Cytoplasmic Fraction (S100)

Cells were grown on YPD and the sphaeroplasts lysate prepared as described in the previous section. The soluble cytoplasmic fraction was then purified as described (Waters and Blobel, 1986). Lysed sphaeroplasts were centrifuged in a Sorval SS34 rotor at 15000rpm for 15mins. The supernatant was removed, placed into a fresh centrifuge tube and centrifuged in a swinging bucket rotor at 33000rpm for 30mins. After centrifugation the upper thin layer of lipid was withdrawn and discarded. The clear supernatant zone referred to as S100, was collected carefully without disturbing the flocculent material at the bottom of the tube. The S100 was then used directly for transport assays or stored at -80°C and used after only one freeze/thaw cycle.

Manipulation of DNA

2.11 Preparation of Plasmid DNA from Bacterial Cells

2.11.1 Small Scale DNA Preparation (Miniprep)

The method of Crouse, *et al.*, (1983) was adapted. 5ml of L-broth was inoculated by a single bacterial colony and grown overnight under appropriate antibiotic selection. The cells were then harvested by centrifugation for 10mins at 3500rpm using a benchtop centrifuge and the sedimented bacterial cells resuspended in 192μl of solution I (1% glucose; 10mM EDTA pH8.0; 25mM Tris.HCl pH8.0; 2mg/ml lysozyme). The suspension was then transferred to a sterile 1.5ml eppendorf tube, vortexed for 30sec and allowed to stand for 5mins at room temperature. Next, the tube was placed on ice for 2mins followed by the addition of 400μl of freshly prepared solution II (0.2N NaOH, 1% SDS) to lyse the cells. The tube was vortexed and returned

to ice for 5mins before adding 300μl of 3M sodium acetate (pH4.8). The solution was mixed and incubated on ice for a further 10mins. Chromosomal DNA and other bacterial debris was pelleted by centrifugation at 12000rpm for 5mins. 750μl of the supernatant was transferred to a new tube and the nucleic acid precipitated by the addition of 500μl of isopropanol and incubation for 15mins at -20°C. The DNA was then sedimented by centrifugation at 12000rpm for 5mins, the supernatant discarded and the pellet washed with 70% ethanol. All traces of ethanol was eliminated by draining the tube and removing any remaining droplets with a glass capillary tube. The pelleted DNA was resuspended in 200μl of TE buffer followed by extraction with 200μl of phenol and then with 200μl of chloroform to remove the protein contaminants. The suspension was centrifuged for 3mins and the aqueous layer removed to a fresh tube and the DNA precipitated as described, (Section 2.14.4). The vacuum dried pellet was resuspended in 100μl of TE buffer.

2.11.2 Large Scale DNA Preparation

10ml of L-broth was inoculated with a single bacterial colony and grown overnight with antibiotic selection . This culture was used to inoculate 1 litre of fresh L-broth and grown with aeration until the culture reached late logarithmic phase ($O.D_{600}$=0.6). The plasmid DNA was then amplified by adding chloramphenicol to a final concentration of 200μg/ml and the incubation continued overnight. The bacterial cells were harvested by centrifugation at 10000rpm for 10mins at 4°C. The supernatant was discarded and the pellet resuspended in 10ml of solution I (previous section) containing 50mg lysozyme. The solution was mixed well and left at room temperature for 30mins. 20ml of solution II was added, mixed by inversion and then kept on ice for 30mins. Precipitation of cell debris and proteins was subsequently achieved by the addition of 15ml of 3M sodium acetate (pH4.8), and leaving on ice for a further 10mins.

Lysed cells and debris were sedimented by centrifugation 25mins at 18000rpm at 4°C.

Nucleic acid precipitation was achieved by adding an equal volume of isopropanol and incubating for 30mins at room temperature. The DNA precipitate was collected by centrifugation for 30mins at 12000rpm at 4°C. The precipitated DNA was then washed with 70% ethanol, dried under vacuum and then resuspended in TE buffer. An exact volume of caesium chloride was added depending on the final volume of sample (20.6g in 27ml or 29g in 39ml). Ethidium bromide was added to a final concentration of 120μg/ml prior to transfer by syringe to quickseal Sorval centrifuge tubes. The tubes were balanced, heat sealed and centrifuged at 44000rpm for 17-22hrs at 15°C in a VTi50 rotor.

The tubes were carefully removed from the rotor and viewed under U.V. illumination. Usually two bands were clearly visible. The lower plasmid DNA band was collected from the gradient and the ethidium bromide removed by repeated solvent extraction using CsCl-saturated isopropanol. The DNA sample was dialysed against TE buffer overnight with four changes and the purified plasmid DNA stored at -20°C.

If complete separation of plasmid from chromosomal DNA had not been achieved, the lower plasmid band was removed and recentrifuged as just described.

2.12 Preparation of Yeast Plasmid DNA

The procedure of Hoffman and Winston (1987) was employed for preparation of yeast plasmid DNA. 5ml of yeast culture was grown overnight in a medium to maintain selection for the plasmid. A 1.5ml eppendorf tube was then filled with this culture and the cells collected by centrifugation for 10mins in a microcentrifuge. The supernatant was discarded and the pellet was vortexed briefly to resuspend the cells in the residual liquid. Next, 0.2ml of 2% TritonX-100, 1% SDS, 100mM NaCl, 10mM Tris.Cl pH8.0 and 1mM EDTA were added. 0.2ml phenol:chloroform:isoamylalcohol

(25:24:1) was added and then 0.3g acid washed glass beads. The suspension was vortexed for 2mins, centrifuged for 5mins at 12000rpm and the aqueous supernatant containing the purified plasmid DNA was collected in a new eppendorf tube. The DNA was recovered by ethanol precipitation.

2.13 Spectrophotometric Estimation of Plasmid DNA Concentration

The optical density of a 1/20 or 1/50 dilution of plasmid DNA was determined at 260 and 280nm. A pure DNA sample has an $A_{260/280}$ ratio of 1.8. An O.D of 1.0 spectrophotometer unit = 50μg DNA/ml (Maniatis, *et al.*, 1982) .

2.14.1 Digestion with Restriction Enzymes

10x stocks of restriction enzyme buffer were made as described in this table (Maniatis, *et al.*, 1982).

Stock solution(μl)	Low	Medium	High (1ml)
NaCl (5M)	0	100	200
Tris, pH7.4 (1M)	100	100	500
$MgSO_4$ (1M)	100	100	100
DTT (1M)	10	10	0
Sterile water	790	690	200

Restriction digests were usually performed in 30μl reaction volumes. A typical restriction digest always consisted of 1-2μl plasmid DNA, 3-5 units of restriction enzyme, 3μl of appropriate 10x restriction enzyme buffer and 1μl of DNAase free RNAase (1mg/ml). Incubation was for 3-4hrs at 37°C. Digestion of DNA with two enzymes having similar buffer requirements were performed at the same time. For

double digests using two enzymes with different restriction buffer requirements, the DNA was extracted with phenol:chloroform and precipitated with ethanol between digests. The reaction was terminated by the addition of 3μl of stop dye (1ml 10% SDS, 2ml 250mM EDTA pH8.0, 0.1ml 1M Tris.HCl pH8.0, 5ml Glycerol, 10mg Bromophenol blue, H_2O up to 10ml).

2.14.2 Ligation of DNA Fragments

Ligation was usually performed by mixing a large excess of donor DNA to be cloned with linearised plasmid DNA (vector DNA) in the presence of T4 DNA ligase enzyme and 10x ligation buffer (660mM Tris.HCl, pH7.6, 50mM $MgCl_2$, 50mM DTT, 10mM ATP pH7.0). The reaction was then left for 4hrs at room temperature or at 15°C overnight. The reaction mixture was normally composed of 15μl (6.0μg) of the DNA to be cloned, 10μl (2.0μg) vector DNA, 3μl 10x ligation buffer, 1-2units T4 DNA ligase and sterile distilled water to 30μl.

2.14.3 Phenol-Chloroform Extraction of DNA

The removal of restriction enzymes, bacterial cell proteins and other protein contaminants during purification of DNA was by sequential extraction with equal volumes of phenol (Re-distilled phenol, 8 hydroxyquinoline), phenol:chloroform and then chloroform, all equilibrated with TE buffer (10mM Tris.HCl, 1mM EDTA pH8.0). Care was taken to leave denaturated protein at the interface between the separated organic and aqueous phases.

2.14.4 Recovery of DNA by Ethanol Precipitation

0.1 volumes of 3M sodium acetate, pH4.8 and 3 volumes of cold 100% ethanol were added to the purified DNA solution, mixed by vortexing and stored at -80°C for 30mins. Precipitated DNA was collected by centrifugation at 12000rpm for 10mins in a microfuge. The DNA pellet was washed with 70% ethanol, recentrifuged again for 5mins to retain the loose pellet and the tube carefully drained over tissue paper before drying under vacuum for 5mins. The dried DNA was resuspended in sterile distilled water or TE buffer.

2.15 Phosphorylation of Synthetic Oligonucleotide Linkers

Prior to ligation the $5'$ ends of synthetic linkers were phosphorylated by the action of polynucleotide kinase. 1μl (approximately 1μg of linker DNA, 7μl of sterile distilled water, 1μl 10x ligase kinase buffer (0.66M Tris.HCl pH7.6, 10mM ATP, 10mM spermidine, 0.1M $MgCl_2$, 150mM DTT, 2mg/ml BSA) and 1μl (2 units) of polynucleotide kinase were mixed and incubated at 37°C for 30mins, followed by the addition of another 2 units of polynucleotide kinase and incubated for a further 30mins. The reaction mixture was then heated at 70°C and allowed to cool slowly to room temperature before an aliquot (Approx. 0.25μg of linker DNA) was removed and used for a ligation reaction, the remainder was stored at -20°C.

2.16 Exonuclease III/Mung Bean Nuclease Deletion of DNA

Deletions of varying lengths of DNA were achieved by performing a single reaction with Exonulease III and removing aliquots at various time intervals. The enzyme specifically degrades at DNA sequences with protruding $5'$ ends and proceeds in a $3'$ to $5'$ direction, creating a population of DNA molecules with single stranded tails at

each end. The tails were removed by the action of Mung Bean nuclease, (Henikoff, 1984) which specifically degrades single stranded DNA, resulting in duplex molecules with deletions.

The reaction mixture normally contained 5μg of restricted DNA, 12.2μl 2x Exo. buffer (100mM Tris.HCl pH8.0, 10mM $MgCl_2$, 20μg/ml tRNA), 2.5μl of fresh 100mM 2-Mercaptoethanol, 1μl exonuclease III (20U/μg) and H_2O to 25μl for each time point of the reaction. The reaction was stopped by the addition of 175μl of Mung Bean stop buffer (40μl 5x Mung Bean buffer (150μl NaOAc pH 5.0, 0.25M NaCl, 5mM ZnCl, 25% glycerol) in 135μl of sterile distilled water). The samples were then heated at 68°C for 15mins and placed on ice. 40 units of Mung Bean nuclease (previously diluted with 1x Mung Bean dilution buffer (10mM NaOAc pH5.0, 0.1mM ZnOAc, 1mM cystine, 0.005% TritonX-100)) were added to each tube and incubated at 30°C for 30mins. The Mung Bean nuclease protein was then removed from the deleted DNA by a modified phenol extraction procedure in which the following solutions were added: 4μl 20% SDS, 10μl 1M Tris.HCl pH9.5, 20μl 8M LiCl., and 250μl of phenol:chloroform:isoamylalcohol (25:24:1). The tubes were then vortexed, followed by centrifugation for 1min and the upper aqueous layer was removed to a new tube and re-extracted with the same volume of chloroform. The aqueous layer was transferred to a new tube and tRNA to a final concentration of 10ng/ml was added, followed by 0.5ml of cold 100% ethanol. The DNA was left to precipitate for 10mins on ice and recovered by centrifugation for 20mins at 12000rpm. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 15μl of TE buffer. 1μl was used in a ligation reaction, performed at 15°C overnight, incorporating a specific oligonucleotide linker and 2μl loaded onto a 0.7% agarose gel for analysis.

2.17 Agarose Gel Electrophoresis

2.17.1 Maxigels

Agarose was at a concentration of 0.7% or 2% for separation and identification of DNA restriction fragments. Gel preparation, pouring and electrophoresis were as described by Maniatis *et al.,* (1982). All DNA samples and suitably restricted bacteriophage λ size markers were mixed with stop dye (Section 2.11.3) prior to loading. A slab gel consisting of 200ml of 0.7% agarose in 1x Alex gel buffer (0.04M Tris-acetate, 0.001M EDTA pH7.7 plus 3μg/ml EtBr) was prepared. The gel was poured into a horizontal 180 x 150mm perspex mould sealed to a glass plate with high vacuum grease. When the gel was set, the mould was removed and the agarose gel immersed into a tank containing 1x Alex buffer. DNA samples were then loaded into wells and electrophoresed for 4-5hrs at 100V or overnight at 30V. After electrophoresis the gels were visualized under long wave length ultraviolet light (300-360nm) and photographed using a red filter and Polaroid 667 film (ISO 3000).

2.17.2 Minigels

Minigels were often used for the rapid detection of DNA, either determining the progress of a restriction digest or to confirm the successful recovery of DNA after phenol extraction or fragment isolation from LMP agarose. 60ml of 0.7% agarose was prepared in the same way as for maxigels except with TBE buffer (216g Tris, 110g Boric acid and 18.6g EDTA plus 3μg/ml EtBr per 2 litre distilled water). Minigels were electrophoresed at a maximum of 90V for 2hrs.

2.18 Isolation of DNA from Agarose Gels

Isolation of DNA Fragment Using Low Melting Point Agarose Gels

A 0.7% agarose LMP gel was prepared as described above for minigels. DNA samples were loaded into wells and electrophoresed at 30V overnight. After electrophoresis the gel was exposed to ultraviolet light and the required fragments were removed from the gel with a sterile scalpel. The slice containing the DNA fragment was melted at 65°C , two volumes of 50mM Tris.HCl, 0.5mM EDTA pH8.0 was added and the mixture placed at 37°C for a few minutes. The sample was phenol extracted (section 2.14.3) and ethanol precipitated (section 2.14.4). Then the sample was resuspended in 20μl sterile distilled water or TE buffer.

Freeze Elution from 0.7% Agarose Gels

A thin gel slice containing the DNA fragment was obtained as described above and placed in a 1.5ml eppendorf tube with 0.9ml of sterile distilled water and 0.1ml of 3M sodium acetate, 10mM EDTA. The tube was left for 15mins in the dark with occasional shaking. The gel slice was then placed in a 0.5ml eppendorf tube that had been plugged with siliconised glass wool, and punctured in both lid and base. The tube was placed inside a larger eppendorf tube, stored at -80°C for 15mins and then immediately centrifuged at 12000rpm for 15mins. The liquid accumulated in the large tube was retained and further elution of the DNA was achieved in a second centrifugation. 5μl of 1M $MgCl_2$ and 10% acetic acid were added to the pooled samples and the DNA was then recovered by ethanol precipitation. The purified DNA was dried under vacuum and then resuspended in 20μl of TE buffer.

2.19 Bacterial Transformation

The modified method of Mandel and Higa (1970) was used. 1ml of an overnight culture was inoculated into 50ml of L-broth and then incubated at 37°C with vigorous shaking until an $O.D_{600}$ of 0.3-0.4 was reached (2-3hrs). Cells were harvested by centrifugation for 7mins at 3600rpm. Next, the supernatant was discarded and the pellet resuspended in 40ml of ice cold 50mM $CaCl_2$,10mM Tris.HCl pH8.0. The cell suspension was kept on ice for 1hr and recentrifuged as before. The pellet was resuspended in 1ml of the same buffer. The suspension was left for at least 1hr at 0°C prior to use.

10μl of ligation mixture was mixed with 100μl of competent cells and the mixture left on ice for 30mins. Controls of unligated vector and unrestricted vector were also performed. The suspension was then heat shocked for 2mins at 42°C. 1ml of L-broth was added and the suspension incubated at 37°C for 1hr to allow for expression of the antibiotic resistance genes. 100μl of 10^0, 10^{-1}, and 10^{-2} dilutions were placed onto selective plates containing 10μl of 100mM IPTG and 20μl of 20% X-Gal. The plates were incubated at 37°C for 12-16hrs.

JM83, GM119 and JM101 transformants bearing a plasmid insert appeared white on IPTG, X-gal agar, due to insertional inactivation of the *lacZ* gene, against a background of blue non-recombinant colonies.

Single white transformants presumed to be bearing chimaeric plasmids were sub-cultured under identical antibiotic selection before recovery of the plasmid DNA by Minipreps (Section 2.11.1).

2.20 Preparation of Yeast Competent Cells and Transformation

The modified method of Ito, *et al.*, (1983) was performed for preparation of yeast competent cells and DNA transformation. 10ml of YPD medium was inoculated with a single yeast colony and left to grow at 30°C until an $O.D_{600}$ was reached 0.4-0.5. The cells were harvested by centrifugation at 3500rpm for 5mins, washed once with distilled water, then once with TE buffer. The pellet was resuspended in 10ml of 0.1M lithium acetate and incubated for 30-60mins at 30°C. The cells were pelleted again and then resupended in 0.4ml of 0.1M lithium acetate. For transformation of DNA, 1μg (10μl) of DNA was added to 50 μl of freshly prepared competent cells in an eppendorf tube and incubated at 30°C for 30mins. Next, the cells were shaken and 0.6ml of 40% PEG-4000 in 10mM Tris-HCl (pH7.6) was added, mixed well and incubated for 1hr at 30°C before heat shock at 42°C for 5mins and the placing immediately on ice. The cells were centrifuged for 10sec, the supernatant discarded and the transformed cells resuspended in 100μl of sterile distilled water, plated on selective yeast minimal agar and incubated at 30°C for 3-4 days.

2.21 Construction of M13 Clones for Mutagenesis and Sequencing

0.1μg of M13 DNA (mp18 or mp19) was digested with the appropriate enzymes and a third of the digest ligated to the desired DNA fragment which had first been isolated using the LMP procedure (Section 2.18).

Competent cells of JM101 were prepared as described in section 2.19. Then 5, 10 and 15μl aliquots of ligation mixture were added to 100μl of competent cells and left on ice for 20-30mins. Cells were heat shocked at 42°C for 2mins and placed on ice. Next, 200μl of fresh log phase JM101 cells, 10μl of 100mM IPTG and 20μl of 10% X-Gal were added and the mixture finally added to 3ml of soft agar. This was

poured on top of an L-agar plate, and when set, incubated at 37°C overnight. White plaques were picked directly for template preparation.

Preparation of Single-Stranded M13 DNA

50ml of 2YT broth was inoculated with 100μl of an overnight culture of JM101 cells and 2ml aliquots added to sterile universal tubes. Single white plaques were picked and inoculated into each tube and a single blue plaque was also taken as a control. The tubes were incubated at 37°C overnight. 1.5ml of each culture was centrifuged in a microcentrifuge for 5mins and the supernatant transferred to a fresh tube, re-centrifuged and 1ml aliquoted into a further clean tube. 200μl of 20% PEG-6000, 2.5M NaCl were added, vortexed and left at room temperature for 30mins before centrifuging for 5mins. The pellet was resuspended in 100μl of TE buffer, then extracted with an equal volume of phenol and then chloroform. The DNA was recovered by ethanol precipitation and finally resuspended in 20μl of TE buffer. 2μl samples were loaded onto a 0.7% agarose gel and electrophoresed with control M13 single-stranded DNA as a size standard.

2.22 DNA Sequencing by Dideoxynucleotide Chain Termination

First, 10μg of purified plasmid DNA was incubated for 30min at 37°C with 20μg of DNAase-free RNAase. The enzyme was removed by phenol, phenol:chloroform, chloroform extraction and the DNA recovered by ethanol precipitation (Section 2.14.4).

For alkaline denaturation and sequencing of plasmid DNA, the method of Hattori and Sakaki (1986) was used. The dried pellet from above, was resuspended in 18μl of sterile distilled water and 2μl of 2N NaOH was added to render the DNA single-stranded. After 5mins incubation at room temperature, 8μl of filter sterilized 5M ammonium acetate (pH7.4) was added and the denaturated DNA precipitated for

15mins in 100μl of ethanol at -80^{o}C, rinsed once with 70% ethanol and vaccum dried before resuspension in 10μl sterile distilled water.

10μl of the denaturated DNA, 2μl reverse primer and 1μl of 10x polymerase reaction buffer (70mM Tri.HCl pH7.5, 70mM $MgCl_2$, 500mM NaCl) were heated to 90^{o}C for 15mins in an eppendorf tube and then allowed to cool at room temperature before placing on ice.

Into four eppendorf tubes labelled A, G, C, and T, 2μl of the corresponding dNTP/ddNTP (0.125mM each) nucleotide mixture were introduced. 1μl of ^{35}S-dATP, 1μl of 0.1M DTT and 1μl (2 units) of Klenow polymerase were added to the template DNA samples, and 3μl of the resultant solution transferred to the nucleotide mixtures in the labelled tubes. The contents were mixed, briefly centrifuged and incubated for 20mins at 37^{o}C. 1μl of 0.5mM dATP was added and incubation continued for a further 20mins. The reaction was terminated by the addition of 5μl of formamide stop dye (10mM EDTA pH8.0, 0.2% bromophenol (w/v), 0.2% xylene cyanol (w/v)). The samples were then stored at -20^{o}C. Before loading, samples were boiled for 3mins and loaded directly onto DNA sequencing gels.

2.23 Urea-Polyacrylamide Electrophoresis

Two glass sequencing plates were washed and one side of each, cleaned with 100% ethanol several times and then treated with "Repelcote" (2% dimethyl dichlorosilane in 1,1,1, trichloroethane). To 20ml solution of 38% acrylamide and 2% bis-acrylamide, 42g urea and 10ml 10x TBE buffer were added and the volume completed to 100ml with distilled water. The acrylamide solution was degassed, 0.8ml 10% ammonium persulphate solution and 20μl of TEMED then added and the mixture poured between the glass plates. A comb was inserted and the gel allowed to set.

Once the gel had set the tape and the comb were removed from the gel. The

gel was then mounted on the electrophoresis apparatus and the reservoirs filled with TBE buffer. An aluminium sheet was clamped to aid heat dissipation and the wells were cleared of urea by flushing with buffer.

The gels were pre-electrophoresed at 50mA (1300V) for 1hr. The boiled samples were loaded in the order A, G, C, and T for each template and the gel electrophoresed for 1-1.5hrs until the lower of the two dye bands reached the bottom of the gel.

2.24 Autoradiography

The two plates were carefully separated, leaving the gel adhering to one plate. The gel was then transferred to a sheet of Whatman 3MM paper, covered with polythene sheeting and dried under vacuum for 3-4hrs at 80°C. The dried gel was overlaid with sensitized Fuji RX X-ray film clamped between two glass plates and exposed for 12-14hrs before being developed.

CHAPTER THREE

3. PREPARATORY STUDIES

3.1 Introduction

Studies on the proteinases in yeast led to the discovery of carboxypeptidase Y (CPY) (Doi, *et al.*, 1967; Hayashi, *et al.*, 1970; Hayashi, *et al.*, 1973a,b; Hata, *et al.*, 1967a; Hayashi, 1976). The enzyme was previously termed proteinase C (Hata, *et al.*, 1967a), but CPY is used to distinguish it from similar enzymes from other sources such as pancreatic carboxypeptidase A.

The broad amino acid specificity of CPY implicated it in a general role in intracellular peptide hydrolysis (Hayashi, *et al.*, 1970) and its possible usefulness in studies on the amino acid sequencing of proteins was realized (Hayashi, *et al.*, 1973a). This led to extensive purification and characterization of the enzyme by different groups (Doi, *et al.*, 1967; Aibara, *et al.*, 1971; Hayashi, *et al.*, 1973a; Kuhn, *et al.*, 1974; Johansen *et al.*, 1976; Hasilik and Tannar, 1976a). The enzyme was found to be composed of one polypeptide chain of 61kD molecular weight of which 10kD is carbohydrate. At pH5.5-6.5 CPY exhibits a high activity in hydrolysing most amino acid residues, including proline from C-termini of proteins and peptides (Hayashi, *et al.*, 1970, 1973a; Kuhn, *et al.*, 1974; Hayashi, 1976). The enzyme also exhibits high activity against ester substrates such as N-acetyl-DL-phenylalanine-β-naphthylester Doi, *et al.*, 1967; Aibara, *et al.*, 1971; Wolf and Fink, 1975; Hayashi, 1976) and anilide substrates (N-benzoyl-L-tyrosine-p-nitroanilide) (Aibara, *et al.*, 1971; Hayashi, 1976). Phenylmethylsulphonylfluoride (PMSF) and Diisopropylfluorophosphate are powerful inhibitors of CPY activity (Doi, *et al.*, 1967; Kuhn, *et al.*, 1974).

Biochemical analysis of a mutant lacking CPY activity (Wolf and Fink, 1975) confirmed the existance of a second enzyme exhibiting carboxypeptidase activity, which was termed Carboxypeptidase S (CPS) (Wolf and Weiser, 1977). This enzyme was strongly inhibited by chelating agents like EDTA or phenanthroline.

Carboxypeptidases (CPY and CPS) have been implicated in the utilization of exogenously supplied peptides (Wolf and Ehmann, 1981; Zubenko, 1981; Jones, 1984), and naturally occurring peptides (Wolf and Ehmann, 1981). CPY also acts on endogenously generated peptides (Zubenko and Jones, 1981; Jones, 1984). It appears to act particularly on peptides generated by proteinase B catalysis (Zubenko and Jones 1981).

The aims of this chapter are as follows:-

1. Purification of CPY to produce a specific anti-CPY antibody.

2. Genetic crosses in order to produce *sec, pep4-3* strains for use in *in vitro* transport studies (Chapter 4).

3.2 Results

3.2.1 Purification of Carboxypeptidase Y

Carboxypeptidase Y was purified as outlined in section 2.4.12. 100g of dried bakers yeast was autolysed with 200ml chloroform for 20hrs at room temperature, the cell debris was removed by centrifugation and the supernatant was made up to 90% saturation with ammonium sulphate. The precipitate was then collected by

centrifugation. The activation of CPY in this fraction was achieved by dialysis with 50mM sodium acetate pH5.0. Activation is due to the activities of proteinase A and proteinase B (Hayashi, *et al.*, 1973a; Hayashi, 1976, Wolf and Fink, 1975; Wolf and Weiser, 1977). The specific activity of CPY in this fraction was determined to be 80.00 nmol/min/mg protein (Section 2.4.4). The fraction was then applied to a DEAE-Cellulose column (2 x 12cm) and the column was eluted with a linear gradient of NaCl (0.1-0.45M) in 10mM sodium phosphate buffer, pH7.0. 20 x 5ml fractions were collected, followed by determination of CPY activity in each fraction as before (Fig 3.2.1.1). The CPY containing fractions located between fraction numbers 11-19 were dark yellow in colour. They were pooled, precipitated again with 90% saturation ammonium sulphate and resuspended in 50mM sodium acetate buffer as before. This fraction was then applied to a DEAE-Sephadex A-50 column for further purification. The Column (0.8x 20cm) was developed with a linear gradient of NaCl (0.1-0.45M) in 10mM sodium phosphate buffer, pH7.0 and 25 x 2ml fractions were collected. The active CPY fractions were then collected and the results presented in fig 3.2.1.2 indicate that two peaks of CPY activity appeared, the first peak located at fraction 18 and the second peak at fraction 21. The active fractions were pooled, made up to 90% saturation with ammonium sulphate and resuspended in 1ml of 70mM sodium acetate buffer, pH7.0 for a gel filtration on Sephadex G-200. The column (1.5 x 100cm) was eluted with 70mM sodium phosphate buffer pH7.0 containing 0.15M NaCl, and 150 fractions of 1ml volume each were collected (Fig 3.2.1.3). Only one peak of active CPY was observed. The specific activity of the purified CPY had increased by at least 420 fold (34 x 10^3 nmol/min/mg protein) compared to that in the crude extract.

The homogeneity of the purified enzyme was demonstrated by SDS-PAGE electrophoresis in the presence of mercaptoethanol and the protein bands detected by

Fig 3.2.1.1: Chromatography on DEAE-Cellulose of the 90% ammonium sulphate fraction of the yeast autolysate. Protein concentration was determined as described in section 2.4.3. CPY assay as described in section 2.4.4.

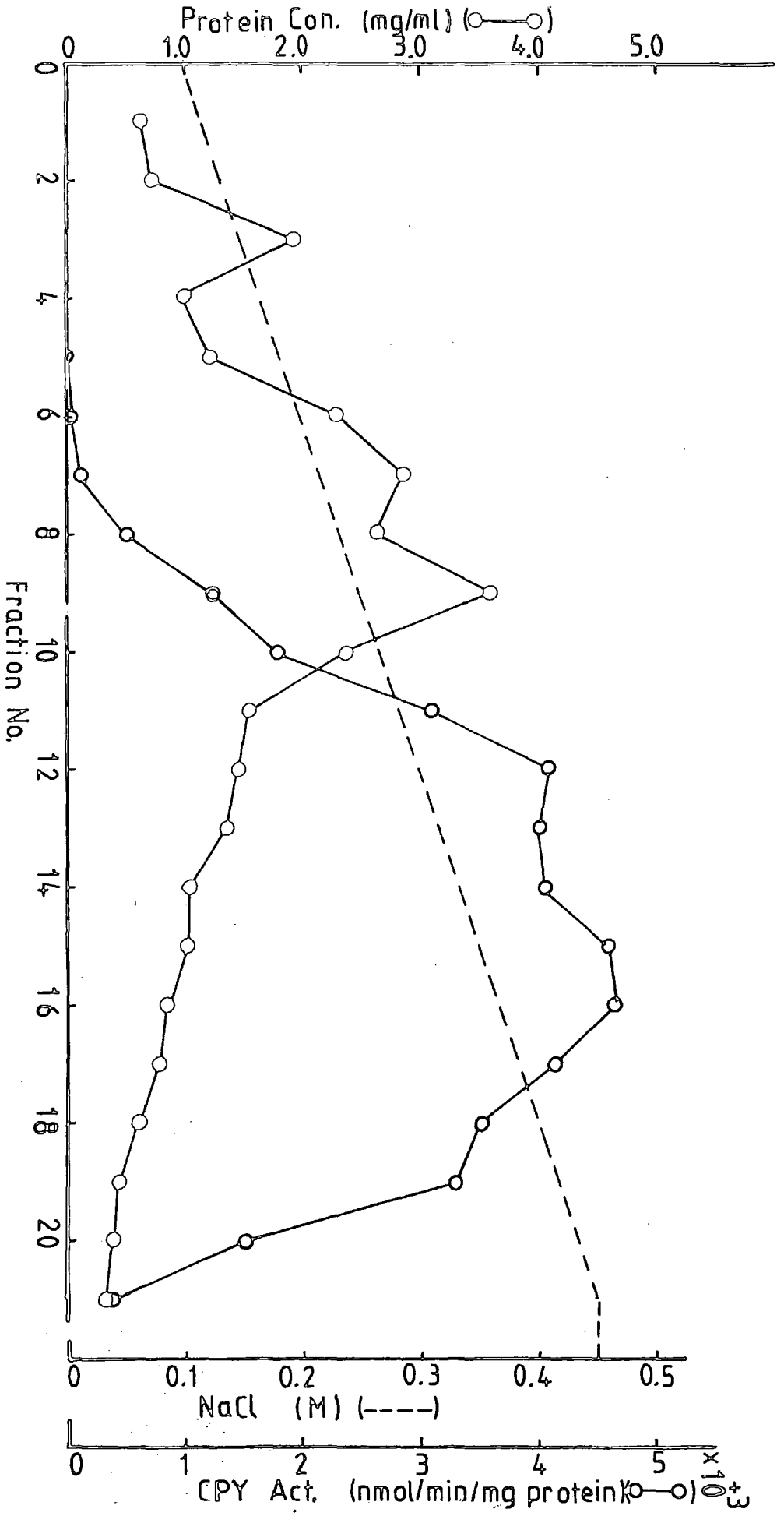

Protein Con. (mg/ml) (o—o)
0
1.0
2.0
3.0
4.0
5.0
Fraction No.
0
2
4
6
8
10
12
14
16
18
20
0
0.1
0.2
0.3
0.4
0.5
NaCl (M) (----)
0
1
2
3
4
5
x10+3
CPY Act. (nmol/min/mg protein) (o—o)

Fig 3.2.1.2: Chromatography on DEAE-Sephadex A-50 of the active CPY fractions eluted from the DEAE-Cellulose column, (Fig 3.2.1.1).

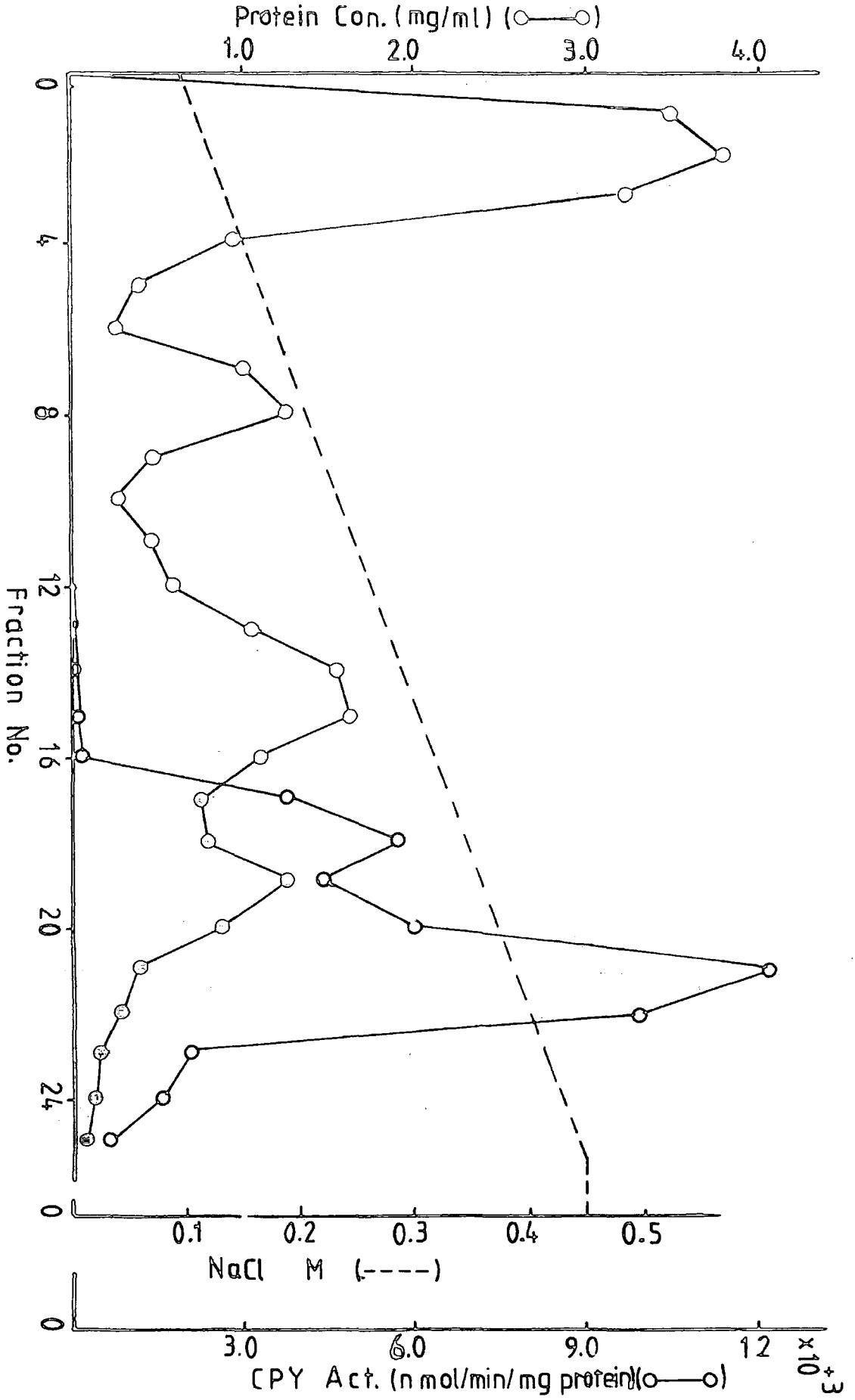
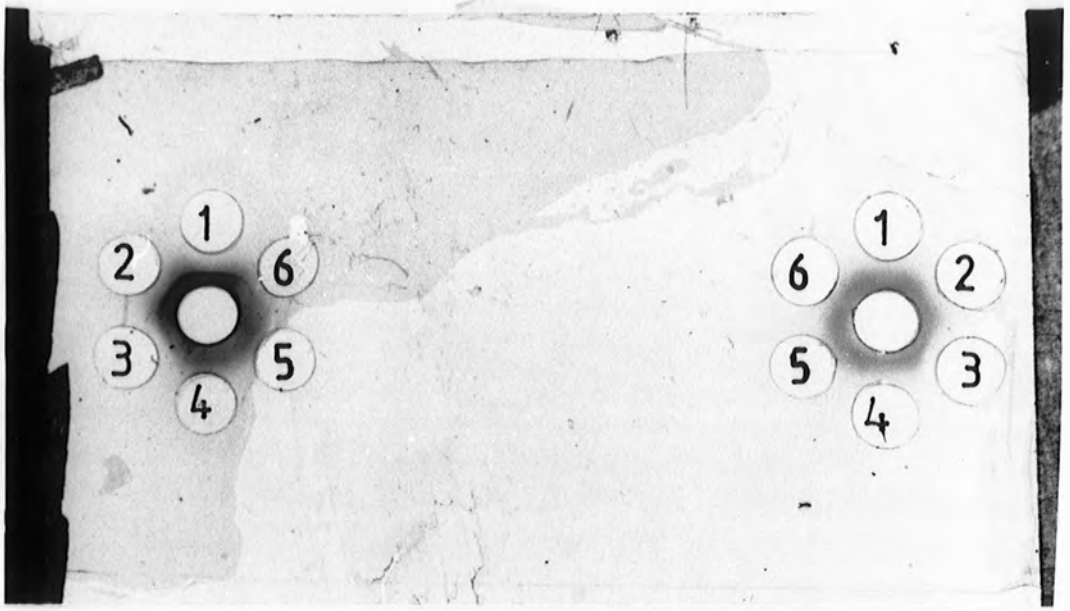

Protein Con. (mg/ml) (○—○)
1.0
2.0
3.0
4.0
0
4
8
12
16
20
24
Fraction No.
0
0.1
0.2
0.3
0.4
0.5
NaCl M (----)
0
3.0
6.0
9.0
12
×10⁻³
CPY Act. (n mol/min/mg protein)(○—○)

Fig 3.2.1.3: Gel filtration on Bio-Gel P-200 of the active enzyme fractions pooled from the DEAE-Sephadex column (Fig 3.2.1.2).

A



B

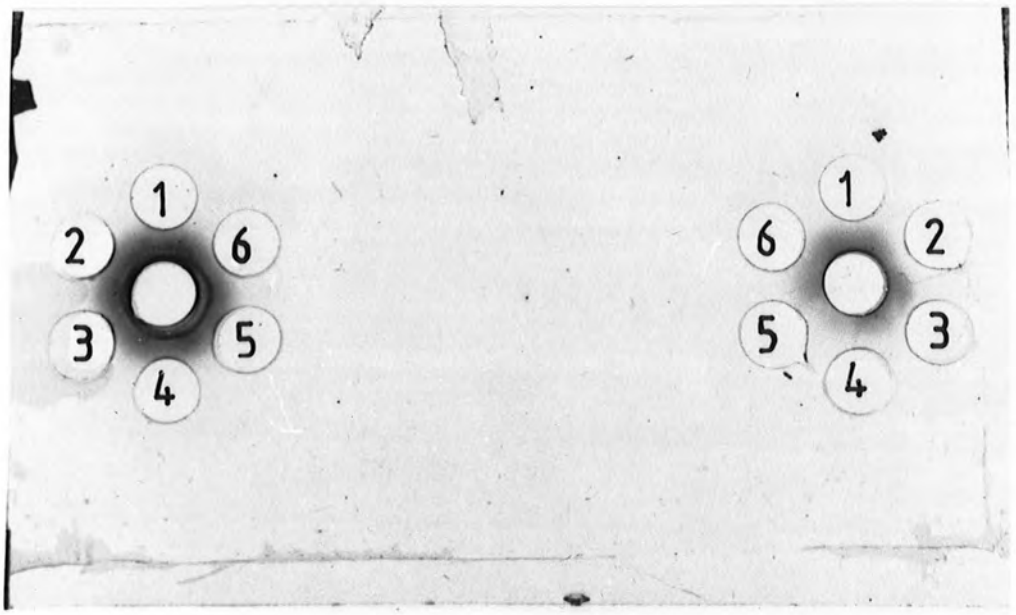
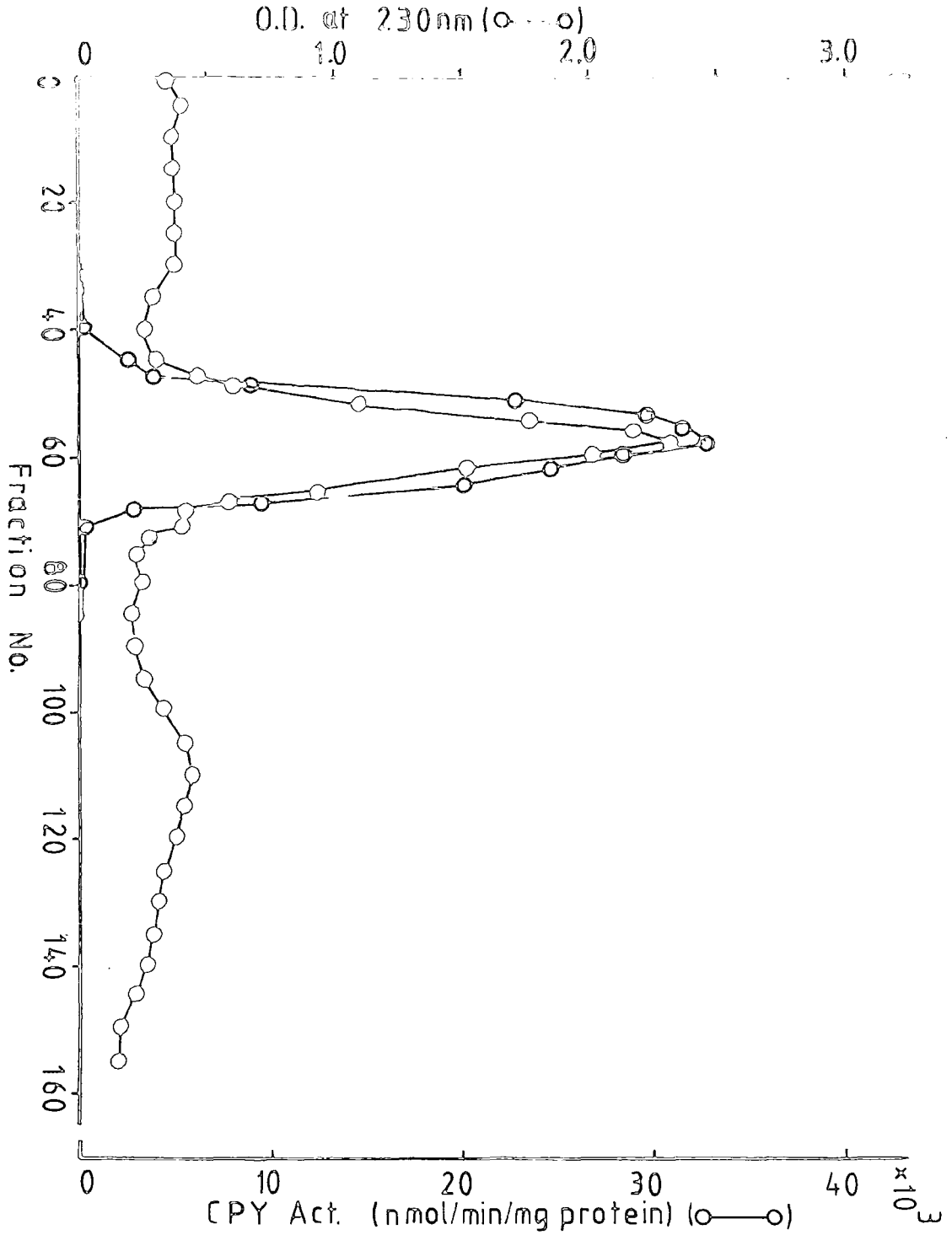


Fig 3.2.3.1,A,B: Ouchterlony immunodiffusion of purified CPY and its specific antisera. Dilution of antigen was started from well no.2 (1/2) to well no.6 (1/32), well no.1 undiluted antigen. (A, first bleeding; B, last bleeding; I, antiserum I; II, antiserum II).


O.D. at 230nm (o - - o)
0
1.0
2.0
3.0
Fraction No.
20
40
60
80
100
120
140
160
0
10
20
30
40
×10³
CPY Act. (nmol/min/mg protein) (o——o)

silver staining. Only one band representing active mature CPY of 61kD molecular weight was visible (Fig 3.2.1.4, lane 1).

3.2.2 Preparation of Anti-CPY Antibody

In order to study the *in vitro* reconstitution of transport of CPY from microsomes to vacuoles we needed to have a specific anti-CPY antibody to determine the shift of molecular weight of proCPY from 69kD (CPY precursor) to mature CPY of 61kD. Therefore, CPY was purified as detailed above and used to raise antibodies. Two rabbits were injected with the purified CPY several times as described in section 2.8. After bleeding of the two rabbits, crude anti-CPY antisera was collected and its specificity against antigen (CPY) was followed as described below.

3.2.3 Immuodiffusion and Precipitation

The specificity of the antisera collected from both rabbits (I and II) after the first and last bleeding was tested against several dilutions of antigen (CPY) using Ouchterlony plates. 50μl of each dilution was placed in the outer wells and then 50μl of antiserum in the centre well (Section 2.7.2). The results presented in fig 3.2.3.1,A and B, indicate that antiserum I was highly specific to CPY due to the appearance of a clear immunoprecipitated band around the central well. Only a faint precipitated band was visible in the case of antiserum II.

The IgG fraction was purified from crude antiserum I by ammonium sulphate precipitation at a concentration of 50% saturation (Section 2.8.1). Its specificity against antigen was also determined as described above and the results are presented

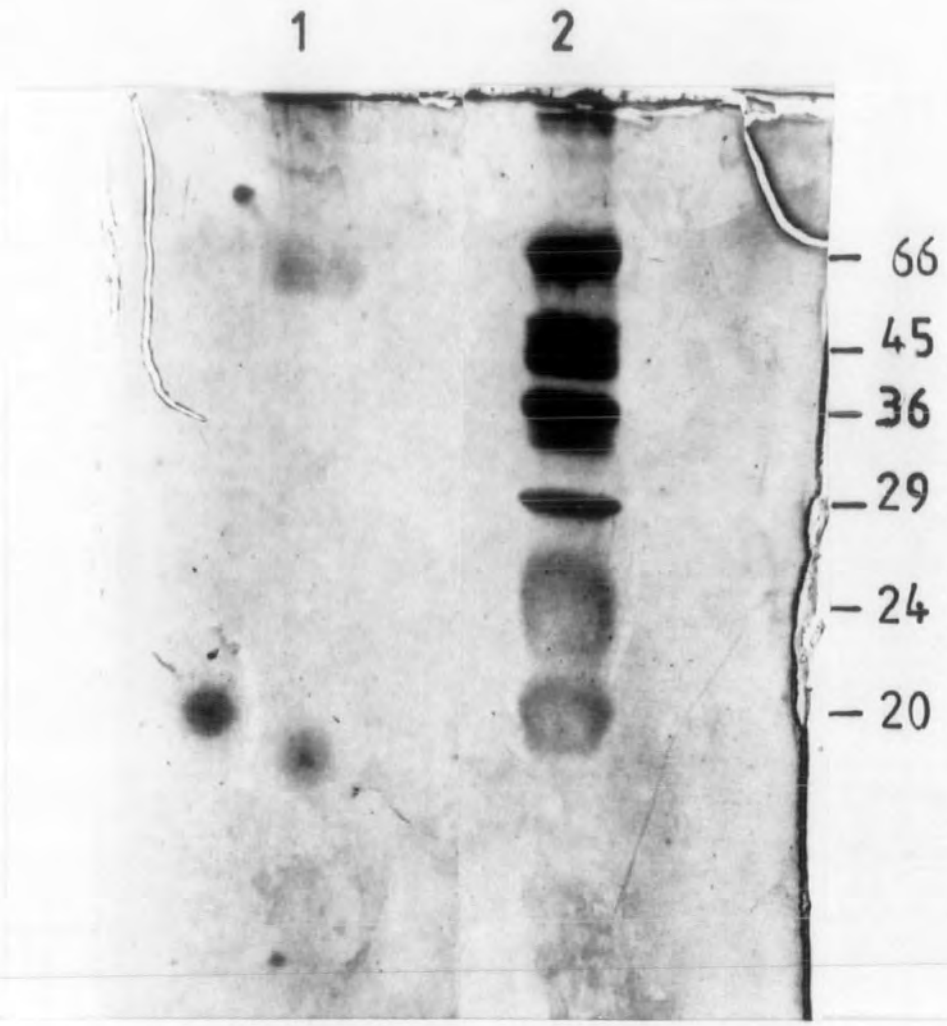


Fig 3.2.1.4: SDS-PAGE electrophoresis and silver staining of purified mature CPY (Lane 1, purified CPY; Lane 2, standard marker protein).

in fig 3.2.3.2. It is clear that the specificity of the antiserum to CPY has increased by comparing the immunoprecipitated band with that obtained with crude antiserum.

3.2.4 ELISAS of Crude Antisera

To improve the titration of the crude antisera (I and II) an immunosorbent assay (ELISA) was adapted. The antisera were used to set up a direct double antibody microtitration plate enzyme-linked immunosorbent assay for the detection of CPY specific antibody. CPY was immobilized onto the microtitration plate. An aliquot of rabbit antisera was added. The amount of the primary antibody bound to the solid phase was detected by the addition of goat anti-rabbit IgG horse radish peroxidase conjugate which was subsequently reacted with 2,2-azinobis (3-ethylbenzthizoline-6-sulphonic acid, ABTS) to give a coloured product. The colour which formed was directly proportional to the level of the specific antibodies present. Results presented in fig 3.2.4 indicate that both antisera are of low concentration but both exhibit high specificity against CPY.

3.2.5 Analysis with Western Blots

The specificity of the antiserum I to antigen was also tested with western blots. 3μg of purified CPY, 3μg of Sigma CPY, and 0.35mg of crude yeast extracts prepared from wild type strains (MC16 and MD40-4C) and a mutant strain lacking CPY activity (BJ1075). The results presented in fig 3.2.5 indicate that the antiserum was not only specific for CPY, but also for other yeast components (Fig 3.2.5, lanes 1, 2, 3).

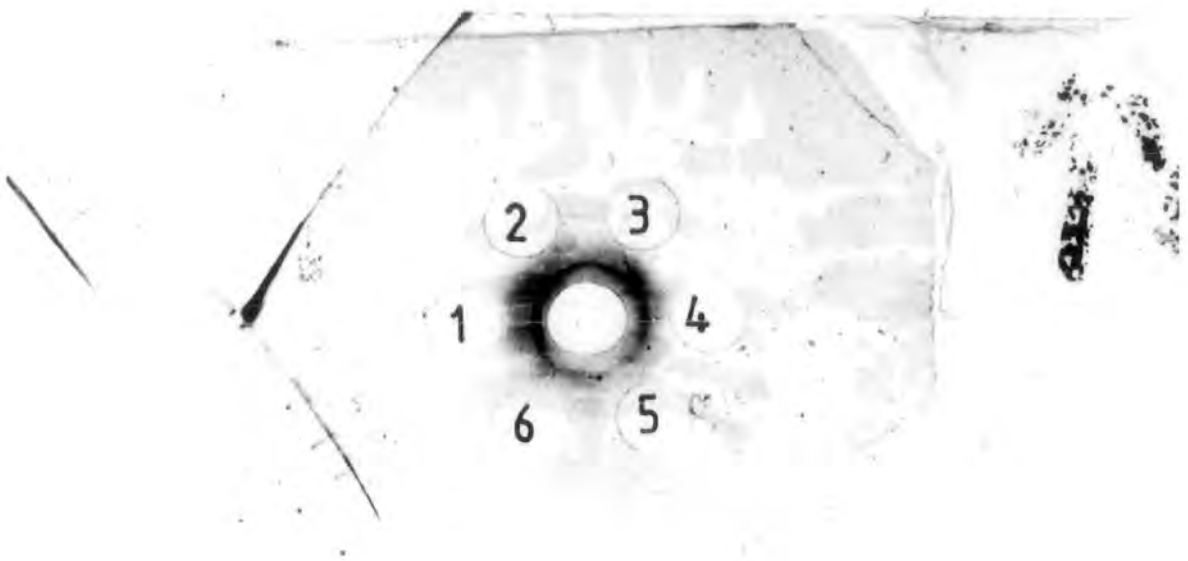
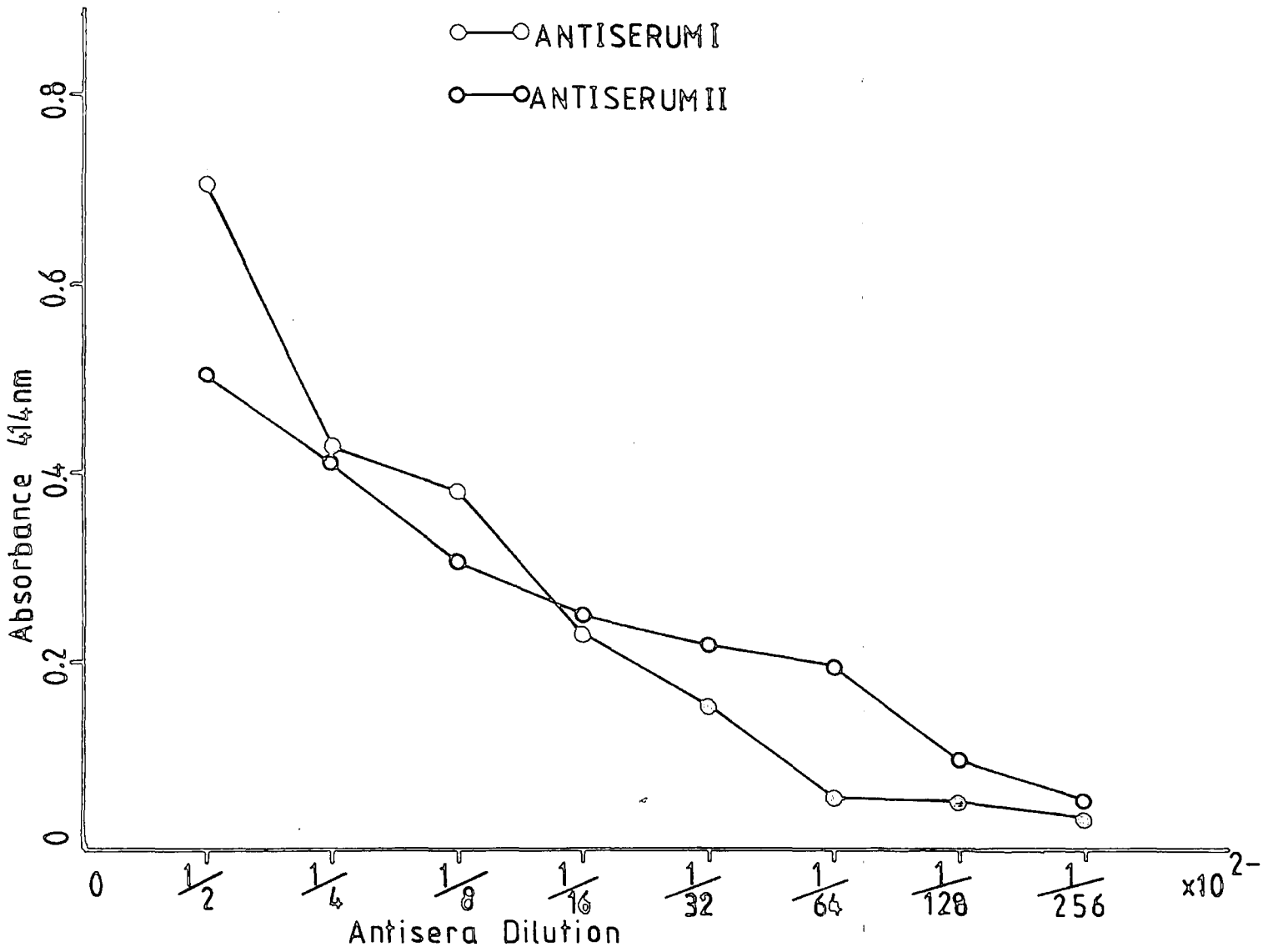


Fig 3.2.3.2: Ouchterlony immunodiffusion of purified CPY against the IgG fraction of the CPY antiserum I. Well no.1 undiluted antigen, dilution of antigen started from well no.2 to well no.6 (1/2-1/32)

Fig 3.2.4: determination of the specificity of crude CPY antisera (Antiserum I and II) against antigen (CPY) using ELISAS as described in section 2.7.3.


ANTISERUM I
ANTISERUM II
Absorbance 414nm
0
0.2
0.4
0.6
0.8
0
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
x10 2-
Antisera Dilution

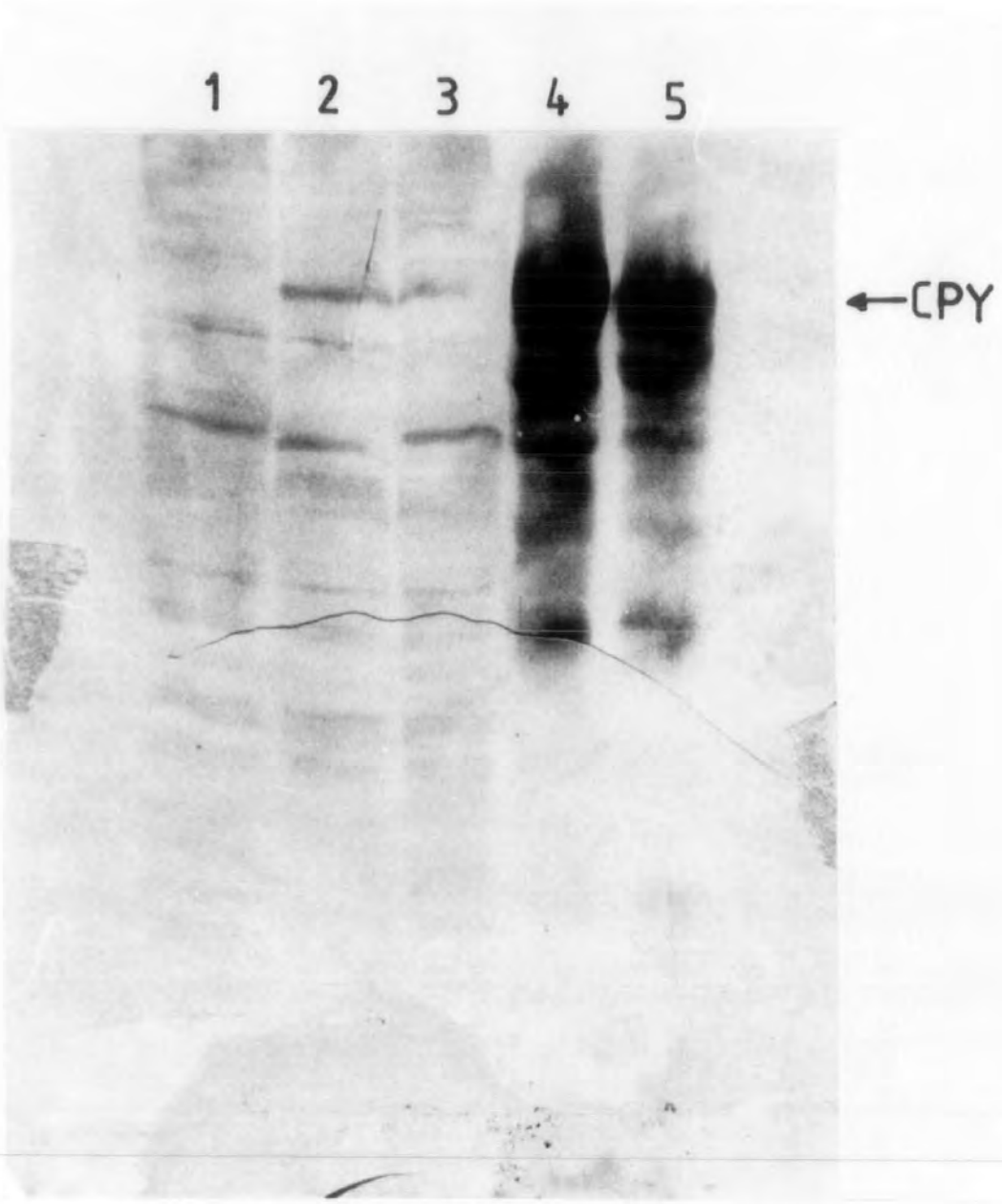


fig 3.2.5: Western blots of CPY using anti-CPY antibody (IgG fraction of antiserum I). Lane 1, BJ1075 lacking CPY activity; Lane 2 and 3, wild type strains (MC16 and MD40-4C); Lane 4, purified CPY; Lane 5, Sigma CPY.

3.2.6 Genetic Crosses

Genetic crosses were used to construct *sec, pep4-3* mutant strains to be used as a source of donor microsomes containing proCPY for *in vitro* transport studies.

However, to produce *sec, pep4-3* mutant strains, the following *sec* strains of a mating type (*sec1-1, sec7-1, sec14-3, sec18-1 and sec53-6*) were mated separately with MC16. This is to produce *sec* strains of α mating type. The diploid cells were separated from haploid cells, transferred to presporulation medium before being transferred to sporulation slant medium. The ascospores were separated from non sporulated diploids, and then germinated to haploid cells, all as described in section 2.9. The *sec* strains were differentiated from wild type by their temperature sensitivity to growth at 37°C. The *sec* strains were then tested for their mating type and their requirement for amino acids. Only the following strains were retained.

1- DUBY1 *sec53-6 his, ade, leu,* α

2- DUBY2 *sec53-6 his, leu,* α

3- DUBY3 *sec53-6 his, ade, leu,* a

4- DUBY4 *sec53-6 his, ade,* a

5- DUBY5 *sec7-1 his, leu,* ~~alpha~~ α

6- DUBY6 *sec7-1 his, leu, ade,* α

7- DUBY7 *sec7-1 leu, ade,* α

8- DUBY8 *sec7-1 his, ade,* α

9- DUBY9 *sec7-1 his, leu,* a

10- DUBY10 *sec7-1 ade, leu, his,* a

11- DUBY11 *sec7-1 ade, leu,* a

12- DUBY12 *sec7-1 his, ade,* a

13- DUBY13 *sec1-1 leu, his,* α

14- DUBY14 *sec1-1 his, leu,* a

15- DUBY15 *sec1-1 his, ade, leu,* α

16- DUBY16 *sec1-1 his, leu, ade,* a

17- DUBY17 *sec18-1 his, leu, ade,* α

18- DUBY18 *sec18-1 his, leu, ade,* a

19- DUBY19 *sec18-1 leu, his,* α

20- DUBY20 *sec18-1 leu, his,* a

21- DUBY21 *sec14-3 leu, his, ade,* α

22- DUBY22 *sec14-3 leu, his, ade,* a

23- DUBY23 *sec14-3 his, leu,* a

24- DUBY24 *sec14-3 his, ade,* α

25- DUBY25 *sec14-3 his, ade,* a.

The following *sec* strains of α mating type, (DUBY2, DUBY8, DUBY15, DUBY17 and DUBY24) were now mated separately with MT302-1C (a *his11-3, arg5-6, leu2.12, his3-5, pep4-3*), to introduce the *pep4-3* allele. Mating and sporulation were carried as described in section 2.9. The *sec* strains were first differentiated from wild type strain as described above, then classified according to their mating types and requirement for amino acids. The *sec* mutant strains were then screened for the inheritance of the *pep4-3* allele by determining the activity of CPY and comparing the activities obtained to those of a wild type (MC16) and a mutant lacking CPY activity (MT302-1C). The following new strains were retained and their respective CPY activities presented in table 3.2.6.1.

1- DUBY26 *sec53-6 leu, his, arg,* α

2- DUBY27 *sec53-6, pep4-3 leu, his, ade,* a

3- DUBY28 *sec53-6, pep4-3 leu, his, ade,* α

4- DUBY29 *sec7-1, pep4-3 leu, his, ade,* a

5- DUBY30 *sec7-1, pep4-3 leu, his, ade,* α

6- DUBY31 *sec14-3, pep4-3 his, arg, ade,* α

7- DUBY32 *sec14-3 leu, his, arg, ade,* α

8- DUBY33 sec14-3, pep4-3 leu, his, ade, a

9- DUBY34 sec14-3, pep4-3 leu, his, arg, ade, a

10- DUBY35 sec14-3, pep4-3 leu, his, arg, a

11- DUBY36 sec1-1 leu, arg, α

12- DUBY37 sec18-1, pep4-3 leu, his, arg, ade, α

13- DUBY38 sec18-1 leu, his, arg, ade, a.

In order to be certain that the low CPY activity exhibited by the new strains is due to the *pep4-3* mutation, they were analysed with western blots against anti-CPY antibody and compared to that of known wild type (MC16) and *pep4-3* mutant (MT302-1C) strains. The results presented in fig 3.2.6.2,A and B indicate that strains showing low CPY activity, accumulated CPY of higher molecular weight than found in the wild type strain and similar to that found in the known *pep4-3* strain (MT302-1C).

Interestingly two strains, DUBY36 and DUBY38, which had intermediate levels of CPY activity accumulated only the wild type form of CPY.

3.3 Discussion

In the autolysate of bakers yeast, carboxypeptidase Y is in an inactive form (Doi, *et al.,* 1967; Hata, *et al.,* 1967a; Wolf and Fink, 1975; Hayashi, 1976). The molecular weight of which is approximately 20000 higher than the active enzyme and is due to the formation of a CPY-inhibitor complex (Hayashi *et al.,* 1968). Activation is brought by the action of copurified yeast proteinase A in an acid environment

Table 3.2.3.1: CPY specific ativities of *sec* and *sec, pep4-3* mutant strains.

Strains	CPY specific activity (nmol/min/mg protein)
MC16	44.50
MT302-1C	7.45
DUBY26	43.40
DUBY27	6.37
DUBY28	6.80
DUBY29	7.84
DUBY30	7.43
DUBY31	7.12
DUBY32	47.43
DUBY33	8.56
DUBY34	9.10
DUBY35	6.78
DUBY36	15.46
DUBY37	8.34
DUBY38	18.23

(Hayashi, *et al.,* 1972; Wolf and Fink, 1975) due to the digestion of CPY inhibitor after partial purification of the crude extract by ammonium sulphate fractionation. During the purification of the crude CPY by DEAE-Cellulose chromatography one broad peak of active CPY was achieved (Fig 3.2.1.1). This step of purification is very effective because the CPY elutes later than the major protein peak. After chromatography on DEAE-Sephdex A-50, the partially purified CPY is resolved into two peaks of CPY activity (Fig 3.2.1.2). Similar results have been previously reported (Kuhn, *et al.,* 1974; Hashimoto, *et al.,* 1981). The carbohydrate content of the first peak was 14.4% and 14.7% for the second peak (Hashimoto, *et al.,* 1981) and they named the first peak CPY-I and the second peak CPY-II. Moreover, they also explained that the appearance of two peaks was due to forms of CPY differing in phosphate content. The molecular ratio of mannose/phosphate is 12.5 in CPY-I and 10.8 in CPY-II. Since carboxypeptidase Y contains 50 mannose units per molecule (Hasilik and Tanner, 1978b), this means that there may be four phosphate residues in CPY-I and five in CPY-II. Chromatography of the pooled CPY-I and CPY-II peaks on Bio-gel P-200 yields only one peak of protein coincident with the peak of CPY activity (Fig 3.2.1.3). Similar results were found by Kuhn,*et al.,* (1974). The degree of purification of CPY was about 420 fold and the yield was 33.1% mg of CPY.

The homogeneity of the enzyme was tested on SDS-PAGE electrophoresis and the protein bands observed by silver staining. The results show only one band of protein of 61kD apparent molecular weight, identical to the published figure for mature CPY (Fig 3.2.1.4).

Results show that antiserum I posseses high specificity towards CPY, whereas the specificity of antiserum II against CPY is low (Fig 3.2.3.1,A,B). The specific activity of antiserum I was increased when the IgG fraction was purified from crude antiserum (Fig 3.2.3.2). However, when ELISAS were used for the titration of the

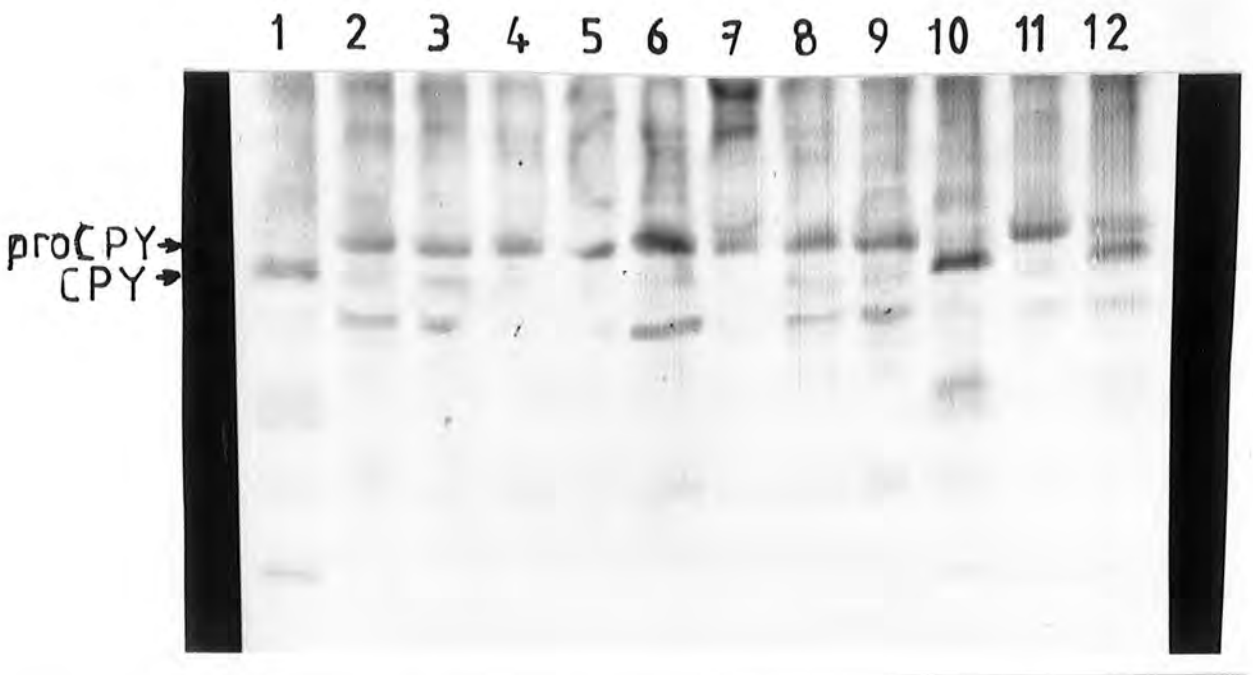


Fig 3.2.6.2,A: Western blots of crude cell extracts using anti-CPY antibody. (Lane 1, MC16; Lane 2, MT302; Lane 3, DUBY28; Lane 4, DUBY29; Lane 5, DUBY30; Lane 6, DUBY31; Lane 7, DUBY33; Lane 8, DUBY34; Lane 9, DUBY35; Lane 10, DUBY36; Lane 11, DUBY37; Lane 12, DUBY38). Wild type (MC16) and *sec* strains accumulate mature CPY. A mutant lacking CPY activity (MT302-1C) and *sec, pep4-3* strains accumulate CPY precursor.

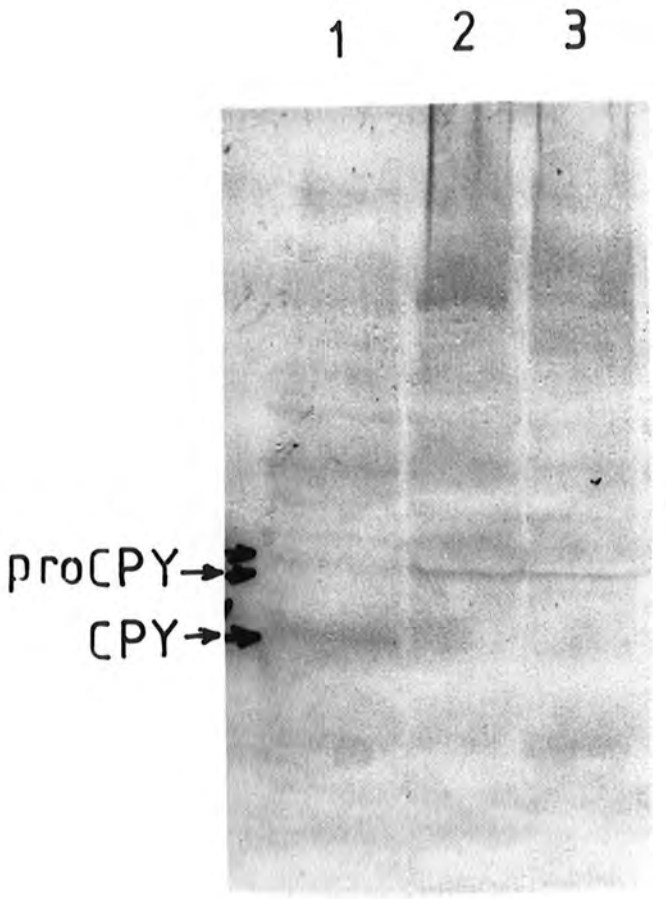


Fig 3.2.6.2,B: Western blots of crude cell extracts against anti-CPY antibody. (Lane 1, MC16; Lane 2, MT302-1C; Lane 3, DUBY27).

antisera both were highly specific to CPY but of low concentration (Fig 3.2.4).

Analysis with western blots of yeast crude extracts probed with the CPY antibody revealed that the anti-CPY antiserum not only reacted with CPY but also cross-reacted with other yeast components (Fig 3.2.5). This cross-reactivity became a major problem in later experiments (See chapter 4). Carboxypeptidase Y is a mannoprotein, it is probable that the polyclonal antibody reacts with both the protein and carbohydrate domains of CPY. Therefore, anti-CPY antibody cross-reacts with any mannoprotein containing carbohydrate similar to that of CPY, such as vacuolar proteinases, invertase and yeast cell wall mannoproteins. Similar results have been previously described (Hasilik and Tanner, 1976b).

CHAPTER FOUR

4. IN VITRO PROTEIN TRANSPORT

4.1 Introduction

A property of all eukaryotic cells is their ability to direct the efficient localization of defined subsets protein from their cytoplasmic site of synthesis to their various noncytoplasmic destinations. A major portion of protein translocation in eukaryotes is routed through the endoplasmic reticulum (ER). The majority of proteins translocated into the ER are glycosylated and then directed to the Golgi apparatus, where further glycosylation occurs. In the Golgi apparatus a sub-set of proteins is then sorted for secretion from the cell. Another sub-set is sorted for transport to the lysosome or vacuole. The transport of protein between these compartments has been extensively described *in vivo* (Pfeffer and Rothman, 1987).

Various parts of this pathway have been reconstituted *in vitro*. Microsomes isolated from canine pancreas have been widely used in experiments showing cotranslational translocation of protein into the lumen of the ER, (Blobel and Dobberstein, 1975). It has been reported that translocation into canine pancreatic microsomes can also occur posttranslationally for a truncated form of the human glucose transporter (Muckler and Lodish, 1986a,b) and for a fusion protein of lactamase and β-globin (Perara, *et al.*, 1986).

In vitro, the reconstituton of intercompartmental transport between the cisternae of the Golgi stack has also been achieved, (Pfeffer and Rothman, 1987). The transport of the vesicular stomatitis virus (VSV)-encoded G protein from the *cis* compartment of a mutant Chinese Hamster ovary (CHO) Golgi stack (donor) to the

medial compartment of a wild type CHO Golgi stack (recipient) was detected, (Fries and Rothman, 1980; Balch, *et al.*, 1984). The movement was measured biochemically by the addition of GlcNAc in a complementation assay during glycosylation that occurs when G protein is transported from the *cis* donor compartment to the *medial* recipient. The mutant donor Golgi are defective in GlcNAc transferase I. Transport was absolutely dependent upon the presence of a crude cytosolic fraction, ATP and the protein from the surface of Golgi membranes, (Fries and Rothman, 1980; Balch, *et al.*, 1984; Balch and Rothman, 1985).

In vitro translocation of the yeast secretory protein prepro-α-factor into the lumen of the ER can occur posttranslationally (Hansen, *et al.*, 1986; Waters and Blobel, 1986). This mechanism was shown to require membrane proteins in the yeast microsomal vesicles (Hansen, *et al.*, 1986), an energy source supplied as ATP (Hansen, *et al.*, 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986) and stimulated by soluble protein factors (Waters, *et al.*, 1986; Baker, *et al.*, 1988; Ruohola, *et al.*, 1988).

Preinvertase synthesised in a yeast lysate was also successfully translocated and glycosylated by yeast microsomes,(Rothblatt and Meyer, 1986a). Recently , Hansen and Walter, (1988) reported that both prepro-CPY and a truncated form of pre-invertase, but not full length of pre-invertase, can be translocated *in vitro* posttranslationally into the ER vesicles.

From the ER to the Golgi stack, Haselbeck and Schekman, (1986) described the *in vitro* translocation of core glycosylated invertase. However, Baker *et al.*, (1988) have developed a promising powerful new method for preparing yeast ghosts for studing intercompartmental transport. They used freeze-thaw lysis of sphaeroplasts

that causes gentle breakage of the plasma membrane, this releases cytosolic proteins but leaves the organelles in an intact form. Utilising such ghosts they showed transport of prepro-α-factor from the cytosol through the ER to Golgi vesicles. The reaction was temperature dependent, required ATP and stimulated 6 fold by the addition of a cytosolic fraction.

A number of yeast proteins are sorted and transported through the secretory pathway to the lysosomal-like vacuole. Certain *sec* mutants accumulate the precursors of these proteins in either ER or Golgi vesicles at the non-permissive temperature (37°C). These precursors are sorted again upon returning to the permissive temperature (24°C).

In this chapter a strategy for detecting protein transport *in vitro* from Golgi vesicles to vacuoles is described. By use of the *sec14-3* or *sec7-1* mutations proCPY is accumulated in Golgi vesicles and purified as a part of the microsomal fraction. The strains used as a source of microsomes have the *pep4-3* mutation and are unable to convert the accumulated proCPY to active CPY. The microsomes are mixed with purified vacuoles from a CPY deficient strain that is PEP4 and capable of processing proCPY to mature CPY.

4.2 Results

4.2.1 Analysis of Transport with Western Blots

The best assay to detect *in vitro* reconstitution of transport of CPY from donor Golgi vesicles to recipient vacuoles is to observe the size-change upon maturation of

proCPY using anti-CPY antibody and western blots. Purified microsomes (section 2.10.2) from strain DUBY35 (*sec14-3, pep4-3*) grown with 2% glucose were mixed with purified vacuoles (Section 2.10.1, method one) from strain BJ1075 (*prc1-229, PEP4*). The reaction mixture was supplemented with an energy source (section 2.4.11) and the incubations carried out at both 24°C and 37°C for one hour. The reaction mixtures were then subjected to SDS-PAGE (Section 2.5) followed by western blotting (Section 2.6) using rabbit anti-CPY antibody (Section 2.8). Comparing the results obtained from the reaction mixtures containing both donor Golgi vesicles and recipient vacuoles to the reaction mixture with either vacuoles or microsomes alone showed no detectable difference. This experiment was repeated several times with minor modifications without a positive result. The anti-CPY antibody cross-reacted with many proteins on the western blot, including vacuoles from strain BJ1075 alone. This strain is devoid of anti-CPY antigen (Hemmings *et al.*, 1981). Therefore, I suggested that transport could be determined biochemically as only mature CPY is enzymatically active.

4.2.2 Measurement of In Vitro Transport by Enzyme Assay

The reaction mixtures for these experiments were as just described (Section 4.2.1). Initially, the activity of the maturated CPY was determined utilising BTPNA as a substrate (Section 2.4.6) as it is specific for CPY, however, no detectable CPY activity was found. As a last alternative the activity of CPY was determined using the dipeptide Cbz-Phe-Leu as a substrate (Section 2.4.4). After preparation of the reaction mixtures, aliquots were taken and analysed for CPY activity at the time intervals shown in fig 4.2.2. The reactions (50μl) were stopped by diluting into the CPY assay reaction mixture (1.1ml). This dilution mixture causes lysis of vacuoles due to osmotic shock and allows access of the Cbz-Phe-Leu substrate to the vacuolar CPY.

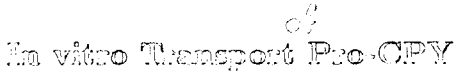
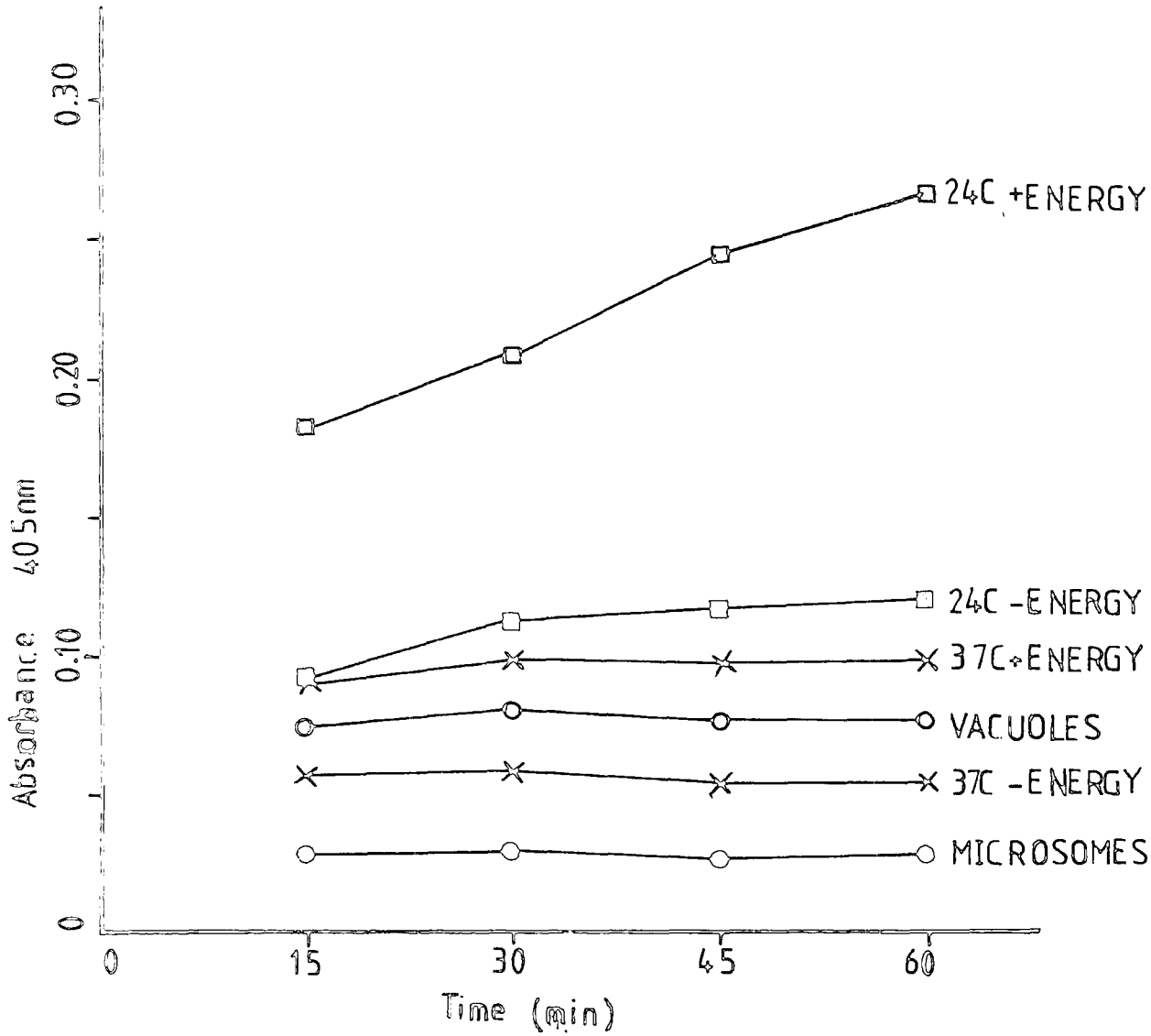


Fig 4.2.2: Temperature sensitivity and energy dependence of *in vitro* transport of CPY from DUBY35 Golgi vesicles (Donor) to purified BJ1075 vacuoles (Recipient). The prepared reaction mixtures (Section 2.4.11) were incubated for 15, 30, 45, and 60mins at 24°C or 37°C.


Absorbance 405nm
0
0.10
0.20
0.30
0
15
30
45
60
Time (min)
24C +ENERGY
24C -ENERGY
37C+ENERGY
VACUOLES
37C -ENERGY
MICROSOMES

Results presented in fig 4.2.2 show transport of CPY was achieved when the reaction mixture was incubated at 24°C. Reaction mixture incubated at 37°C produced values more barely different from the microsomes or vacuoles alone, thus reflecting the *in vivo* temperature sensitivity of the donor microsomes.

4.2.3 The Energy Requirement of Transport

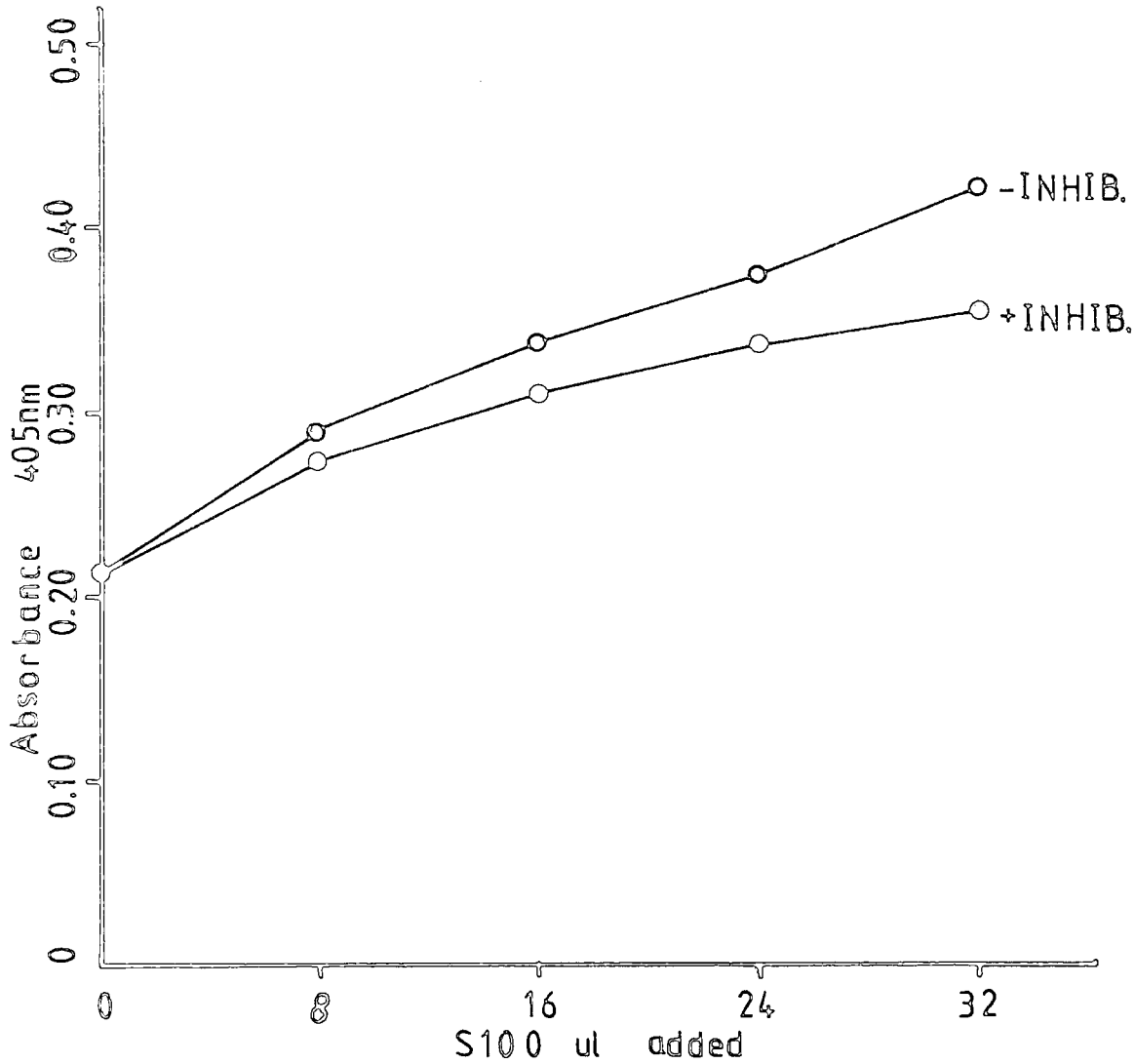
As outlined in the introduction (Section 4.1) transport of proteins between many compartments of the secretory pathway requires energy. To investigate the effect of energy upon the transport of proCPY from Golgi vesicles to the vacuoles the above experiment was repeated (Section 4.2.2) leaving out the energy regeneration system of ATP, creatine phosphate and creatine kinase and replacing it with buffer A. The results shown in fig 4.2.2 indicate that the lack of a suitable energy source reduces the rate of transport at 24°C, by at least two fold, down to the background level displayed by the vacuoles alone.

4.2.4 The Effect of Soluble Factors on Transport

A further series of experiments were performed to see whether the transport of CPY could be stimulated by the addition of a crude cytoplasmic fraction. The source of the soluble proteins was an S100 fraction purified from the donor strain (Section 2.10.3). The S100 protein concentration was determined (28.65mg/ml) and also assayed for any CPY-like activity. No detectable CPY-like activity was found (less than 0.025nmole/min/mg protein). Results plotted in fig 4.2.4 show that increasing amounts of the S100 stimulate CPY transport by up to 2 fold compared to the reaction mixture supplemented with the energy source alone.

The Effect of Soluble Factors on Transport

Fig 4.2.4: The stimulation of transport by soluble factors. Increasing amounts of the S100 fraction in buffer A were added to the standard reaction mixtures (Fig 4.2.2). The mixtures were incubated for 1hr at 24°C. The reaction was stopped by diluting 50μl into the CPY assay mixture (Section 4.2.2). The total reaction volume for all assays was 75μl.


Absorbance 405nm
0
0.10
0.20
0.30
0.40
0.50
0
8
16
24
32
S100 ul added
-INHIB.
+INHIB.

4.2.5 The Effect of Proteinase Inhibitors on Transport

Direct mixing of proCPY with proteinase A and proteinase B in a membrane free system results in its conversion to active CPY (Mechler, *et al.*, 1987). To test that our observations are truly due to translocation and not just mixing of free donor proCPY and the proteinases A and B, present in the recipient fraction, the above experiments in section 4.2.4 were repeated in the presence of pepstatin and chymostatin which are specific peptide inhibitors of proteinase A and proteinase B respectively. The concentrations of 1mM and 4μg/ml respectively were used. The addition of these inhibitors should block maturation of CPY if the observations are due to direct mixing. The results presented in fig 4.2.4 indicate that the addition of inhibitors have a negligible effect on the production of CPY.

4.2.6 The Effect of Detergents upon the Effect of Proteinase Inhibitors

The results described in the previous section 4.2.5, could be explained by one of two mechanisms. Firstly, proCPY and proteinase A and B are in separate membrane bound vesicles and do not have direct contact until after transport, or secondly, there is direct mixing but the inhibitors are inactive. To distinguish between these two possibilities, the transport system (microsomes and vacuoles) was deliberately disrupted with a detergent (TritonX-100) and the experiment repeated using just the final reaction (32μl S100) described in fig 4.2.4. The results of this experiment are presented in table 4.2.6. As can been seen the forced mixing of the contents of the Golgi vesicles and the vacuoles by the addition of TritonX-100 greatly stimulates the production of active CPY. When the inhibitors were added to this disrupted system,

Table 4.2.6: The effect of detergents upon the action of proteinase inhibitors. The reaction mixtures were as described in the legend to fig 4.2.4. The standard mixture without S100 contained Golgi fraction, vacuoles, energy and 32μl of buffer A. The reaction mixture with S100 was identical to the final point in fig 4.2.4, with 32μl of S100 instead of buffer A. The concentration of inhibitors was 1mM pepstatin and 5μg/ml chymostatin. The final concentration of TritonX-100 was 1%.

Conditions	CPY absorbance 405nm	% inhibition by protease inhibitors (absolute values CPY absorbance in brackets)
Standard mixture	0.237	
Standard mixture plus S100	0.401	12.46 (0.351)
Standard mixture plus S100 plus TritonX-100	0.555	58.19 (0.232)

the production of active CPY was greatly reduced. The absolute recorded value of the disrupted, inhibited reaction (0.232) was well below that of the undisrupted complete translocation system (0.401) and even lower than the basal level recorded by simply mixing the two membrane components without the addition of the soluble fraction (0.237). Once again the addition of the inhibitors to the complete intact system gave only a small (12.4%) reduction.

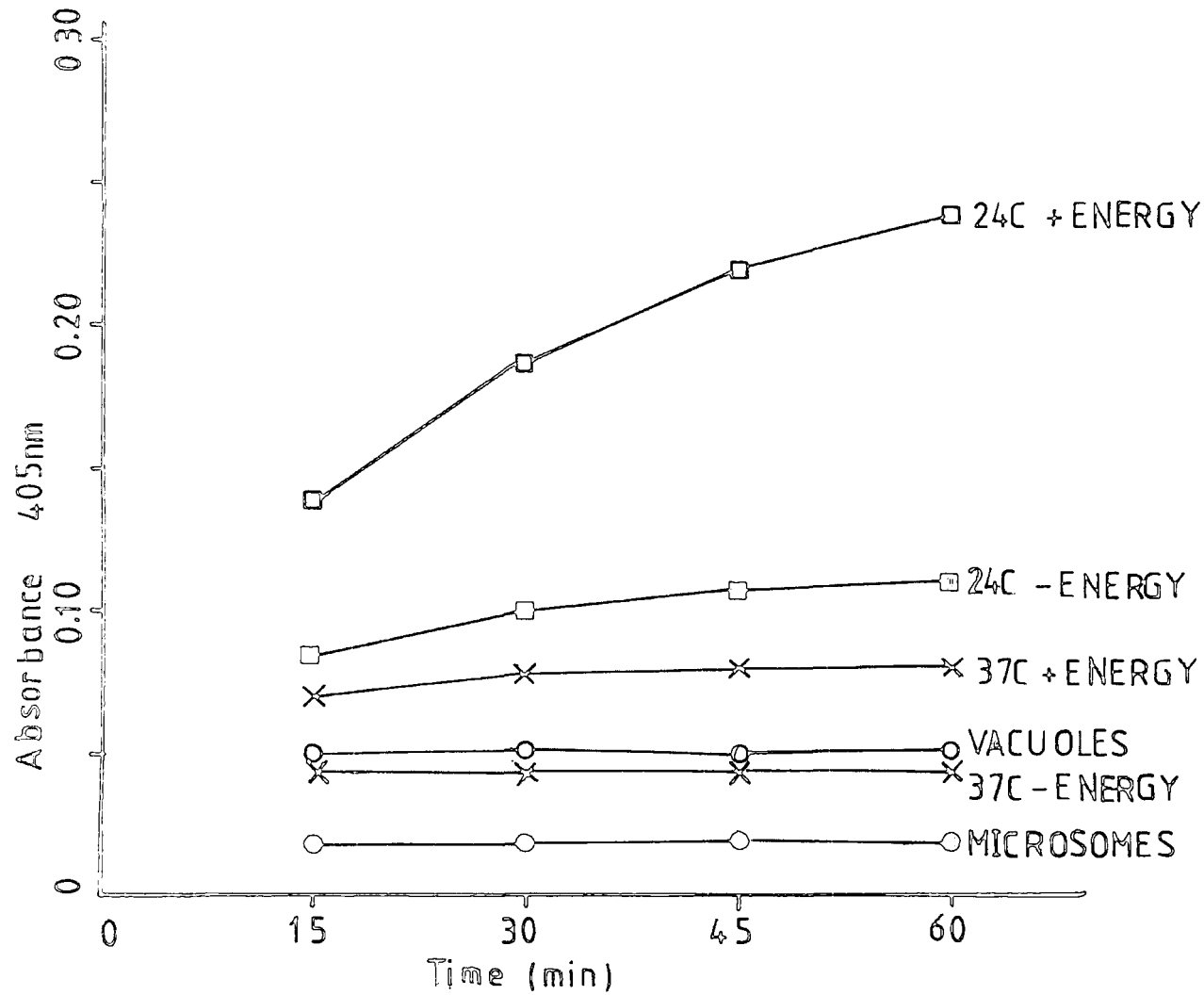
4.2.7 A Carboxypeptidase S Lacking Strain as a Source of Recipient Vacuoles

The results of the experiments described above (Section 4.2.2 and 4.2.6) indicate that translocation of proCPY occurs when the reaction mixtures are supplemented with a suitable energy source and by the addition of soluble factors. All of these experiments utilised Cbz-Phe-Leu to assay for mature CPY. Cbz-Phe-Leu not only assays for CPY but also for carboxypeptidase S, (CPS) (Wolf and Weiser, 1977). Therefore, the high background obtained in these assays, particularly with the vacuole fraction, is at least in part due to the presence of contaminating CPS. To reduce the background, the above experiments were repeated using vacuoles purified from strain BYS 232-31-42. This strain lacks both CPY and CPS. It also lacks proteinase B, but proCPY can be maturated by proteinase A alone.

The results (Fig 4.2.7.1 and 4.2.7.2) show that previous data is repeatable with a different recipient strain. The back ground level produced by the vacuoles alone is reduced by approximately 30% (See discussion, section 4.3). The stimulation produced by the addition of energy (Fig 4.2.7.1) is approximately 2.2 fold, similar to the value obtained previously (Section 4.2.2). The system also still shows temperature

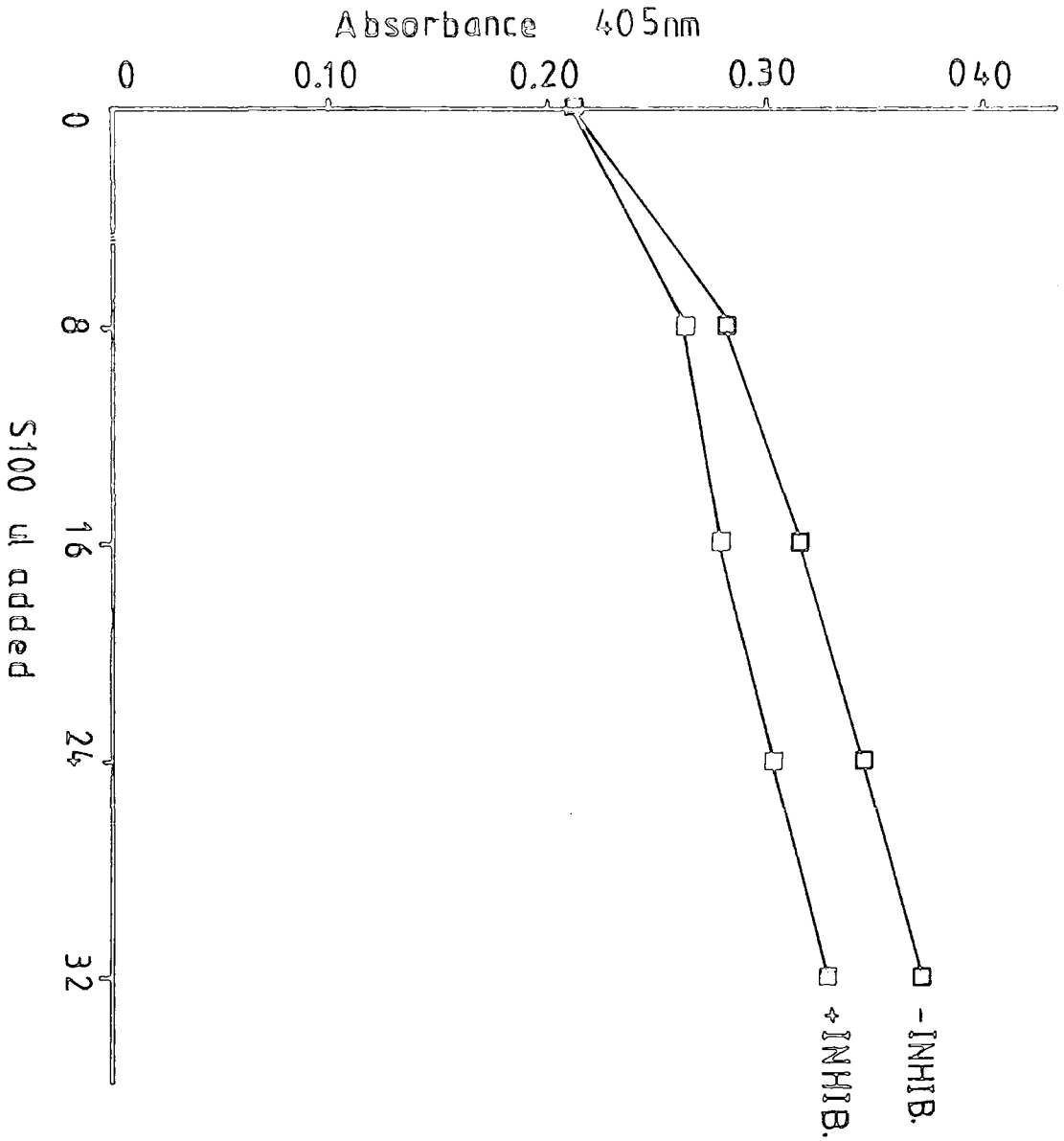
In Vitro Transport of Pro-CPY

Fig 4.2.7.1: Temperature sensitivity and energy dependence of transport. The method used was identical to that described in fig 4.2.2 except that vacuoles were purified from strain BYS 232-31-42.


Absorbance 405nm
0
0.10
0.20
0.30
0
15
30
45
60
Time (min)
24C + ENERGY
24C - ENERGY
37C + ENERGY
VACUOLES
37C - ENERGY
MICROSOMES

The Effect of Soluble Factors on Transport

Fig 4.2.7.2: The stimulation of transport by a soluble factors. 8, 16, 24, and 32μl of S100 were added to the standard reaction mixtures and the method described in fig 4.2.4 was followed except that the vacuoles were purified from BYS 232-31-42.


Absorbance 405nm
0
0.10
0.20
0.30
040
0
8
16
24
32
S100 µl added
- INHIB.
+ INHIB.

sensitivity and is stimulated by the addition of an S100 fraction (Fig 4.2.7.2).

The experiments with proteinase inhibitors and TritonX-100, again show that the the maturation of CPY occurs in membrane bound vesicles which confer resistance to proteinase inhibitors (Fig 4.2.7.2 and table 4.2.7).

4.2.8 Transport with Sec7 Donor Vesicles

All of the previous experiments have utilized a *sec14-3* strain grown with 2% glucose as a source of donor vesicles. As well as *sec14-3*, it has been shown that *sec7-1* strains accumulate proCPY in the Golgi apparatus when grown at the restrictive temperature (Stevens, *et al.*, 1982). This strain when grown at the restrictive temperature on rich media containing 2% glucose accumulates toroidal shaped vesicles termed Berkeley bodies. When the temperature is shifted back to the permissive temperature (24°C), *sec7-1* strains do not return to the wild type phenotype. If, however, these are grown on rich media containing only 0.2% glucose, only 80-100nm vesicles accumulate and the phenotype is reversible upon return to the permissive temperature, (Novick, *et al.*, 1981; Schekman, 1982).

To determine whether the *in vitro* transport of proCPY can be demonstrated with a *sec7-1* strain and whether the transport is affected by the concentration of the glucose present in the growth media of the donor strain, the previous experiments were repeated using microsomes purified from strain DUBY29 (*sec 7-1, pep4-3*) grown with either 2% or 0.2% glucose. The source of the recipient vacuoles was BYS 232-31-42 (Section 4.2.7).

The two reaction mixtures were as described in section 4.2.2 except just the

Table 4.2.7.: The effect of detergents upon the action of proteinase inhibitors. The reaction mixtures and the conditions of the experiments are as described in the legend to table 4.2.6.

Conditions	CPY absorbance 405nm	% inhibition by protease inhibitors (absolute values CPY absorbance in brackets)
Standard mixture	0.221	
Standard mixture plus S100	0.352	10.79 (0.314)
Standard mixture plus S100 plus TritonX-100	0.467	56.95 (0.201)

final time point (1hr) was used for the incubation of the reaction mixtures. All of the incubations were carried out at 24°C. The results presented in fig 4.2.8 show that maturation of proCPY only occurred with reactions using the donor microsomal membrane fraction purified from cells grown on media containing 0.2% glucose. No detectable maturation of proCPY was found with reaction mixtures using microsomes prepared from cells grown with 2% glucose.

The *in vitro* transport of CPY is energy dependent. Transport is considered to have occurred when values greater than that with vacuoles alone were obtained. The addition of DUBY29 S100 (30.15mg/ml) to the reaction mixtures stimulates CPY transport by nearly 2 fold in the reaction mixtures with the 0.2% glucose derived microsomes (Fig 4.2.8). Again there is no detectable transport in the reaction mixtures with the 2% glucose derived microsomes. As before the addition of proteinase inhibitors had a negligible effect.

4.2.8.1 The Effect of Detergents and Poteinase Inhibitors

The reasoning and experimental design of this experiment was as described in section 4.2.6. The results of this experiment (Table 4.2.8.1) show that the addition of TritonX-100 again stimulates the production of mature CPY in a protease sensitive manner. The TritonX-100 stimulated maturation of CPY occur with 0.2% and 2% glucose derived microsomes. This shows that the lack of appearance of CPY activity in the undisrupted 2% derived reaction mixtures, is not due to a lack of accumulated proCPY.

In Vitro Transport of Pro-CPY with *sec7-1* Golgi Vesicles

Fig 4.2.8: *In vitro* transport of CPY from donor *sec7-1* vesicles to recipient vacuoles. The method used was identical to that in fig 4.2.2, except that the incubation conditions for all of the transport assays was for 1hr at 24°C. Donor microsomes were prepared from DUBY29 grown with either 0.2% or 2% glucose. Vacuoles were purified from BYS 232-31-42.

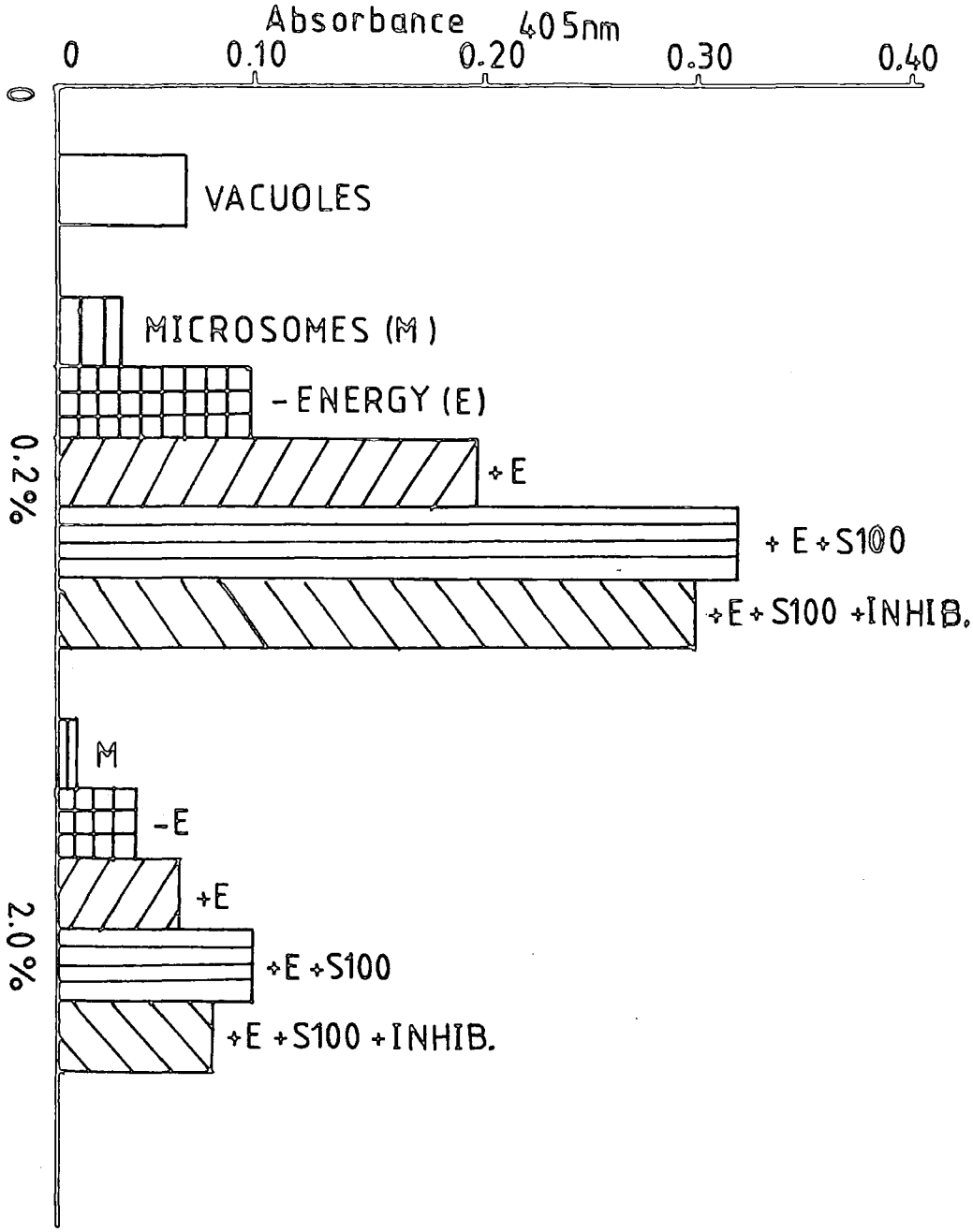

Absorbance 405nm
0
0.10
0.20
0.30
0.40
0
VACUOLES
MICROSOMES (M)
-ENERGY (E)
+E
+E+S100
+E+S100 +INHIB.
0.2%
M
-E
+E
+E+S100
+E+S100+INHIB.
2.0%

Table 4.2.8.1: The effect of detergents and proteinase inhibitors upon *in vitro* transport. Experimental method and preparation of the reaction mixtures was as described in table 4.2.6.

Conditions	CPY absorbance 405nm	% inhibition by protease inhibitors (absolute values CPY absorbance in brackets)
0.2% glucose		
Standard mixture	0.183	
Standard mixture plus S100	0.321	5.60 (0.303)
Standard mixture plus S100 plus TritonX-100	0.473	55.60 (0.210)
2.0% glucose		
Standard mixture	0.061	
Standard mixture plus S100	0.082	15.80 (0.069)
Standard mixture plus S100 plus TritonX-100	0.213	50.70 (0.105)

4.2.9 The Mature CPY Is Sedimentable

All of the results in this chapter obtained so far, indicate that maturation of proCPY is due to transport of proCPY from donor Golgi vesicles to the recipient vacuoles. Therefore, mature CPY should reside in the intact vacuoles. Control experiments show that vacuoles can be sedimented by centrifugation at 12000rpm for 5mins in a microcentrifuge. The experiments described in section 4.2.8 were repeated using microsomes from DUBY29 (*sec7-1*) grown with 0.2% glucose only. BJ1075 was used as a source of recipient vacuoles. The reaction mixtures were incubated for one hour at 24^{o}C. Next, the reaction mixtures were centrifuged, the supernatant transferred to fresh eppendorf tubes and the pellets (Vacuoles and microsomes) resuspended in an equal volume of buffer A. The presence of active CPY was tested in both the supernatant and the pellet fractions as before.

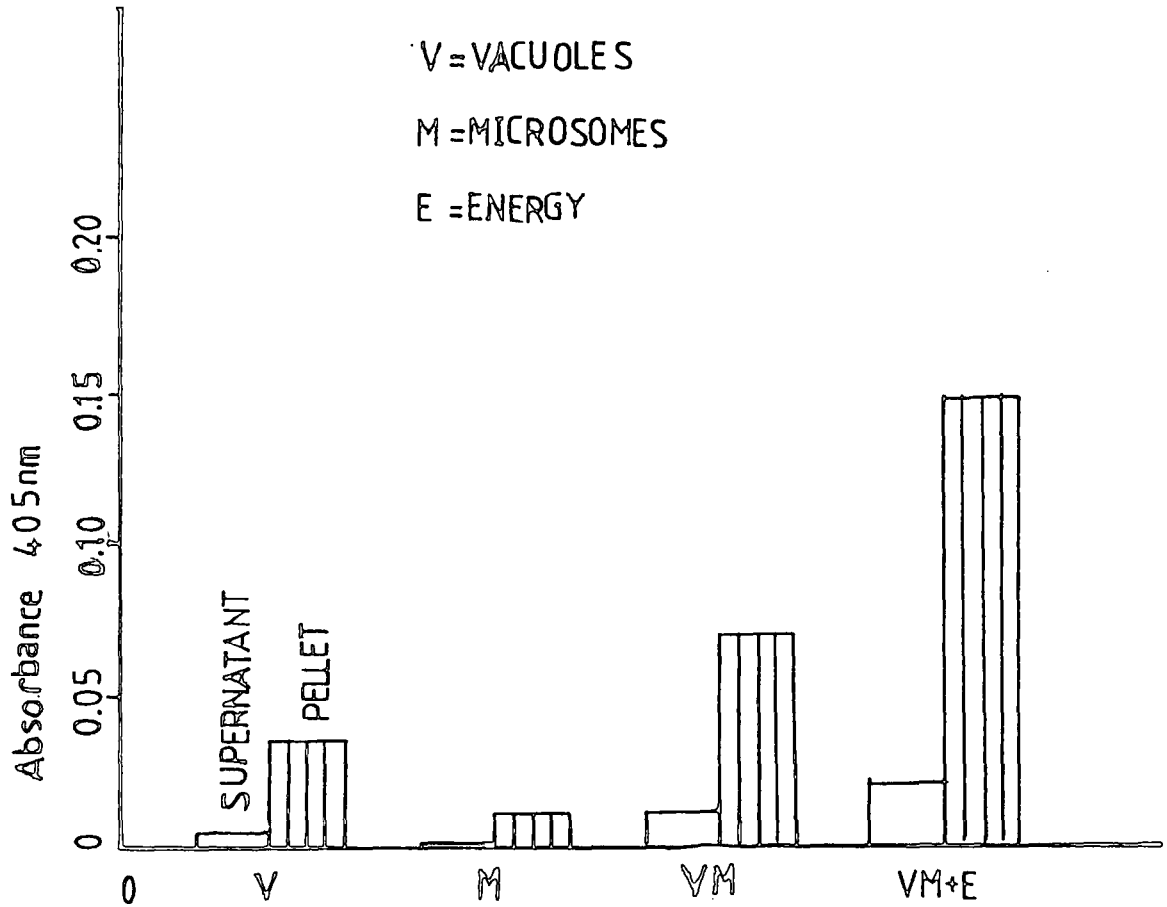
The results of these experiments are presented in fig 4.2.9. They show that the mature CPY is located in the sedimented fraction of the reaction mixtures. Again the requirement for ATP and the soluble fraction can be seen.

4.2.10 Western Blots with New Antibody

All of the results obtained so far indicate that *in vitro* transport of CPY has been achieved. However, all of experiments are based upon enzyme assays with Cbz-Ph-Leu as a substrate. To conclusively show that translocation is occurring, an alternative method to detect mature CPY is desirable. To achieve this the western blot experiments (Section 2.4.1) were repeated with modifications using anti-CPY

The Sedimentation of Mature CPY

Fig 4.2.9: The method used was as described in fig 4.2.8 except that after one hour incubation of the reaction mixtures, but before determination of CPY activity, the reaction mixtures were centrifuged for 5mins at 12000rpm. The supernatants and pellets were assayed separately for CPY activity.


V = VACUOLES
M = MICROSOMES
E = ENERGY
Absorbance 405nm
0
0.05
0.10
0.15
0.20
SUPERNATANT
PELLET
0
V
M
VM
VM+E

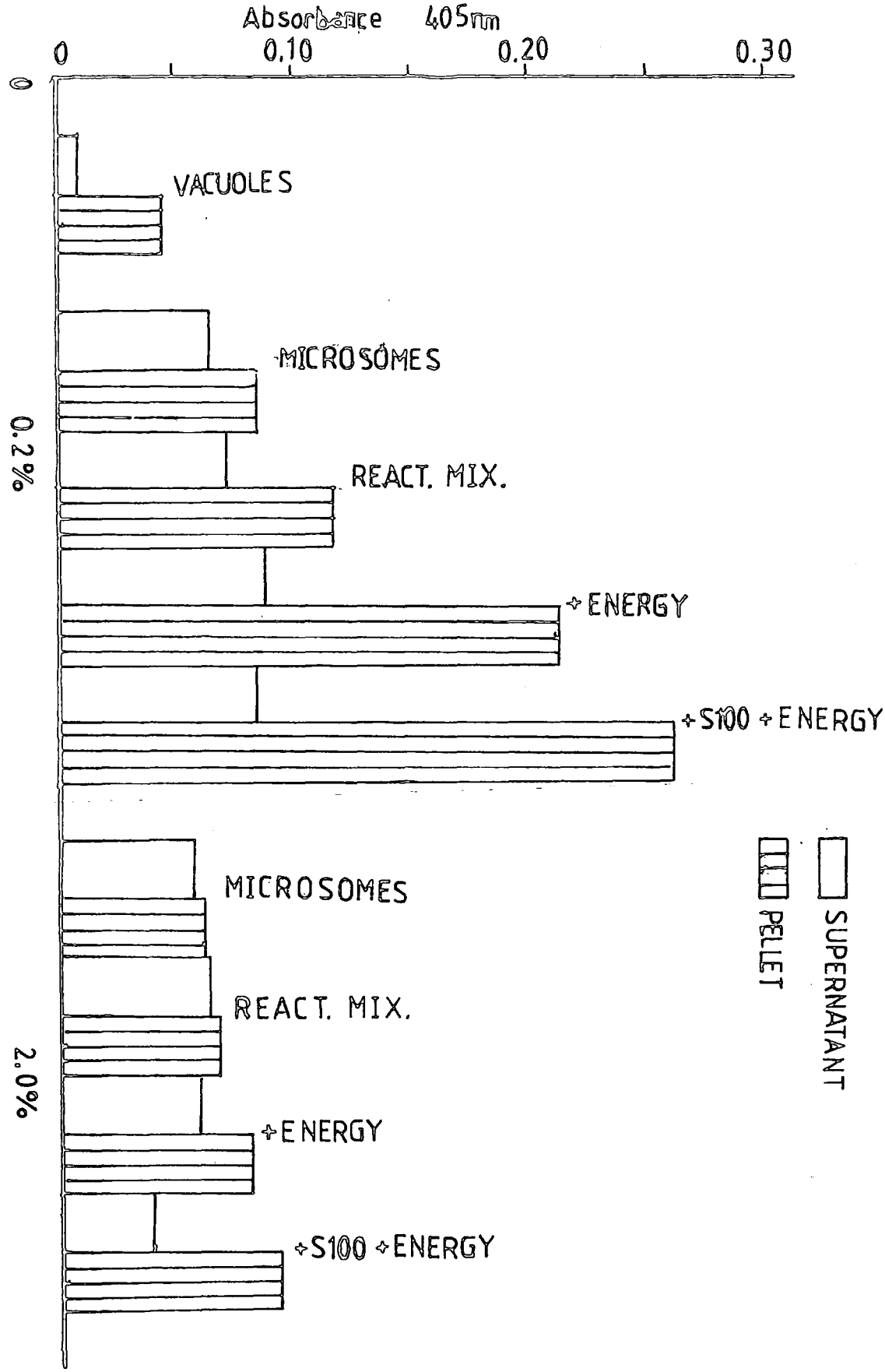
antibody kindly given by Prof. D. H. Wolf.

The experiments in this section were designed as in section 4.2.9 but donor microsomes were purified from DUBY29 *sec7-1, pep4-3* grown with both 0.2% and 2% glucose. Vacuoles were purified from BJ1075 which, although having CPS activity and giving rise to higher background levels, has no CPY antigen. The reaction mixtures were done in triplicate and incubated at 23°C for 3hrs. The supernatant and the pellet fractions were separated by centrifugation. Firstly, translocation of CPY was assayed biochemically by using two sets of the reaction mixtures. In one set the substrate Cbz-Phe-Leu was omitted and substituted with 0.2M potassium phosphate buffer, pH7.0, and the other set was assayed as normal. The difference between the values of the absorbances of the two assay sets is the measure of the activity of CPY alone and discounts any contaminating CPS. In theory this modification should reduce the back ground level of the assay. CPY and CPS liberate leucine from Cbz-Phe-Leu, which is then determined by L-amino acid oxidase. Any containing endogenous amino acids, particularly in the vacuoles, will interfere here with this assay. The modification introduced here is intended to compensate for this endogenous background level.

The results of the biochemical assays are presented in fig 4.2.10.1, they again show that maturation of proCPY occurs in a sedimentable fraction and that transport is stimulated by ATP and by the addition of soluble factors. Transport is only observed when the donor microsomes are purified from cells grown with 0.2% glucose.

For the analysis with western blots, 50μl from each fraction (Supernatant and re-suspended pellet) was precipitated with 10% TCA at 4°C overnight. The precipitates were recovered by centrifugation and then resuspended in 50μl of 2x sample buffer, followed by SDS-PAGE electrophoresis (Section 2.5) and western blots (Section 2.6).

Fig 4.2.10.1: The effect of energy, soluble factors and sedimentation on *in vitro* transport of CPY from DUBY29 Golgi vesicles to BJ1075 vacuoles. The methods used were as described in fig 4.2.8 and sedimentation as described for fig 4.2.9. Two sets of reaction mixtures were prepared and incubated for 3hrs at 23^{o}C. One set of reaction mixtures was assayed without added Cbz-Phe-Leu and the values substracted from the corresponding values assayed with Cbz-Phe-Leu.


Absorbance 405nm
0
0.10
0.20
0.30
0
0.2%
2.0%
VACUOLES
MICROSOMES
REACT. MIX.
+ENERGY
+S100 +ENERGY
MICROSOMES
REACT. MIX.
+ENERGY
+S100 +ENERGY
PELLET
SUPERNATANT

with anti-CPY antibody.

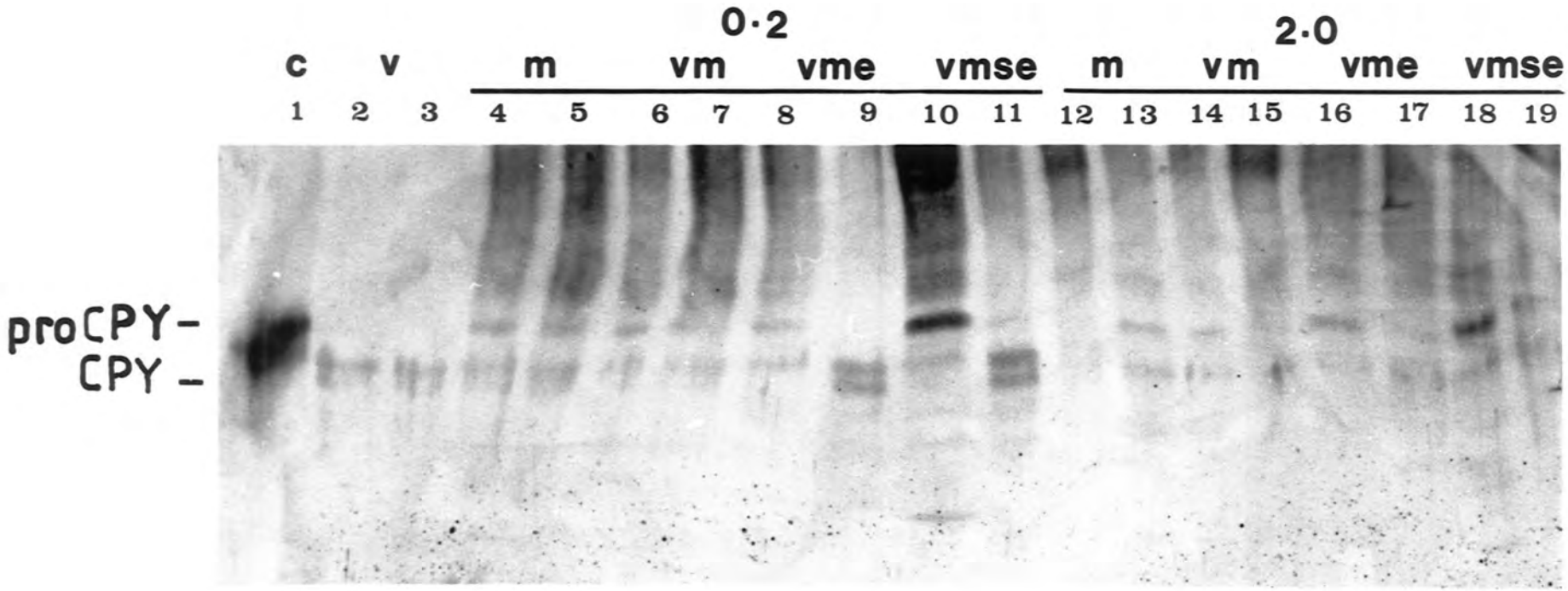
Analysis of the western blot (Fig 4.2.10.2) shows clearly the maturation of proCPY to CPY (Lane 9 and 11). This only occurs with microsomes derived from cells grown with 0.2% glucose (Lanes 4-11), require energy (Lanes 8-11) and is in a sedimentable form (Lanes 9 and 11). Microsomes from cells grown with 2% glucose show no evidence of maturation of CPY (Lanes 12-19).

4.3 Discussion

The results presented here strongly suggest that *in vitro* reconstitution of transport of carboxypeptidase Y from Golgi vesicles to vacuoles has been achieved. In order for active CPY to be formed, the proCPY in the donor vesicles must be mixed with the contents of the recipient vacuoles. Only recipient vacuoles contain active proteinases A and B necessary for the processing of proCPY to the active mature form. The experimental design depends on the fact that any contaminating vacuoles from the donor strain will not interfere because they lack the necessary proteinases due to the *pep4-3* mutation. The recipient vacuoles do not contain active CPY, due to the *prc1-229* and *prc1-1* mutations for BJ1075 and BYS 232-31-42 respectively, except that transported from the donor vesicles. The retained temperature sensitivity of the transport reaction (Fig. 4.2.2, and 4.2.7.1) reflects the *in vivo* temperature sensitivity of the *sec* strains. This is in agreement with the results described by Haselbeck and Scheckman (1986) for the transport of core glycosylated invertase from the ER to the Golgi complex using microsomes purified from a *sec18* strain. However, *in vitro* translocation is also inherently temperature sensitive (Baker, *et al.*, 1988). The system developed by Baker *et al.*, uses yeast ghosts produced by gentle Freeze-thaw. It

Analysis of In Vitro Transport of Pro-CPY with Western Blots

Fig 4.2.10.2: The methods used were as described in fig 4.2.10.1. 50μl of the supernatant and pellet fractions of the reaction mixtures were TCA precipitated before subjecting them to SDS-PAGE. Lane 1 (c), purified CPY. Lanes 4-11, microsomes from DUBY29 grown with 0.2% glucose. Lanes 12-19, microsomes from DUBY29 grown with 2% glucose. Even numbered lanes (2-18) are the supernatent fractions. Odd numbered lanes (3-19) are the pellet fractions of the reaction mixtures. v, vacuoles; m, microsomes; e, energy regeneration system; s, S100 fraction of DUBY29.


0·2
2·0
c
v
m
vm
vme
vmse
m
vm
vme
vmse
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
proCPY-
CPY-

is not dependent on a pre-accumulated product, but uses prepro-α-factor synthesised *in vitro* from a coupled transcription-translation system. The ghosts can be prepared from both wild type and *sec* strains and a direct comparison made. They show that *sec* ghosts are temperature sensitive at 30°C compared to wild type ghosts, but both are sensitive at 37°C. The system described in this chapter is dependent upon *sec* accumulation of proCPY in Golgi vesicles. Therefore, a direct comparison between *sec* and wild type Golgi vesicles cannot be made. Thus it is difficult to conclude that the observed temperature sensitivity in this system is indeed due to the *sec* mutations.

The results presented here show that ATP is essential for Golgi to vacuole transport (Figs 4.2.2, 4.2.7.1, 4.2.8, 4.2.9, 4.2.10.1 and 4.2.10.2). This is not suprising given the complex nature of intercompartmental protein traffic and the almost universal requirement for energy shown by *in vitro* translocation systems, such as the *E. coli* plasma membrane (Chen and Tai, 1985; Geller, *et al.*, 1986), the microsomal membrane (Hansen, *et al.*, 1986; Rothblatt and Meyer, 1986a,b; Waters and Blobel, 1986; Mueckler and Lodish, 1986a,b; Hansen and Walter, 1988; Baker, *et al.*, 1988), chloroplast (Grossman, *et al.*, 1980; Flugge and Hinz, 1986), mitochondria (Pfanner and Neupert, 1986; Eilers, *et al.*, 1987) and intercisternal transport in the Golgi apparatus (Balch, *et al.*, 1984; Dunphy, *et al.*, 1985; Dunphy and Rothman, 1985; Wattenberg, *et al.*, 1986; Dunphy. *et al.*, 1986; Pfeffer and Rothman, 1987)

According to the results otained by Waters, *et al.*, (1986), there are certain factors present in the cytoplasmic fraction (S100) of *Saccharomyces cerevisiae* that stimulate posttranslational translocation of prepro-α-factor. Moreover, it has recently been shown that translocation and glycosylation of a truncated form of bovine opsin was also dependent on the presence of a cytoplasmic fraction (Greenburg and Blobel, unpublished observations). The yeast ghost system developed by Baker, *et al.*,

(1988) shows a clear reguirement for soluble cytoplasmic proteins in transport from the ER to the Golgi. One of these proteins is coded for by the *sec 23* gene. The system is inhibited by the GTP analogue GTPγS and N-ethylmaleimide (NEM) as is intercisternal transport in the Golgi apparatus (Melancon, *et al.*, 1987; Malhotra, *et al.*, 1988). In this system vesicle transport from one cisterna to the next has been shown to be exceedingly complex. Budding of the donor vesicle is sensitive to GTPγS. The vesicle is then transported to the next cisternae where it attaches. The vesicle is then uncoated. The subsequent fusion events can be divided into at least three stages. The first requires the NEM-sensitive factor, ATP and other cytosolic proteins. The second requires a partially characterized component called factor B, ATP and the other cytosolic proteins. The final stage requires only ATP. Recently Chirico, *et al.*, (1988) were able to detect the presence of two distinct activities in the cytoplasmic fraction of yeast that stimulates translocation across the microsomal membrane. They stated that one activity consisted of two constitutively expressed 70kD heat shock related proteins. In my system a cytoplasmic fraction stimulated transport of proCPY from Golgi vesicles to vacuoles, a process likely to be as complex if not more than intercisternal transport in the Golgi.

The high background of apparent CPY activity in the transport assays is due to the use of Cbz-Phe-Leu as a substrate and the presence of CPS which can also use Cbz-Phe-Leu as a substrate. However, in crude extracts of wild type strain it has been found that over 95% of the Cbz-Phe-Leu hydrolysing activity is due to CPY (Wolf and Weiser, 1977; Wolf and Ehmann, 1978a). Strain BJ1075 was used as the source of recipient vacuoles in the initial experiments. As this strain has CPS activity, BYS 232-31-42 was subsequently used because it lacks CPS. However, the reduction in background activity was only about 30%, less than anticipated . The reason for this

is probably due to the endogenous free amino acids stored in the vacuoles (Huber-Walchli and Wiemken, 1979). These are substrates for L-amino acid oxidase used in the coupled assay for CPY. Attempts to compensate for the background level by omitting Cbz-Phe-Leu from the assay, so as to measure that fraction of activity due to endogenous free amino acids were not particularly successful (Fig 4.2.10.1). In these results there is still a high level of background produced by the vacuoles alone even after the compensation for the endogenous activity. However, this particular experiment is again complicated by CPS activity in the vacuoles. It was necessary to use BJ1075 as the recipient strain for the western blots. This does not account for the high values obtained with the microsomes alone, both in the supernatant and pellet fractions.

The sensitivity of *sec7* thermoreversibility to glucose concentration is well documented (Novick, *et al.*, 1981; Schekman, 1982). Cells grown with 2% glucose accumulate aberrant organelles called Berkeley bodies at 37°C and accumulate secretory protein e.g invertase. Upon return to the permissive temperature the accumulated invertase is not secreted, (i.e. the block is irreversible). However, cells grown with 0.2% glucose accumulate secretory proteins and elaborate Golgi-like structures. Upon return to the permissive temperature the accumulated invertase is secreted. This affect of glucose upon *in vivo* thermoreversibility is reflected in *in vitro*. Maturation of proCPY only occurs with microsomes derived from cells grown with 0.2% glucose. This not due to the failure of cells grown with 2% glucose to accumulate proCPY, as can be seen from the western blot of microsomes derived from cells grown under such conditions (Fig 4.2.10.2, lanes 12-19) and the stimulation of CPY maturation upon disruption of microsomes and vacuoles membranes by TritonX-100 (Table 4.2.8.1). This *in vitro* reflection of an *in vivo* property gives strong support to the conclusion

that *in vitro* transport of proCPY has indeed been demonstrated.

Additional support for the conclusion that the maturation of CPY is due to the correct transport of proCPY from microsomal membrane to vacuoles, comes from the following three observations. Firstly, the addition of proteinase A and proteinase B inhibitors have a negligible effect on the processing of proCPY (Figs 4.2.4, 4.2.7.2 and 4.2.8). When the transport system was deliberately disrupted with TritonX-100, production of active CPY was stimulated due to the direct mixing of donor proCPY with recipient proteinase A and proteinase B, (Tables 4.2.6, 4.2.7 and 4.2.8.1). This activation effect was abolished upon the addition of the proteinase inhibitors, and the value of CPY-like activity obtained was below that of the background level shown by the undisrupted complete translocation system. Secondly, high activity of mature CPY was observed only in the sedimented fraction of the reaction mixtures compared to that of the supernatant fraction (Results of sections 4.2.9 and 4.2.10). Both of these first two points argue strongly that the mature CPY is found in intact membranous compartments. Spontaneous lysis of these membranous compartments to mix proCPY with proteinase A and B would mean that maturation would be sensitive to the inhibitors. Spontaneous fusion of donor and membrane components would also be resistant to inhibitors, but would not be energy dependent and would also occur in microsomes derived from cells grown with 2% glucose. Lastly, analysis of transport with western blots shows a very clear band of mature CPY only in the sedimented fraction of the reaction mixtures supplemented with ATP and using microsomes derived from cells grown with 0.2% glucose (Fig 4.2.10.2, lanes 9, 11). The appearance of two bands representing mature CPY may be due to the separate activities of proteinase A and proteinase B which process the pro-sequence of proCPY in a sequential manner giving rise to two species of slightly different molecular weight (Mechler, *et*

al., 1987).

The result obtained with the western blot is important as it analyses transport by a different technique (Change in molecular form rather than change in activity) and is not dependent upon the use of Cbz-Phe-Leu as a substrate.

The use of this *in vitro* translocation system for the investigation of the molecular events involved in the sorting of proteins from the Golgi apparatus to vacuoles may prove to be invaluable. The ability to complement for a defective gene product *in vitro* may enable the purification and identification of that product. This may prove difficult with some of the *sec* and *vpl* mutations which are possibly defective in membrane bound proteins and which cannot always be complemented *in vitro* by the simple addition of a crude preparation of normal protein. This has proven to be the case with *sec18* mutation (Haselbeck and Schekman, 1986), and the *sec 12* mutation (Nakano, *et al.*,, 1988). However, successful complementation has been used to identify the *SEC23* gene product (Baker, *et al.*, 1988). It should, prove possible to purify the cytoplasmic factors that stimulate transport in this system. The characterization of these proteins will surely lead to an understanding of the processes by which membrane bound vesicles from different orgenelles are able to recognize and interact with each other.

One of the major uses of an *in vitro* transport system such as this is for the analysis of the component parts. For this it is important to have a low background (signal to noise ratio) and a clean response. It is doubtful whether the assay systems employed here will prove sensitive enough for a complete fractionation. The assays that employ Cbz-Phe-Leu as a substrate have a high background probably due to the endogenous levels of amino acids in the vacuole. The western blot also shows a high

background level, due to cross-reaction with other cell components, and also does not show the increase in CPY transport found on addition of the soluble fraction.

Adaptations of the methods of Backer, *et al.,* (1988) and Ruohola, *et al.,* (1988) may improve the signal to noise ratio and allow the development of a more sensitive assay. Both of these methods utilise prepro-α-factor transcribed and translated *in vitro* to S^{35} labelled protein. This is then mixed with yeast ghosts (Baker, *et al.,* 1988) or permeabilized cells (Ruohola, *et al.,* 1988) to achieve *in vitro* transport. Ruohola, *et al.,* showed that whole cells are not needed for this reaction and that transport to the Golgi apparatus can be achieved in a totally cell-free system using microsomes prepared in a manner similar to those used in my assay. This observation is important. PreproCPY can be translocated posttranslationally into yeast microsomes (Hansen and Walter, 1988), so at least CPY should be a suitable substrate for the *in vitro* system of Ruohola, *et al.,*. Transport through the entire vacuolar localizing pathway can be monitored by the changing forms of CPY. *In vitro* translated preproCPY, without glycosylation, has a Mr of 53.2kD. The ER forms are 51kD (Unglycosylated) and 67kD (Core glycosylated, P1). The Golgi form is 69kD (P2) and the mature vacuolar form 61kD. These can be detected by immunoprecipitation and autoradiography with very low background (Valls, *et al.,* 1987). The disadvantage of the yeast ghost and permeabilized cell systems is that they retain the original vacuoles. In a totally cell free system, vacuoles from different strains, such as aberrant vacuoles in *vpt* mutants, can be added and the effects observed. The other advantage of these systems is that they use wild type strains and not *sec* strains, so avoiding the temperature sensitivity of my system.

CHAPTER FIVE

5. EXPRESSION OF A CPY-GUS GENE FUSION

5.1 Introduction

The use of fusions between a gene of interest and a reporter gene with an easily detectable product gives several advantages for the study of gene expression and protein processing. Analysis of mutationally altered genes in organisms accessible to transformation is greatly enhanced by the use of a sensitive reporter enzyme. By using a reporter gene that encodes an enzyme activity not found in the organism being studied, the sensitivity by which the expressed gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the assays available for the enzyme.

There are several reporter genes that have been used to study gene expression in eukaryotes. Some bacterial reporter genes have been used in higher plants, such β-galactosidase (Helmer, *et al.,* 1984), chloramphenicol acetyl transferase (CAT), neomycin phosphotransferase (NPTII) (Bevan, *et al.,* 1983; Fraley, *et al.,* 1983; Herrera-Estrella, *et al.,* 1983a,b) and leuciferase (Ow, *et al.,* 1986). These reporter genes are not widely used because of either their endogenous activity in plants e.g. β-galactosidase (Helmer, *et al.,* 1984), or they are very difficult to quantitate and expensive to assay, e.g. CAT and NPTII (Gorman, *et al.,* 1982; Reiss, *et al.,* 1984), or very difficult to assay (luciferase) (Deluca and McElroy, 1978).

However, the work of Jefferson, *et al.,* (1987b) led to the development of the *E. coli* Gus gene (β-glucuronidase) as a reporter gene system for translocation in plants. The product of this enzyme is very easy to quantitate and has no background ac-

tivity in most of the higher plants and yeast (Jefferson, 1985, 1987; Jefferson, *et al.*, 1986, 1987a,b). β-glucuronidase is a hydrolase that catalyzes the cleavage of a wide variety of β-glucuronides (Jefferson, *et al.*, 1987a,b; Jefferson, 1987). This enzyme can be assayed either, spectrophotometrically, fluorometrically or histochemically to determine gene product localization in particular cell types. The Gus gene has been cloned, sequenced and characterized, the encoded enzyme is stable and exhibits desirable properties for the construction and analysis of gene fusion (Jefferson, 1985; Jefferson, *et al.*, 1986, 1987b). The activity of β-glucuronidase is also maintained when fused with other proteins at its amino terminal end (Jefferson, *et al.*, 1987b; Jeferson, 1987).

5.2 Results

The series of experiments detailed below were designed to generate a CPY-Gus gene fusion encoding the promoter and prepro-sequence of carboxypeptidase Y (CPY) and the full length form of the Gus gene (β-glucuronidase).

5.2.1 Isolation of the Promotor and Prepro-Sequence of CPY

The *PRC1* gene including the promoter and entire coding sequence has been mapped to a 3.3kb SalI-PuvII fragment in pTSY3 (Stevens, *et al.*, 1986a). The nucleotide and corresponding amino acid sequence has been determined (Valls, *et al.*, 1987). Stevens, *et al.*, (1986b) have shown that the first 111 amino acids of the CPY precursor encodes the signal sequence and propeptide. The coding region for these amino acids together with the promoter and 1400bp of $5'$ upstream sequence, are carried on a 1862bp SalI-BamHI fragment within the pTSY3 insert (Fig 5.2.1.1). To

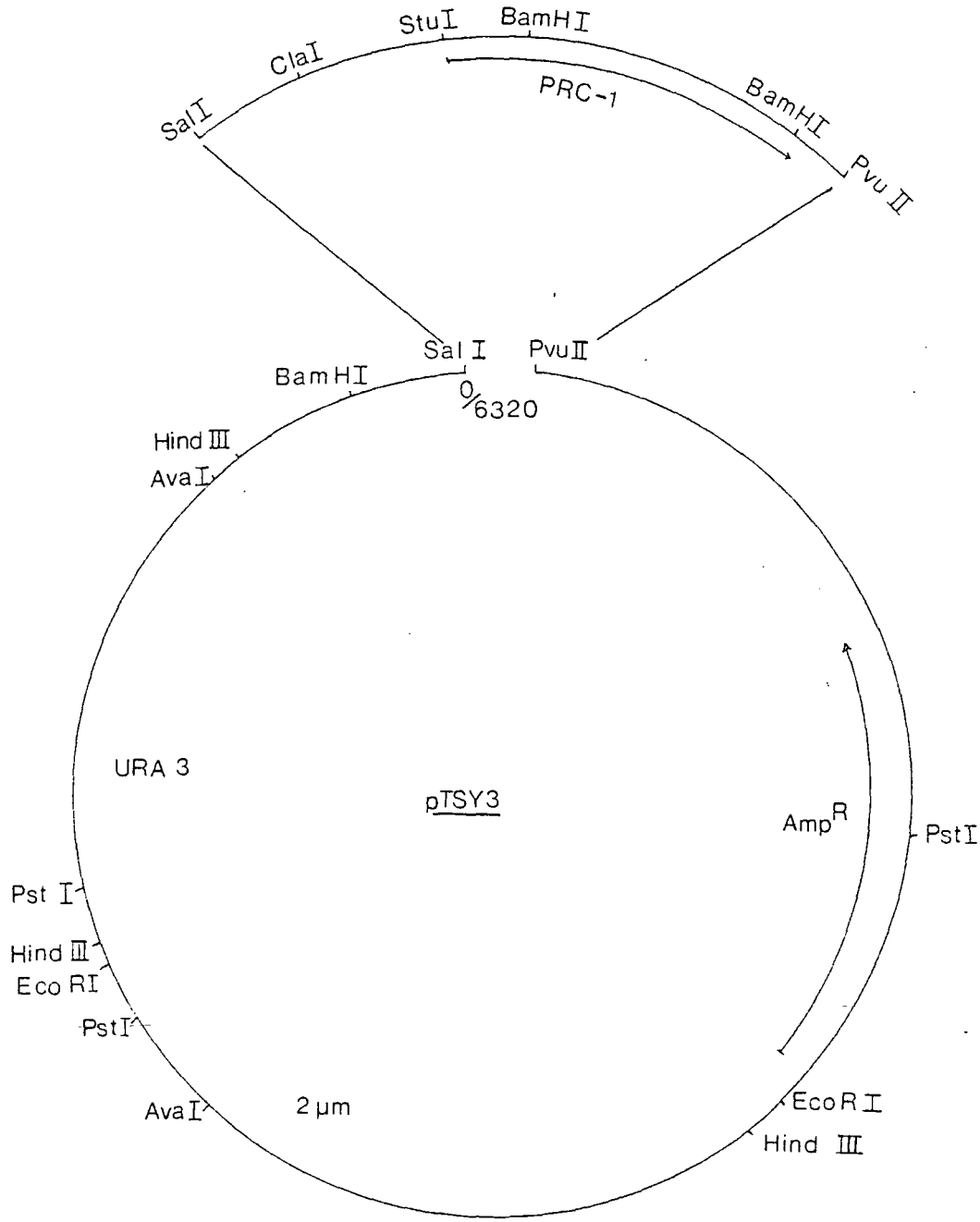


Fig 5.2.1.1: Plasmid pTSY3 encoding *PRC1* (Stevens, *et al.*, 1986).

isolate a *PRC1*-subclone encoding the promoter and preprofragment, purified pTSY3 was restricted in a double digest with SalI and BamHI (see Fig. 5.2.1.1b). The resultant fragments were separated by agarose gel electrophoresis as shown in Fig 5.2.1.2 and the required fragment, termed CPY, was isolated using the freeze elution method (Section 2.18).

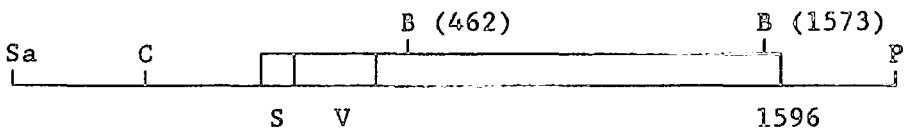
The pTSY3 derived restriction fragment (CPY) was ligated with pUC19 vector and then transformed into *E. coli* (JM83). The white transformant colonies bearing the inserted CPY fragment were streaked onto duplicate selective plates to confirm the purity of the sub-culture.

The colonies were then screened for the correct SalI-BamHI insert by digestion of miniprep plasmid DNA (Section 2.11.1) with these two enzymes in a double digest. Three transformants bearing the SalI-BamHI (CPY) fragment (Fig 5.2.1.3, lanes 3, 5, 10) were identified, the new plasmid is termed pDUB2500.

5.2.2 Construction of a CPY-Gus Gene Fusion

In order to construct a CPY-Gus gene fusion, pDUB2500 and pBI101 encoding a BamHI-EcoRI full length Gus gene, were digested with BamHI plus EcoRI (see Fig. 5.2.2.1). The fragments were separated and purified as described in section 5.2.1. The purified fragments were ligated together and then transformed into JM83. Ten white transformants were purified. The transformants were then screened for SalI-EcoRI insert (CPY-Gus gene fusion). It is clear that all transformants bear the CPY-Gus gene fusion (Fig 5.2.2, lanes 2-11). The constructed plasmid was designated pDUB2503.

A.



Restriction Map of PRC1. After Stevens *et al*, 1987. The boxed area shows the coding sequence for CPY, including the signal sequence (S) and the vacuolar sorting domain or pro sequence (V). The distances are in base pairs numbered from ATG. The signal cleavage site is at position 60, and the pro sequence cleavage site is at position 333.

Sa = Sal I; C = Cla I; B = BamHI; P = PvuII.

B.

GAA AGT AGA AAC GAT CCT GCA AAG GAT CCG GTC ATC CTT
Glu Ser Arg Asn Asp Pro Ala Lys Asp Pro Val Ile Leu

The predicted amino acid sequence near the 1st BamHI site of PRC1.

Fig. 5.2.1.1b. Restriction map and predicted amino acid sequence of PRC1

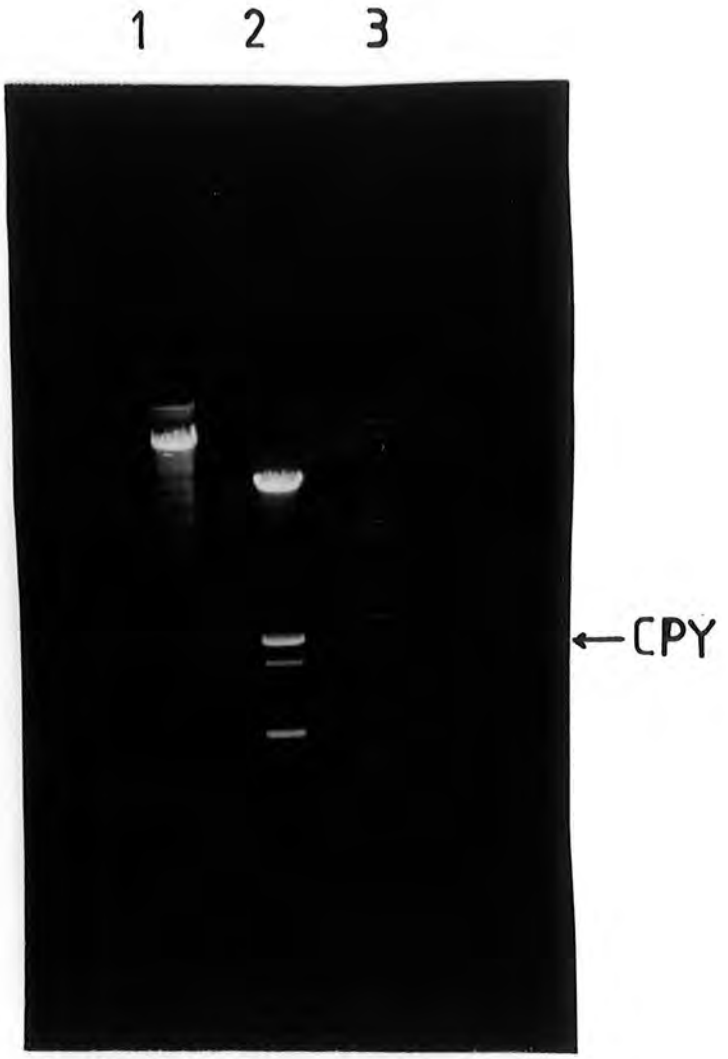


Fig 5.2.1.2: Agarose gel electrophoresis of pTSY3 plasmid DNA restricted with SalI (Lane 1), and SalI-BamHI (Lane 2). Lane 3, bacteriophage λ PstI size marker.

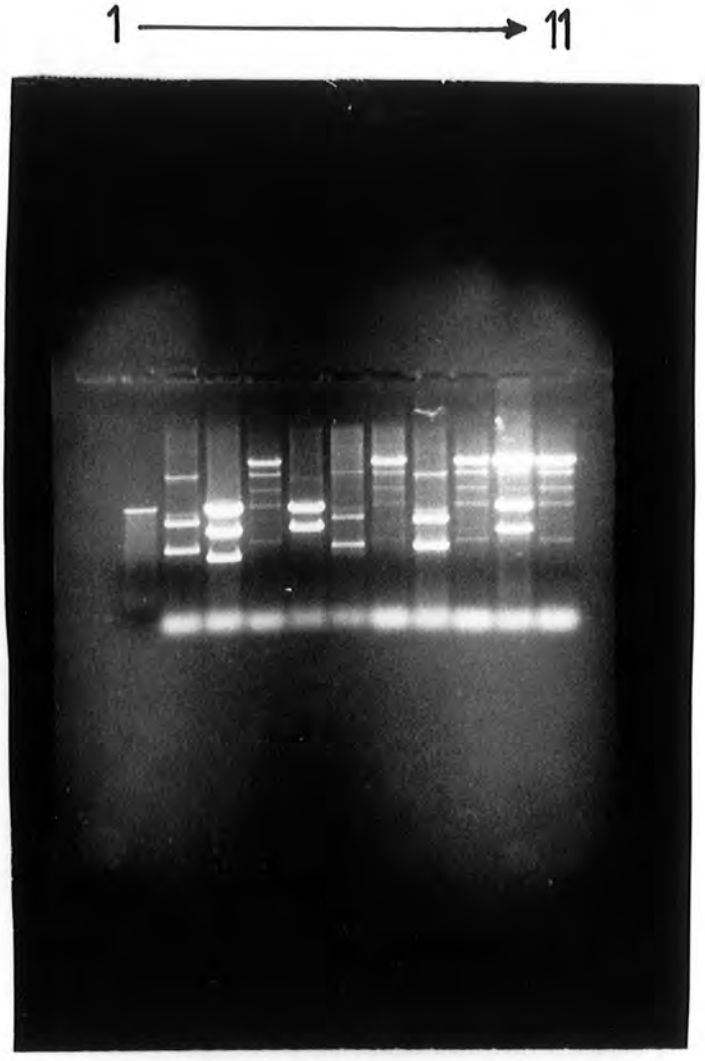
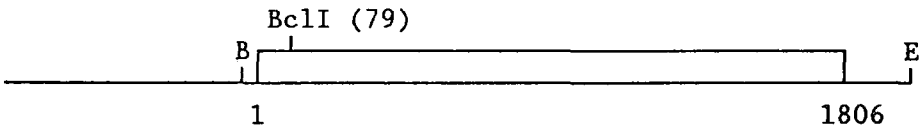


Fig 5.2.1.3: Agarose gel electrophoresis of minipreps of ten transformants digested with SalI-BamHI (Lanes 2-11). Lane 1, purified pUC19 previously restricted with SalI-BamHI.

A.



Restriction map of the GUS gene showing the major restriction sites. The ATG codon is position 1. Distances in base pairs. B = BamHI; E = EcoRI.

B.

CTA GAG GAT CCC CGG GTA GGT CAC TCC CTT ATG TTA
Met Leu

The DNA sequence and reading frame at the 5' end of the GUS gene. The BamHI site is indicated.

C.

BclI

GAA AAC TGT GGA ATT GAT CAG CGT TGG TGG GAA AGC
Glu Asn Cys Gly Ile Asp Gln Arg Trp Trp Glu Ser

The nucleotide sequence and the predicted amino acid sequence adjacent to the BclI site in the GUS gene.

Fig. 5.2.2.1 Restriction map of the GUS gene in plasmid pBI101. The sequence adjacent to the intitiation codon and the BclI are illustrated

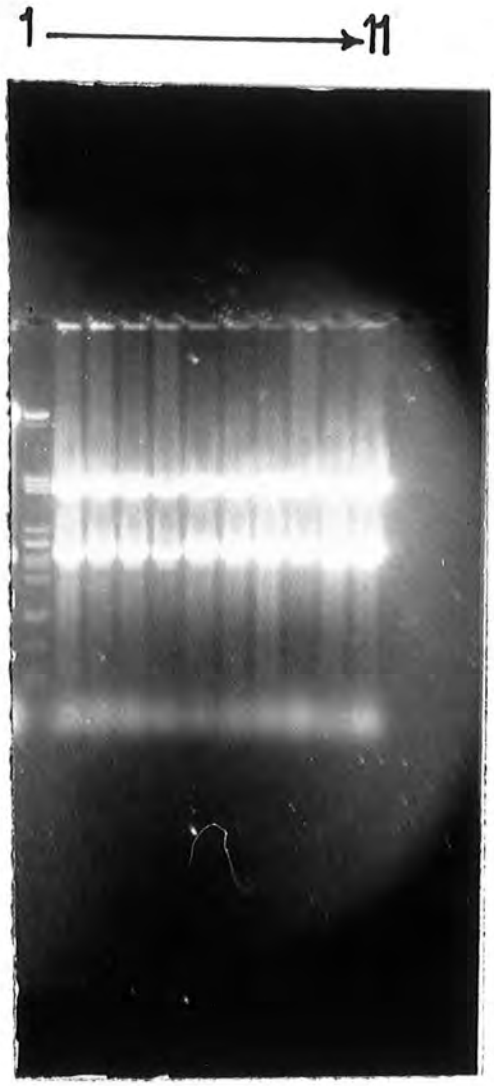


Fig 5.2.2: Agarose gel electrophoresis of pUC19 plus CPY-Gus gene fragment, recovered by DNA miniprep and digested with SalI-EcoRI (Lanes 2-11). Lane 1, bacteriophage λ PstI size marker.

5.2.3 Cloning of CPY-Gus Gene into pEMBLYe31

The yeast shuttle vector pEMBLYe31 was first completely digested with HindIII to obtain a linearised form of the plasmid, then partially digested with EcoRI (Fig 5.2.3.1). The EcoRI-HindIII linearised plasmid was isolated as described in section 5.2.1. pDUB2503 was also digested with the same enzymes and the CPY-Gus fragment isolated as above. The vector and fragment were ligated together, transformed into JM83 and 15 colonies selected randomly and plated again onto selective medium.

These colonies were then screened by HindIII-EcoRI digestion of the recovered plasmid DNA. Results presented in fig 5.2.3.2 show that 13 transformants have the correct fragment and plasmid (Fig 5.2.3.2, lanes 3-18). The new plasmid was termed pDUB2505.

5.2.4 Transformation of pDUB2505 into Yeast

Yeast competent cells prepared from MD40-4C were transformed by pDUB2505 plasmid DNA (Section 2.20). The transformants were plated onto yeast selective minimal agar and incubated at 30°C for 3-4 days. The transformants were then subcultured on the same medium to purify the culture. After two days incubation they were tested for β-glucuronidase activity as described below.

5.2.5 Determination of β-Glucuronidase Activity

The subcultured colonies were grown in 10ml of yeast selective minimal me-

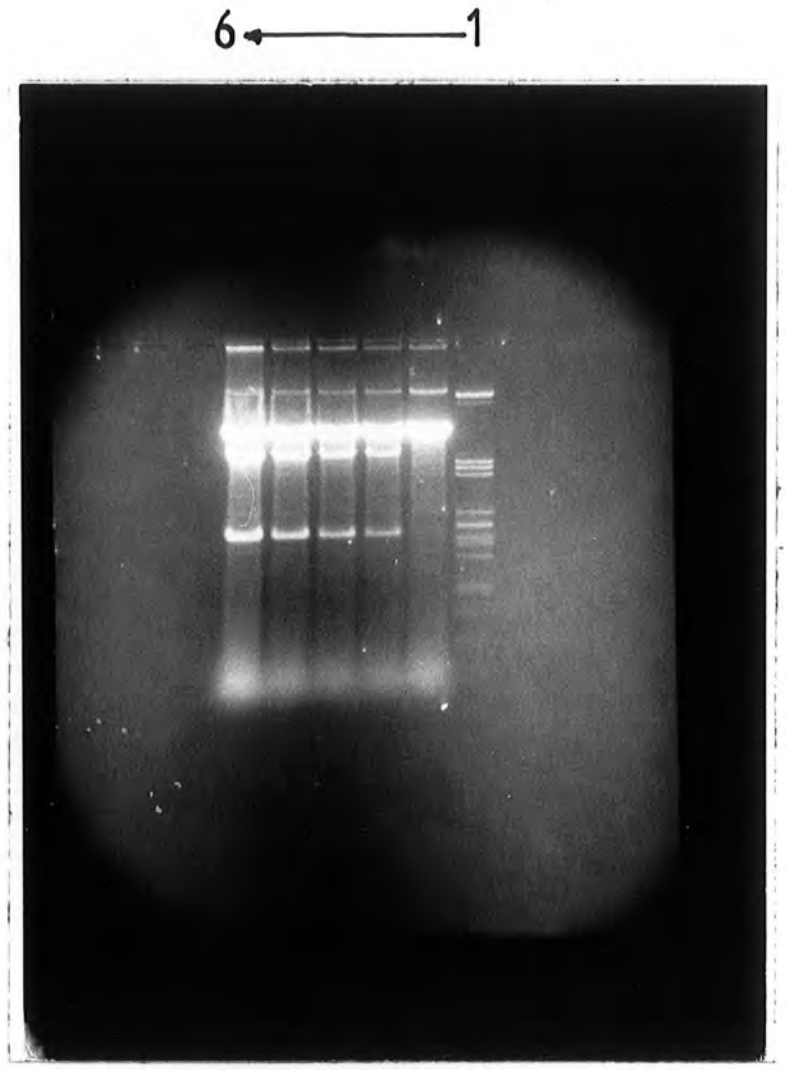


Fig 5.2.3.1: Restriction of pEMBLYe31 plasmid with HindIII to linearise the plasmid (Lane 2) plus partial digestion with EcoRI for 30, 60, 90 and 120mins (Lanes 3-6). Lane 1, bacteriophage λ PstI marker.

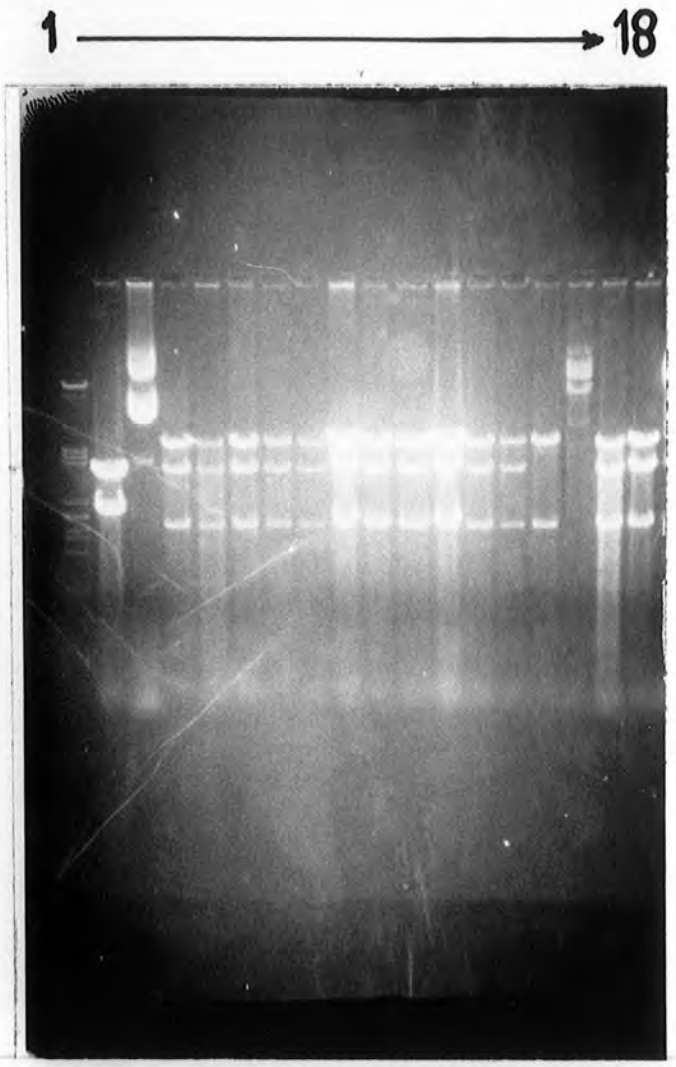
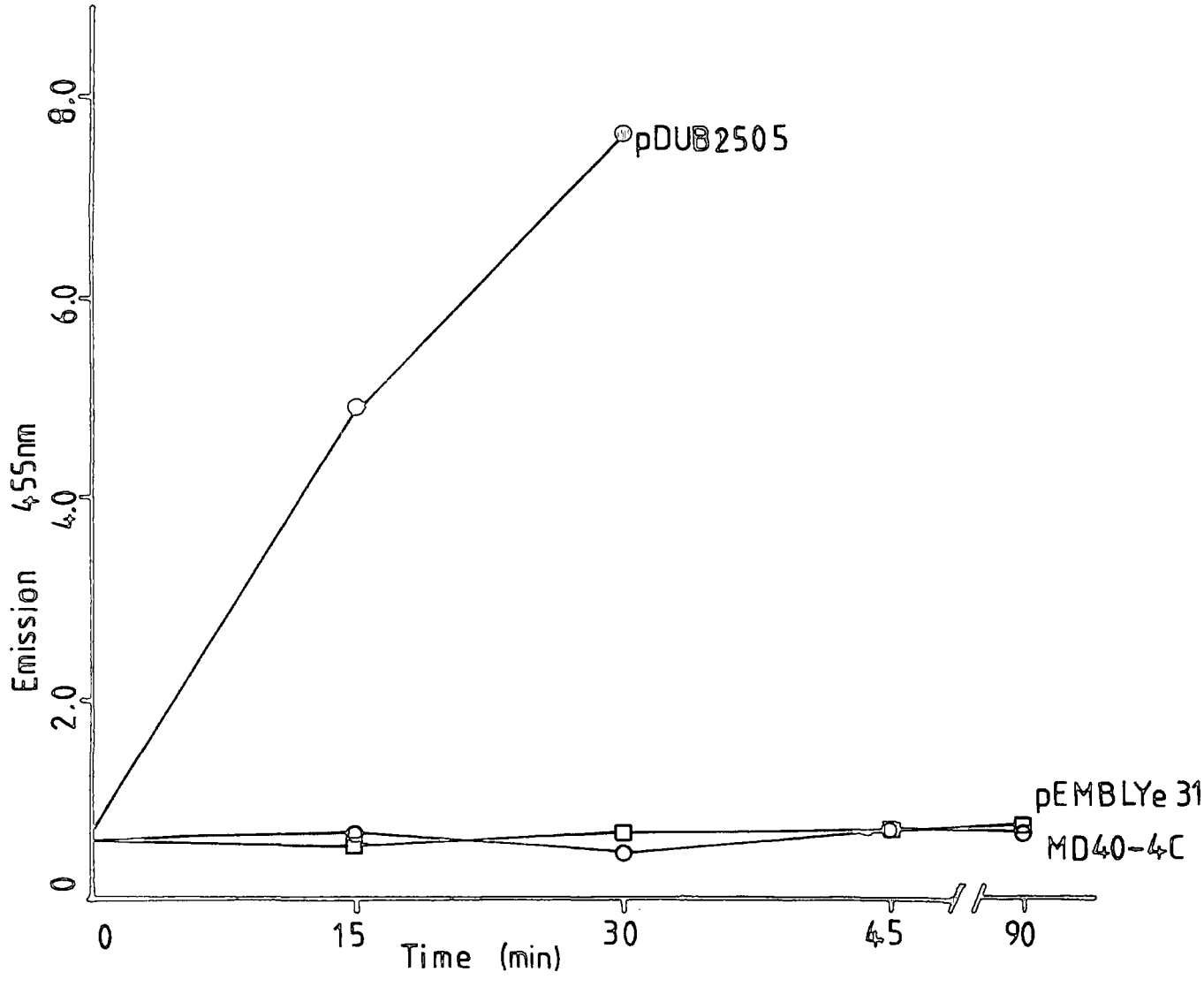


Fig 5.2.3.2: Agarose gel electrophoresis of pEMBLYe31 plus CPY-Gus gene recombinants by digestion of miniprep DNA with HindIII and EcoRI (Lanes 3-18). Lane 1, bacteriophage λ PstI marker and lane 2, pEMBLYe31 restricted with HindIII-EcoRI.


Emission 455nm
0
2.0
4.0
6.0
8.0
pDUB2505
pEMBLYe 31
MD40-4C
0
15
30
45
90
Time (min)

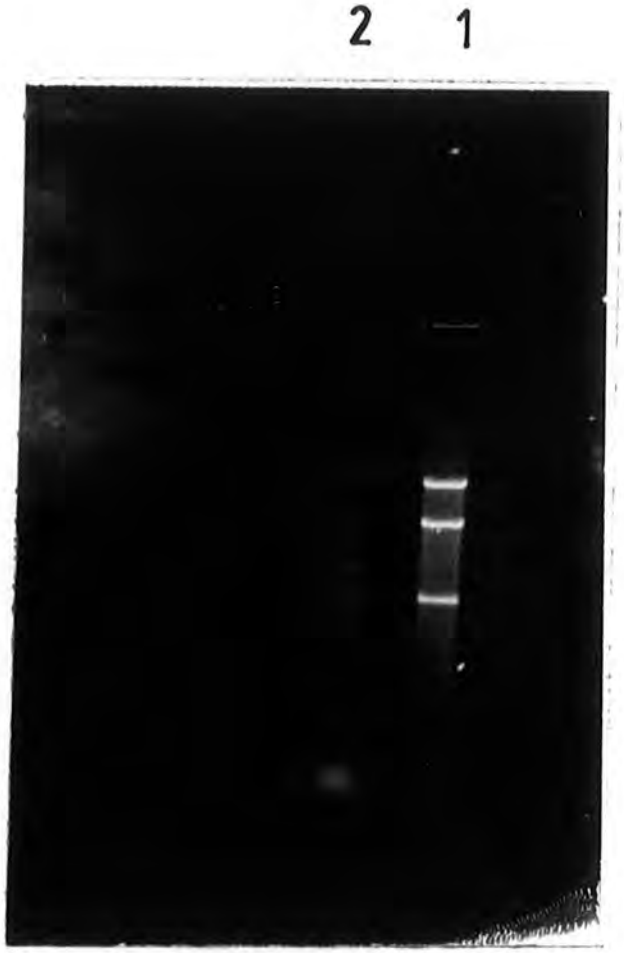


Fig 5.2.5.2: Agarose gel electrophoresis of pDUB2505 transformed into yeast (MD40-4C). The plasmid DNA of the transformants was recovered as described in section 2.12 and restricted with HindIII-EcoRI (Lane 2). Lane 1, control pDUB2505 digested with HindIII-EcoRI.

dia. MD40-4C cells transformed with only pEMBLYe31 vector and non-transformed cells were also grown for comparison. After overnight growth of these cultures, the cells were extracted as described in section 2.4.2 but using Gus extraction buffer and the β-glucuronidase activity of the crude yeast cell extracts was determined fluorometrically (Section 2.4.10). Very little Gus activity was obtained. However, when growth in YPD was substituted for minimal medium, high levels of Gus activity were obtained from the cells transformed with pDUB2505 (Fig 5.2.5.1). No detectable β-glucuronidase activity was found in the case of non-transformed cells or those transformed with pEMBLYe31 vector even after 90mins of incubation.

To be certain that pDUB2505 yeast transformants do not have rearranged plasmid DNA, was prepared as described in section 2.12 and digested with HindIII plus EcoRI and compared to that of authentic pDUB2505. Results presented in fig 5.2.5.2 show that the transformants do not have rearranged plasmids.

5.2.6 Fractionation of Cell Components

Transport of the preproCPY to the endoplasmic reticulum (ER) and delivery of proCPY to the vacuole have separate signals (Johnson, *et al.*, 1987; Valls, *et al.*, 1987). The first 20 N-terminal amino acids, the signal peptide, is responsible for sorting of preproCPY to the ER lumen and an additional signal, the vacuole targeting signal consisting of the next N-terminal 30 amino acids, is necessary for targeting of proCPY to the vacuole.

According to these observations, the CPY-Gus product should be located in the yeast vacuole. To determine this, cell fractionation of pDUB2505 transformed cells was performed as described in section 2.10.1. Fractions (1.0ml each) were taken from

Fig 5.2.5.1: Determination of β-glucuronidase activity in transformed and untransformed MD40-4C cells. 1mg protein was used from each per 200μl of reaction mixture. Incubation of the reaction mixtures was carried out for 15, 30, 45 and 90mins at 37^{o}C. The reaction was stopped after each specified interval by the addition of 0.8ml of 0.2M Na_2CO_3. β-glucuronidase activity was measured fluorometrically at 455nm and a gain setting of 30nm as described in section 4.2.10.

the ficoll gradient, with the vacuoles in the upper layer and the cytoplasmic and cell debris fraction, lower in the gradient. The graph in fig 5.2.6.1 shows the specific activity of CPY and the absolute value of Gus activity at 455nm emission and a gain setting of 30nm after one hour of incubation of the reaction mixtures at 37°C. It is clear that most of the β-glucuronidase activity is located in the cytoplasmic fractions rather than vacuole fractions, which show only low Gus activity, compared to the activity of the vacuolar CPY enzyme in the same fractions.

Both microsomes (Section 2.10.2) and S100 (Section 2.10.3) were also prepared and β-glucuronidase activity in both fractions determined after incubation of the reaction mixture for 15, 30 and 45mins at 37°C. Results plotted in fig 5.2.6.2 show that Gus activity is present in both fractions, but in the S100 about 2 fold more than in microsomes. Gus activity sometimes was also found in a secreted form but at a low level.

5.2.7 Cloning of the CPY-Gus Fusion into YCp50

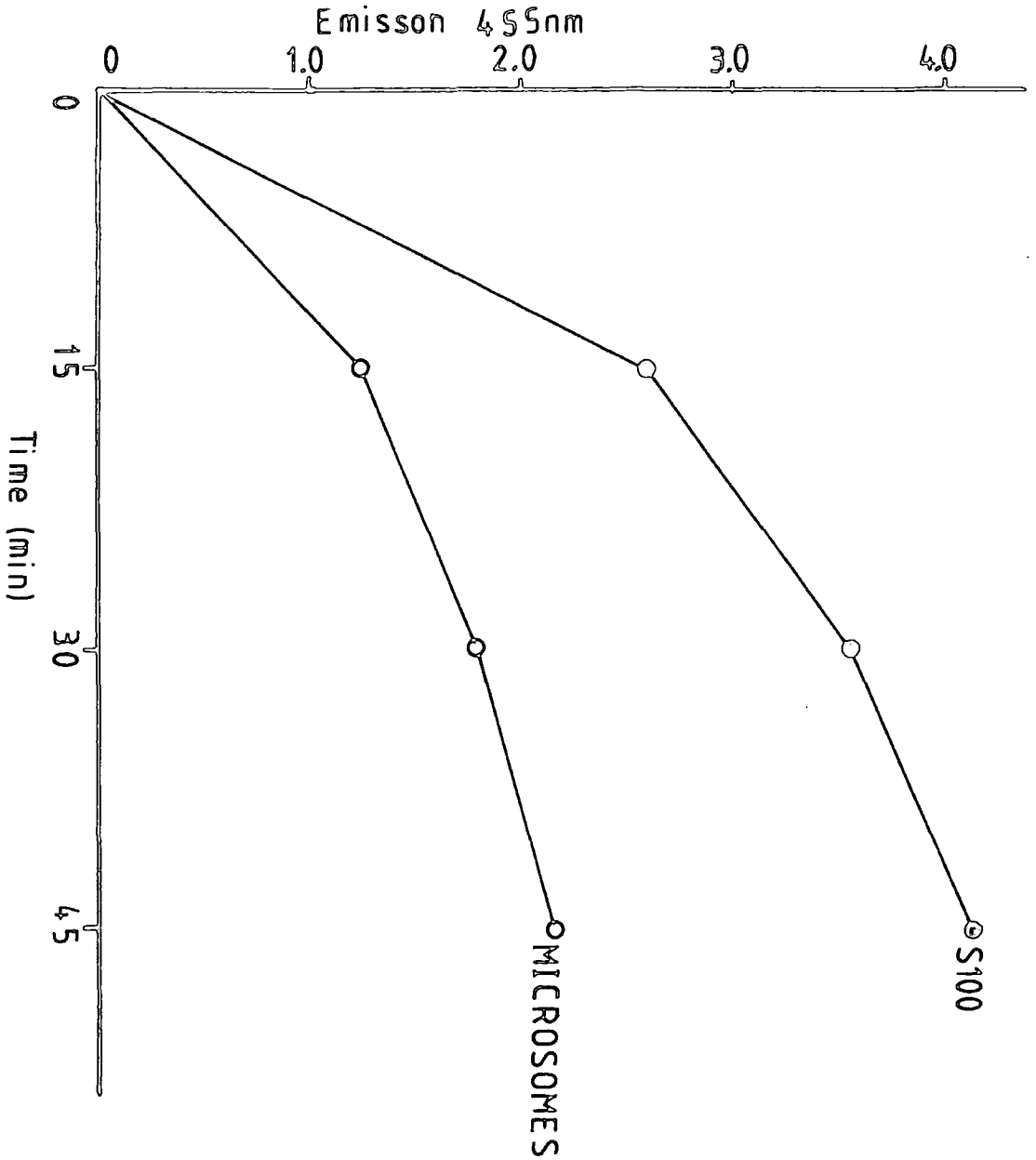
The above results (Section 5.2.5 and 5.2.6) show that Gus expression in yeast is efficient but it is found to the same degree in all cell components. This may be due to the presence of Gus on a multicopy plasmid. Stevens, *et al.*, (1986a,b) have shown that the presence of *PRC1* (CPY) on a multicopy plasmid causes saturation of the vacuolar pathway and mislocalization of CPY. Therefore, to exclude over-expression of the CPY-Gus fusion as an explanation of these results, a centromeric plasmid was used. This was the shuttle vector YCp50 encoding CEN4.

YCp50 and pDUBY2503 (Section 5.2.2) were separately digested with SalI plus EcoRI overnight, then the plasmid and the CPY-Gus fragment were isolated using low

Fig 5.2.6.1: Determination of β-glucuronidase activity and CPY specific activity in the cell fractions of MD40-4C (pDUB2505). The methods described in fig 5.2.5.1, were used except the reaction mixtures were incubated for one hour and CPY activity determined as described in section 2.4.4.

CPY Activity (nmol/min/mg protein)
0
3
6
9
12
15
18
×10
Emission 455nm
0
1.0
2.0
3.0
4.0
Fraction No.
0
3
6
9
12
15
GUS
CPY
Spheroplast
CPY
GUS
Pellet
CPY
GUS

Fig 5.2.6.2: Determination of β-glucuronidase activity in S100 and microsome fractions of pDUB2505 transformed cells. The method in Fig 5.2.5.1 was used exactly with the exception that incubation of the reaction mixture was carried out for 15, 30 and 45mins.


Emisson 455nm
0
1.0
2.0
3.0
4.0
0
15
30
45
Time (min)
MICROSOMES
S100

melting point agarose gels (Section 2.18). They were then ligated and transformed into JM83. The transformants were screened for the CPY-Gus fragment and plasmid by digestion of miniprep DNA with SalI-EcoRI. Three transformants bearing the fragment and plasmid were identified as shown in fig 5.2.7, (Lanes 6, 7, 8). The new plasmid was termed pDUB2512.

5.2.8 Transformation of pDUB2512 into Yeast

pDUB2512 was transformed into *S. cerevisiae* strain YHH19 by the same method as described in section 5.2.4. The transformants that grew on selective minimal medium were subcultured again and then assayed for any β-glucuronidase activity.

5.2.9 Determination of β-Glucuronidase Activity

The growth of cells using YPD broth medium and preparation of cell extracts were achieved using the methods described in section 5.2.5. The Gus activity was determined fluorometrically after incubation of the reaction mixtures for 30, 60 and 90mins at 37^{o}C. Results shown in fig 5.2.9 indicate that the transformants exhibit β-glucuronidase activity but at lower levels to that in pDUB2505 transformed cells. Again both non-transformed YHH19 cells and those transformed with YCp50 vector alone have no detectable β-glucuronidase activity.

5.2.10 The Sub-Cellular Fractionation

To determine the compartmental localization of β-glucuronidase activity, sphaeroplasts of pDUBY2512 transformed YHH19 were fractionated as described in section

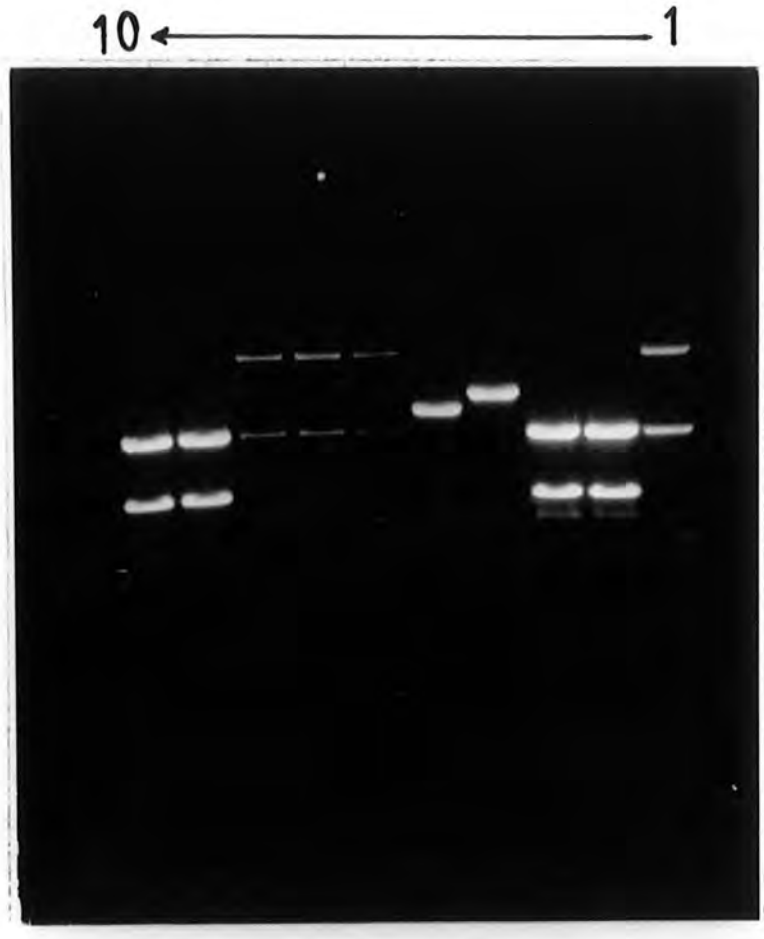
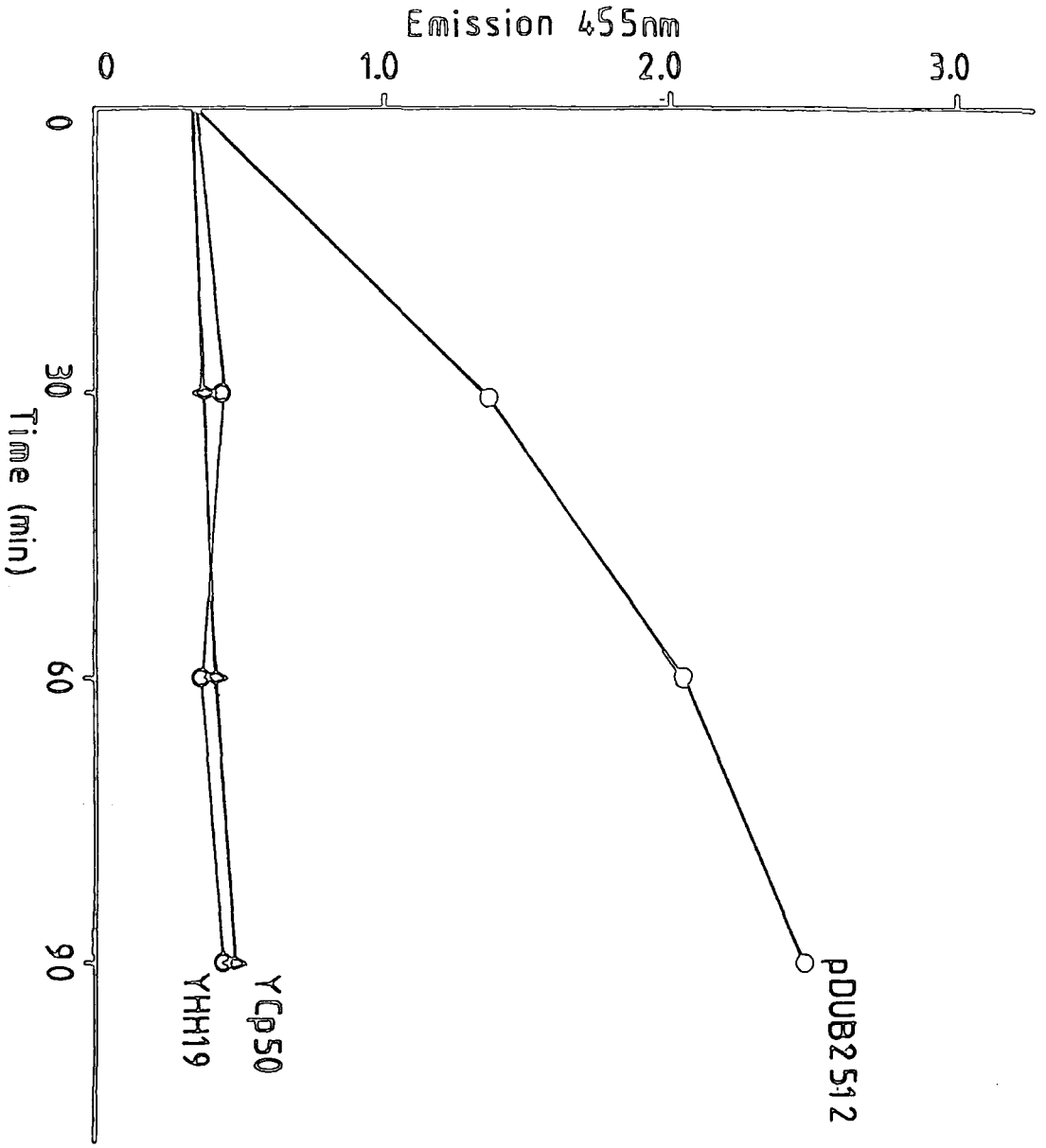


Fig 5.2.7: Agarose gel electrophoresis of YCp50 plus CPY-Gus gene recombinants, recovered by miniprep and digested with SalI plus EcoRI (Lanes 2-10). Lane 1, upper band purified SalI-EcoRI cut YCp50, lower band purified SalI-EcoRI CPY-Gus fragment.

Fig 5.2.9: Determination of β-glucuronidase activity in pDUBY2512 and YCp50 transformed and non-transformed YHH19 cells. The method in fig 5.2.5.1 was followed with the exception that the incubation of the reaction mixtures was for 30, 60, and 90mins.


Emission 455nm
0
1.0
2.0
3.0
0
30
60
90
Time (min)
YHH19
YCp50
pDUB2512

5.2.6 and the results are presented in fig 5.2.10. It is clear that the results of β-glucuronidase activity localization are essentially as described in section 5.2.6 but with lower activities in all tested fractions.

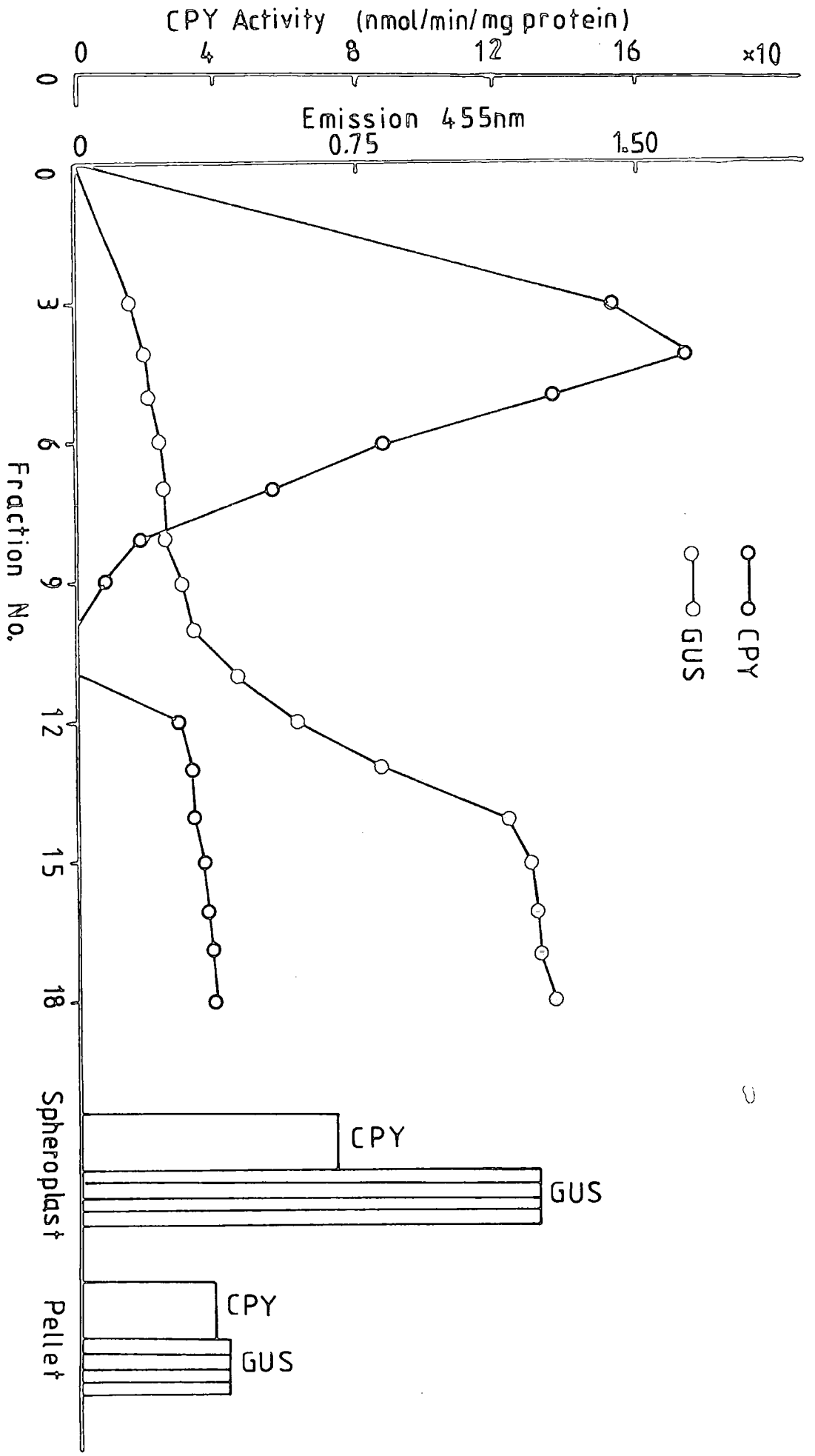
5.2.11 Cloning of CaMV 35S-Gus Gene Fusion into pEMBLYe31

It has previously been reported that some promoters from one organism can function in another (Jabbar, *et al.*, 1985; Bergh,*et al.*, 1987; Bird, *et al.*, 1987). Therefore, a series of experiments were performed to determine the expression of Gus in *S. cerevisiae* (MD40-4C) under the control of the CaMV 35S (Cauliflower mosaic virus) promoter. An 800bp HindIII-BamHI CaMV 35S promoter cloned into pBI101 (Guilley, *et al.*, 1982) was fused with the full length Gus gene approximately 15bp away from the Gus start codon creating pBI121 (Jefferson, *et al.*, 1987b). To isolate the HindIII-EcoRI (CaMV 35S-Gus gene) fragment, pBI121 was digested with these enzymes and the fragment was isolated as described in section 5.2.7. Digestion of pEMBLYe31 with HindIII-EcoRI was also achieved as above. They were ligated together overnight and then transformed in JM83. The transformants were subcultured and plasmid DNA recovered from these transformants was digested with HindIII plus EcoRI. Results presented in fig 5.2.11 show that 13 transformants bear the correct fragments. The new plasmid was designated pDUB2507.

5.2.12 Transformation of pDUB2507 into Yeast

The methods described in section 5.2.4 were followed for preparation of MD40-4C competent cells and transformation. Transformants were then assayed for β-glucuronidase activity as described in section 5.2.6. The incubation of the reaction

Fig 5.2.10: Determination of β-glucuronidase and CPY activities in pDUBY2512 transformed cells. The method described in fig 5.2.6.1 was used.


CPY Activity (nmol/min/mg protein)
0
4
8
12
16
×10
Emission 455nm
0
0.75
1.50
Fraction No.
0
3
6
9
12
15
18
CPY
GUS
Spheroplast
CPY
GUS
Pellet
CPY
GUS

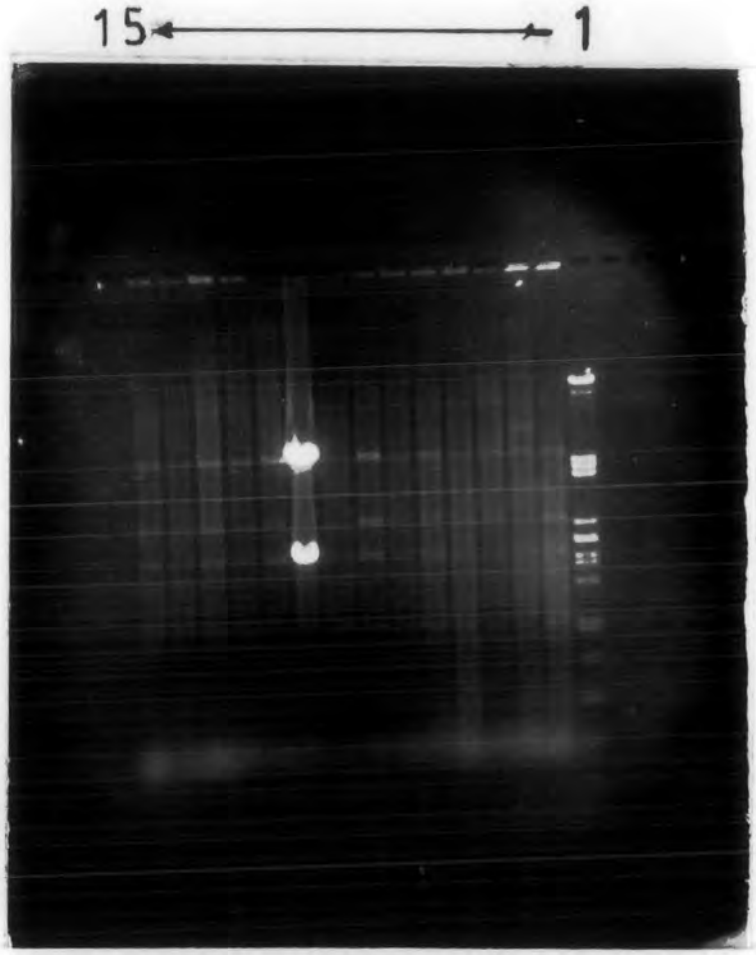


Fig 5.2.11: Agarose gel electrophoresis of pEMBLYe31 plus CaMV 35S-Gus gene transformants. Miniprep DNA was digested with HindIII plus EcoRI (Lanes 2-15). Lane 1, bacteriophage λ PstI marker.

mixtures was carried out for 15, 30, 60 and 90mins at 37°C. Comparing the results of β-glucuronidase activity obtained in transformed cells to that of non-transformed cells, it is clear that Gus expression in yeast under the control of the CaMV 35S promoter was achieved (Fig 5.2.12.1). However, the level of expression was low compared to the expression of Gus in yeast under the control of the CPY promoter (pDUB2505).

However, the determination of β-glucuronidase activity was in S100 and microsomal fractions of pDUB2507 transformed cells indicates that β-glucuronidase activity was absolutely located in the cytoplasmic fraction (Fig 5.2.12.2).

5.2.13 Deletion of the Gus Initiation Codon

The results obtained in sections 5.2.6 and 5.2.10 indicate that most of the β-glucuronidase activity was located in the cytoplasmic fractions when the expression of Gus is under the control of the CPY promoter and when Gus is fused to the CPY vacuole targeting signal. As the Gus in this construct still has its own initiation codon, it is possible that most of the Gus activity observed was initiated separately from that fused preproCPY. To overcome this problem, the Gus initiation codon was deleted.

To isolate the BamHI-EcoRI fragment containing the full length Gus gene, pDUB2503 (Section 5.2.2) was digested with BamHI plus EcoRI and the correct fragment isolated as described in section 5.2.7. This fragment was ligated with purified BamHI plus EcoRI cut pUC19 plasmid, and transformed into JM83. The transformants were purified and tested for the correct restriction pattern by analysing miniprep DNA. The results in fig 5.2.13.1 show that all the transformants have the Gus fragment and plasmid creating pDUB2511.

Fig 5.2.12.1: Determination of β-glucuronidase activity in pDUB2505, pDUB2507 and non-transformed MD40-4C cells. The method in fig 5.2.9 was used.

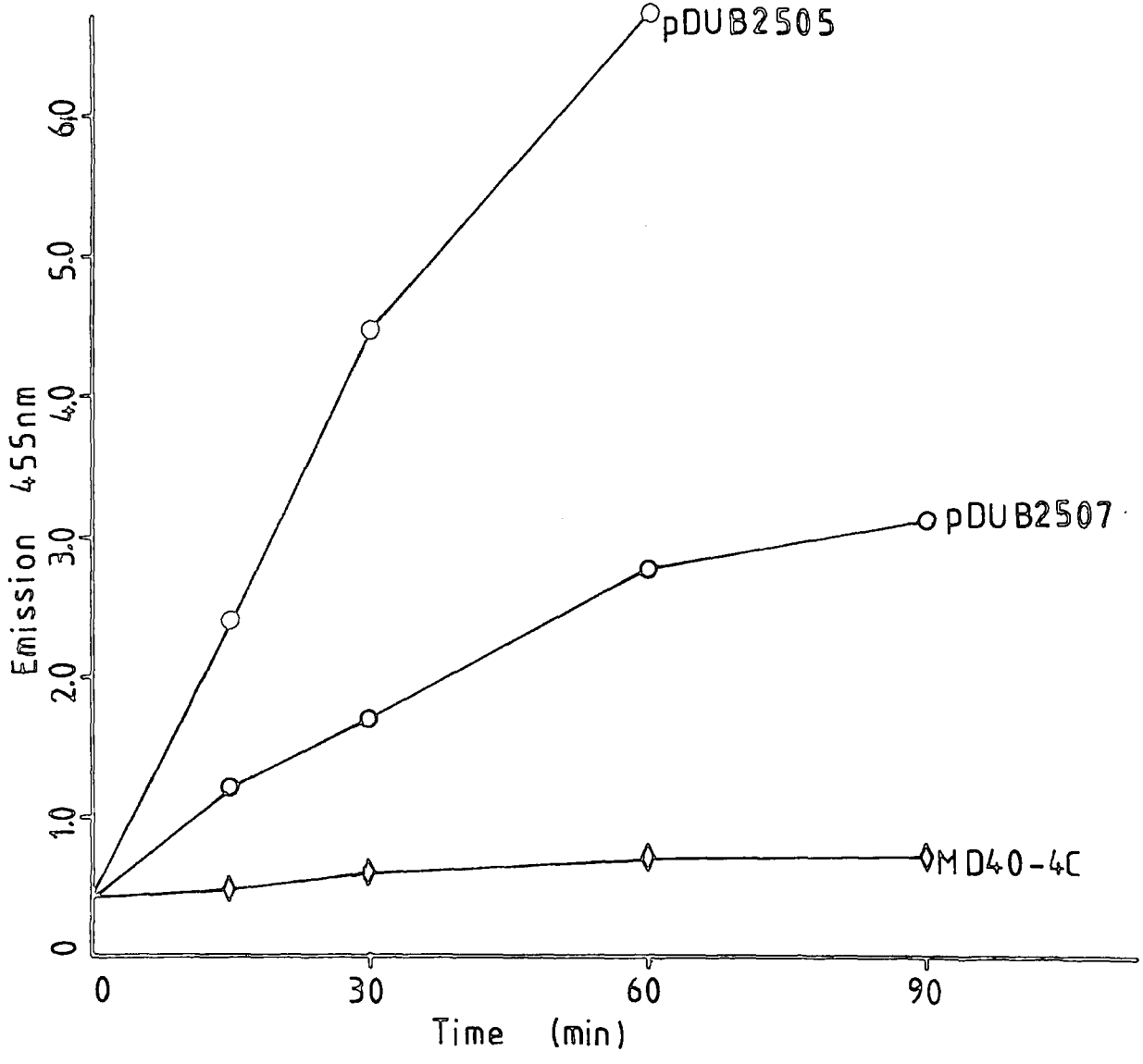
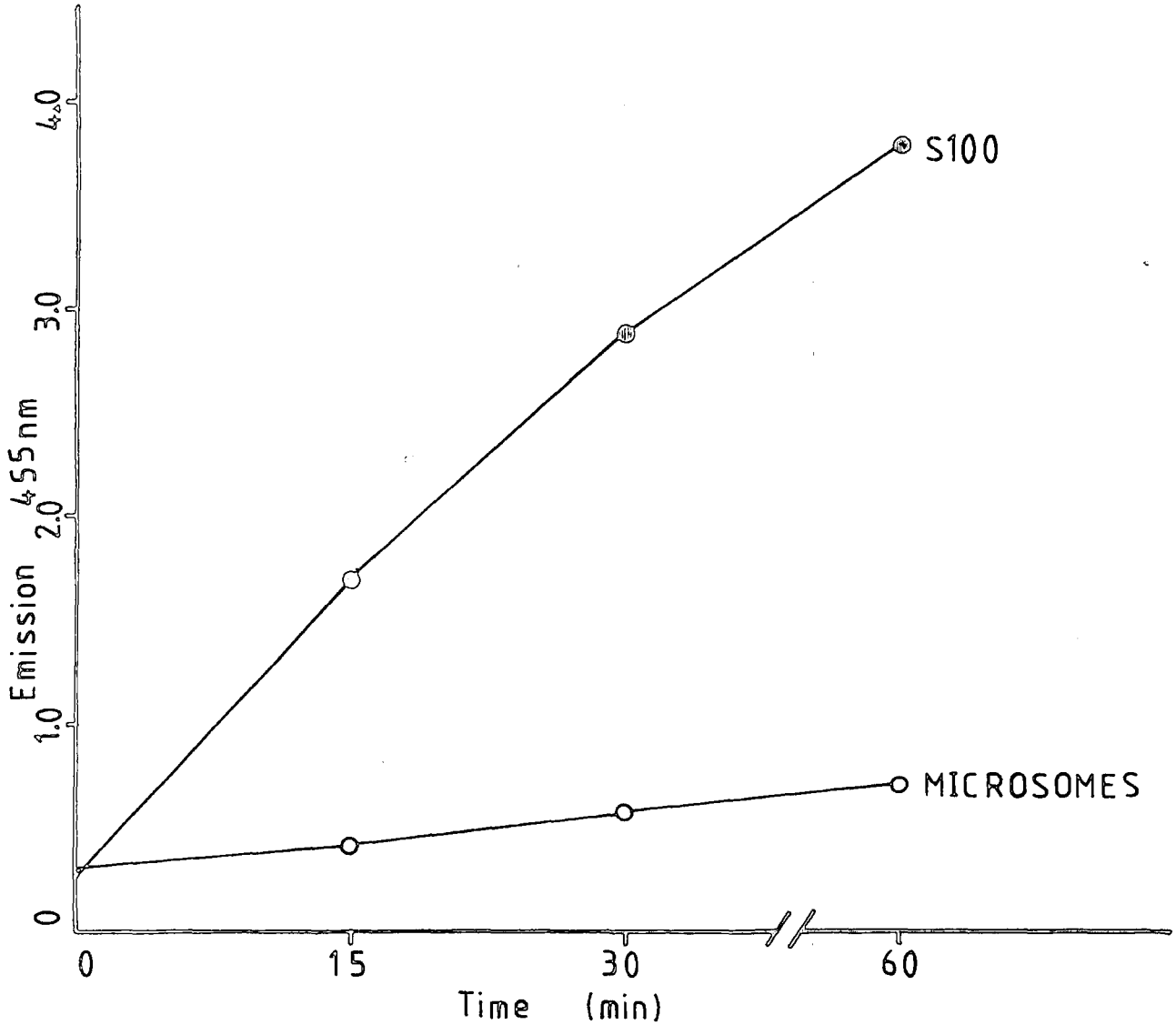
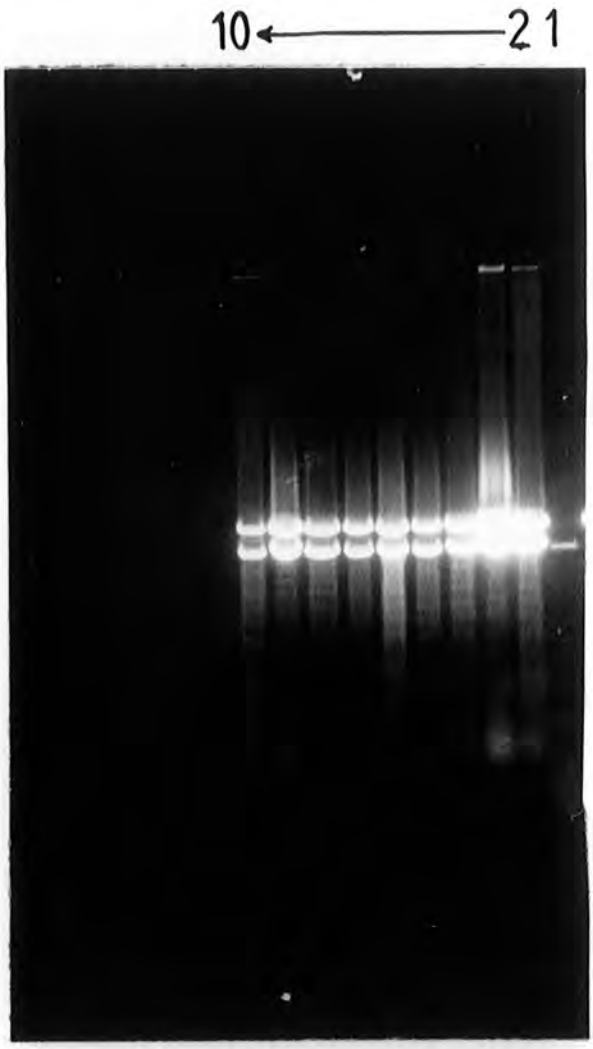

pDUB2505
pDUB2507
MD40-4C
Emission 455nm
0
1.0
2.0
3.0
4.0
5.0
6.0
0
30
60
90
Time (min)

Fig 5.2.12.2: Determination of β-glucuronidase activity in S100 and microsomal fractions of pDUB2507 cells. The method described in fig 5.2.12.1 was used except the incubation of the reaction mixtures was carried out for 15, 30 and 60mins.


Emission 455nm
0
1.0
2.0
3.0
4.0
S100
MICROSOMES
0
15
30
60
Time (min)



5.2.13.1: Agarose gel electrophoresis of pUC19 encoding the full length BamHI-EcoRI Gus gene. Miniprep DNA was restricted with BamHI plus EcoRI (Lanes 2-10). Lane 1, isolated pUC19 previously digested with BamHI-EcoRI.

In order to delete the Gus initiation codon using ExoIII/Mung bean nuclease, pDUB2511 plasmid was purified using the large scale method (Section 2.11.2). The plasmid was digested with BamHI. The BamHI restriction site is about 15bp away from the Gus initiation codon (Section 5.2.11). Therefore, at least 18bp needed to be removed to include the Gus initiator. ExoIII nuclease treatment was performed for 1min at 15°C followed by the Mung bean nuclease treatment as described in section 2.16. The deleted pDUB2511 was then ligated to a synthetic phosphorylated oligonucleotide bearing three restriction sites, namely, BamHI, BglII, and BclI. The ligated molecules were then digested with BamHI (Excess) plus EcoRI. The fused Gus gene-Linker was isolated from agarose gels, ligated with pUC19 vector and transformed into GM119 (Dam^-). The plasmid DNA recovered from the transformants was screened for the fused Gus-Linker by digesting separately with BamHI-EcoRI, BglII-EcoRI and BclI-EcoRI in double digests. The results presented in fig 5.2.13.2,A,B and fig 5.2.13.3 show that the transformants appear to have the correct fragment and plasmid. It is also clear that the size of fused Gus-Linker fragment when digested with BclI-EcoRI appears shorter than the normal size (Fig 5.2.13.3). The new constructed plasmid was termed pDUB2516.

5.2.14 Dideoxynucleotide Sequencing of Fused Gus-Linker

In order to be certain that the Gus initiation codon had been removed by the action of ExoIII/Mung bean nuclease, pUC19 reverse primer was used to sequence the alkaline denaturated DNA of the putative Gus linker fragment starting from the HindIII side as described in section 2.22. The DNA sequence obtained from the autoradiograph (Section 2.24) is shown in fig 5.2.14. The sequence indicates that the Gus initiation codon was not removed and the BclI restriction site in the linker is

Fig 5.2.13.2,A,B: pUC19 recombinant plasmids with putative Gus gene-Linker fragments restricted with BamHI-EcoRI and BglII-EcoRI. (In fig A and B even numbers, DNA digested with BamHI-EcoRI, odd numbers, DNA digested with BglII-EcoRI). Lane 1, in fig A and B, upper band purified BamHI-EcoRI pUC19 plasmid and lower band purified BamHI-EcoRI Gus gene fragment.

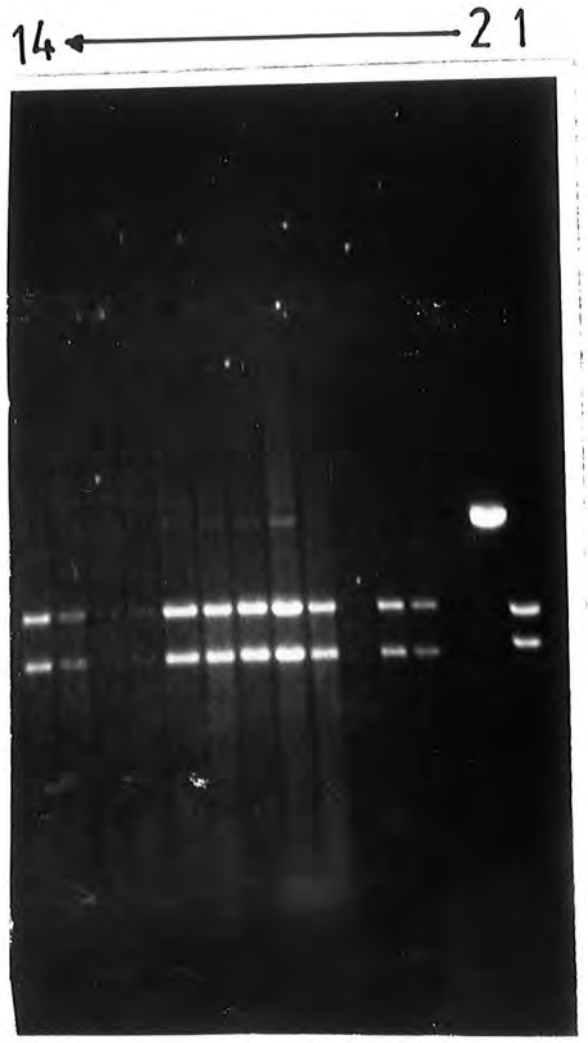
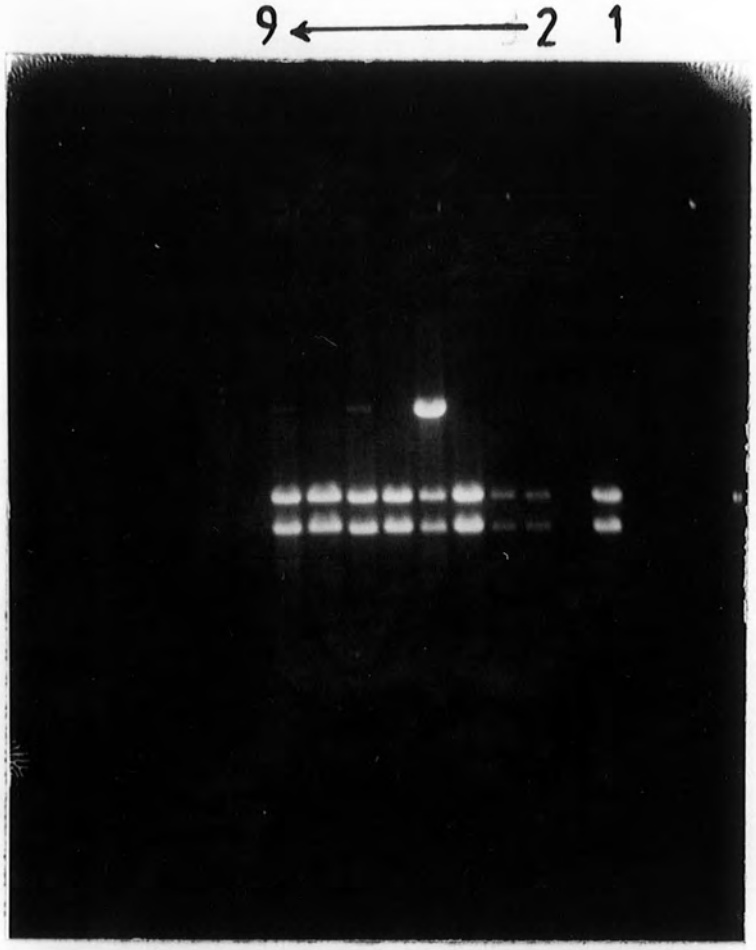


Fig 5.2.13.3: As fig 5.2.13.2 except digested with BclI-EcoRI (Lanes 2-14). Lane 1, upper band purified BamHI-EcoRI pUC19 plasmid and lower band purified BamHI-EcoRI Gus gene fragment.


9
2
1

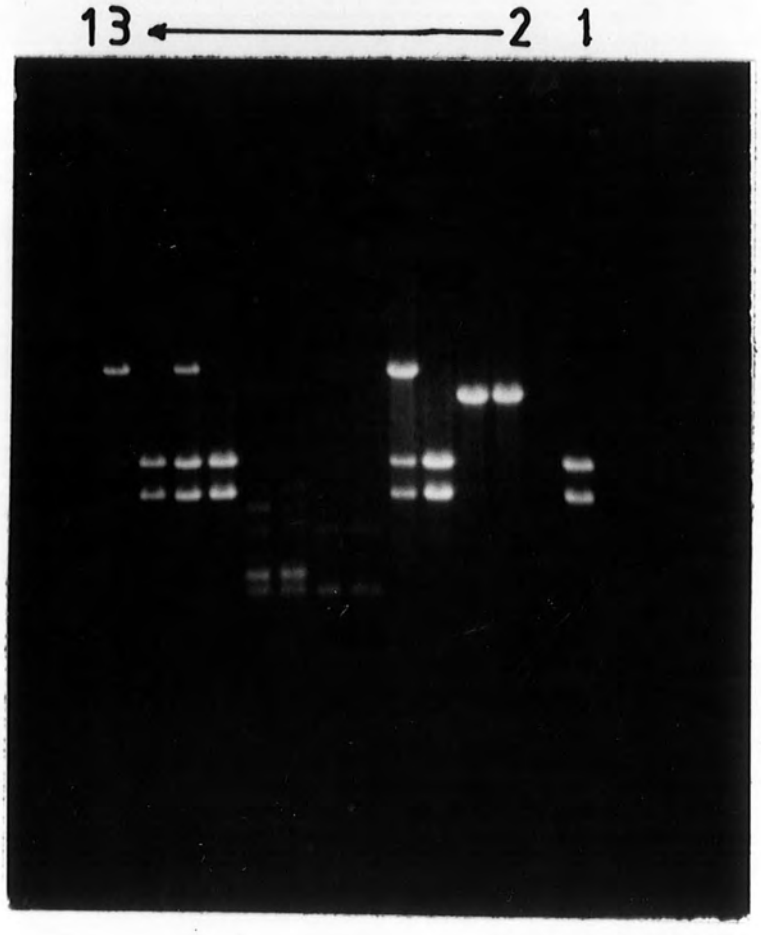
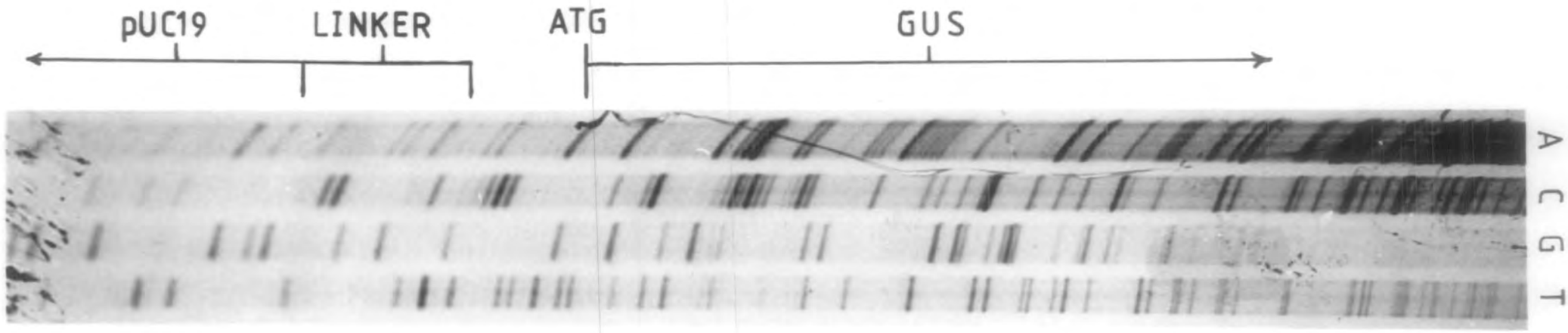

13
2
1

Fig 5.2.14: Sequencing gel autoradiograph. Sequencing the Gus-Linker fusion construct.


pUC19
LINKER
ATG
GUS
A
C
G
T

not there. By examining the Gus sequence (Jefferson ,*et al.*, 1986) it is clear that a BclI site exists in Gus, 90bp after the initiation codon. This observation explains the results shown in section 5.2.13 (Fig 5.2.13.3).

5.2.15 Restriction of Gus Gene with BclI

The BclI restriction site in Gus is compatible with a BamHI site. If Gus is restricted with BclI and ligated with the CPY prepro-fragment (Section 5.2.1) then an in-frame fusion is created. However, to be sure that removal of 90bp does not affect the expression of Gus in bacteria or in yeast, pDUB2516 was digested with BclI plus EcoRI in a double digest. The Gus fragment was isolated as before and ligated with BamHI-EcoRI cut pUC19 and transformed in JM83. This creates an in-frame *lacZ*-Gus fusion in pUC19. Miniprep DNA of transformants was then tested for the BamHI/BclI-EcoRI fragment as before by digestion with BamHI-EcoRI. Eleven transformants are correctly carrying the fragment since the BamHI/BclI hybrid restriction site is not affected by BamHI digestion (Fig 5.2.15.1). The new plasmid is pDUB2521. β-glucuronidase activity in pDUB2521 (Deleted Gus gene), pDUB2511 (Containing full length Gus gene) and non-transformed JM83 cells, was determined by growing them separately in 10ml L-broth plus selection overnight. The cells were digested with lysozyme in β-glucuronidase extraction buffer (section 2.4.10). The incubation of the reaction mixtures was carried out for 45mins at 37^{o}C and the results are presented in table 5.2.15.2. It is clear that the deleted Gus gene is active even when fused to β-galactosidase. This observation was also confirmed by exposing the reaction mixtures to U.V. light (Fig 5.2.15.3). The efficiency of Gus expression is about 2/3 of that found in the case of pDUB2511, the full length gus gene. Gus in both pDUB2511 and pDUB2521 is under the control of the *lacZ* promoter.

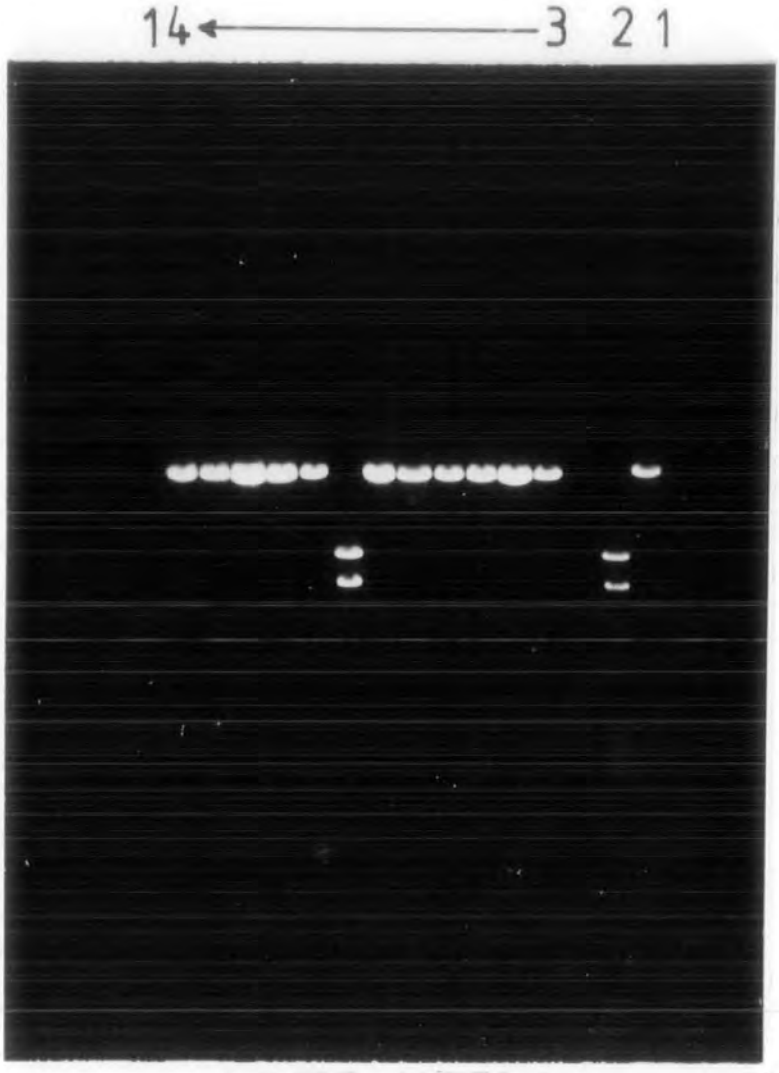


Fig 5.2.15.1: Agarose gel electrophoresis of pUC19 recombinants with the BcI-EcoRI Gus gene fragment recovered by DNA miniprep and digested with BamHI-EcoRI (Lanes 3-14). Lane 1, pDUB2511 encoding BamHI-EcoR1 Gus gene fragment digested with EcoRI only and lane 2, pDUB2511 restricted with these two enzymes.

Table 5.2.15.2: β-glucuronidase activities from 1mg protein of non-transformed JM83, pDUB2511 and pDUB2521 transformed JM83 obtained by dividing the absolute emission value at 455nm by the gain setting. Incubation of the reaction mixtures was carried out for 45mins at 37^{o}C.

Conditions	β-glucuronidase recorded value (1mg protein)
JM83	1.7
pDUB2511	30.0
pDUB2521	20.0

Fig 5.2.15.3: β-glucuronidase reaction mixtures of 1- blank, 2- non-transformed JM83, 3- pDUB2511 and 4- pDUB2521 exposed to U.V light.

5.2.16 Construction of a CPY-Deleted Gus Gene Fusion

DUB2500 (containing the CPY promoter and preproCPY fragment) was digested with BamHI plus EcoRI and the plasmid was isolated as described before (Section 5.2.1). It was then ligated to the purified BclI-EcoRI deleted Gus gene fragment from section 5.2.15 and transformed into JM83. The plasmid DNA recovered from subcultured colonies was screened for the BamHI/BclI-EcoRI fragment by restriction with BamHI-EcoRI. All tested transformants had the correct fragment and plasmid (Fig 5.2.16). The new plasmid is termed pDUB2522.

5.2.17 Cloning of a CPY-Deleted Gus Gene into pEMBLYe31

The methods described in section 5.2.1 were exactly followed for the HindIII plus EcoRI digestion, separation and isolation of pEMBLYe31. pDUB2522 was also restricted with the above enzymes and the CPY-Deleted Gus gene fragment isolated as above. They were ligated together and then transformed again in JM83. Plasmid DNA obtained from transformants was then digested with HindIII and EcoRI. The results presented in fig 5.2.17. show that seven transformants have the correct fragment and plasmid. The constructed plasmid was designated pDUB2523.

5.2.18 Transformation of pDUB2523 into Yeast

pDUB2523 DNA was transformed into MD40-4C using the method described in section 5.2.4. β-glucuronidase activity of the transformants was determined as described in section 5.2.5. Incubation of the reaction mixtures was carried out for 30,

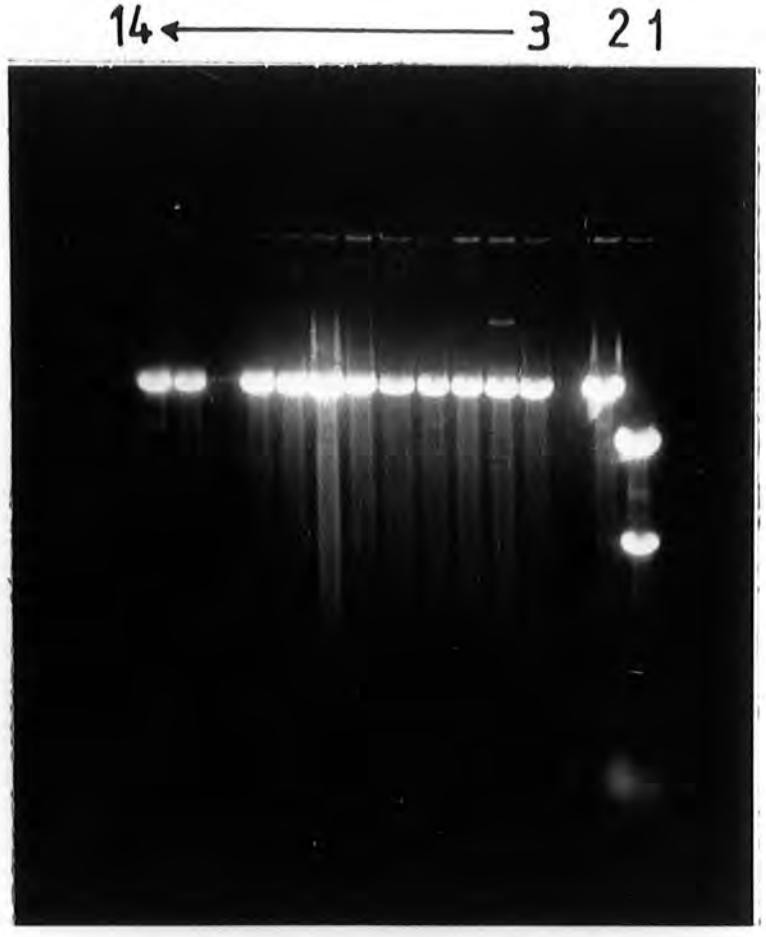


Fig 5.2.16: Agarose gel electrophoresis of pDUB2500 recombinants with the BclI-EcoRI deleted Gus gene. Plasmid DNA was digested with BamHI plus EcoRI (Lanes 3-14). Lane 1, pDUB2503 encoding full length CPY-Gus fusion digested with HindIII-EcoRI and lane 2, pDUB2503 restricted with BamHI only.

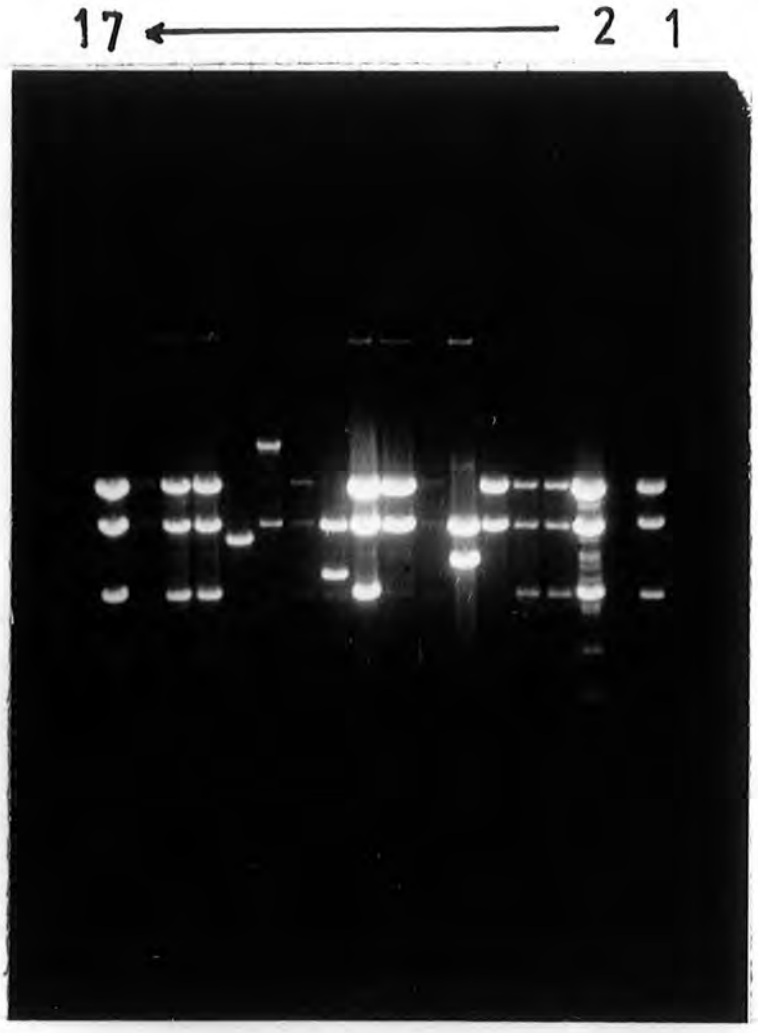


Fig 5.2.17: Agarose gel electrophoresis of pEMBLYe31 recombinants with the HindIII-EcoRI CPY-Deleted Gus gene fragment. DNA was recovered by minipreps and restricted with these enzymes (Lanes 2-17). Lane 1, pDUB2505 restricted with HindIII-EcoRI.

60 and 90mins at 37°C and the results of this experiments are shown in fig 5.2.18. As can be seen the transformants exhibit high β-glucuronidase activity compared to that of non-transformed MD40-4C cells.

5.2.19 Fractionation of Cell Components

The methods described in section 5.2.6 were followed for the growth of pDUB2523 transformed cells, preparation of sphaeroplasts and fractionation of cell components. In this case only three fractions were used for the determination of CPY and β-glucuronidase activities, namely, a cytoplasmic fraction , vacuole fraction and pellet plus another sample preserved from the sphaeroplast lysate. After fractionation, the vacuole fractions were pooled and the vacuoles precipitated by centrifugation. The supernatant was discarded and the vacuoles resuspended in 125μl of β-glucuronidase extraction buffer. CPY specific activity and β-glucuronidase absolute values were determined as described in section 5.2.10. The results presented in table 5.2.19.1 show that the highest CPY activity was located in the vacuole fraction and the total value obtained was greater than 10 times the CPY activity in other fractions. Determination of β-glucuronidase activity was achieved by using 25μl from each fraction separately per 200μl of reaction mixture. The incubations were carried out for 90mins at 37°C and the results are presented in table 5.2.19.2. It clear that the highest β-glucuronidase activity was located in the vacuole fraction and about 10-11 fold greater in the vacuole fraction compared to the sphaeroplast lysate.

To confirm the validity of the above observations, the determination of β-glucuronidase activity was repeated using equal amounts of protein from each fraction (0.012mg protein/200μl of reaction mixture). It can be seen from the results presented in ta-

Fig 5.2.18: Determination of β-glucuronidase activity in pDUB2523 transformed MD40-4C cells. The method in fig 5.2.9 was used.

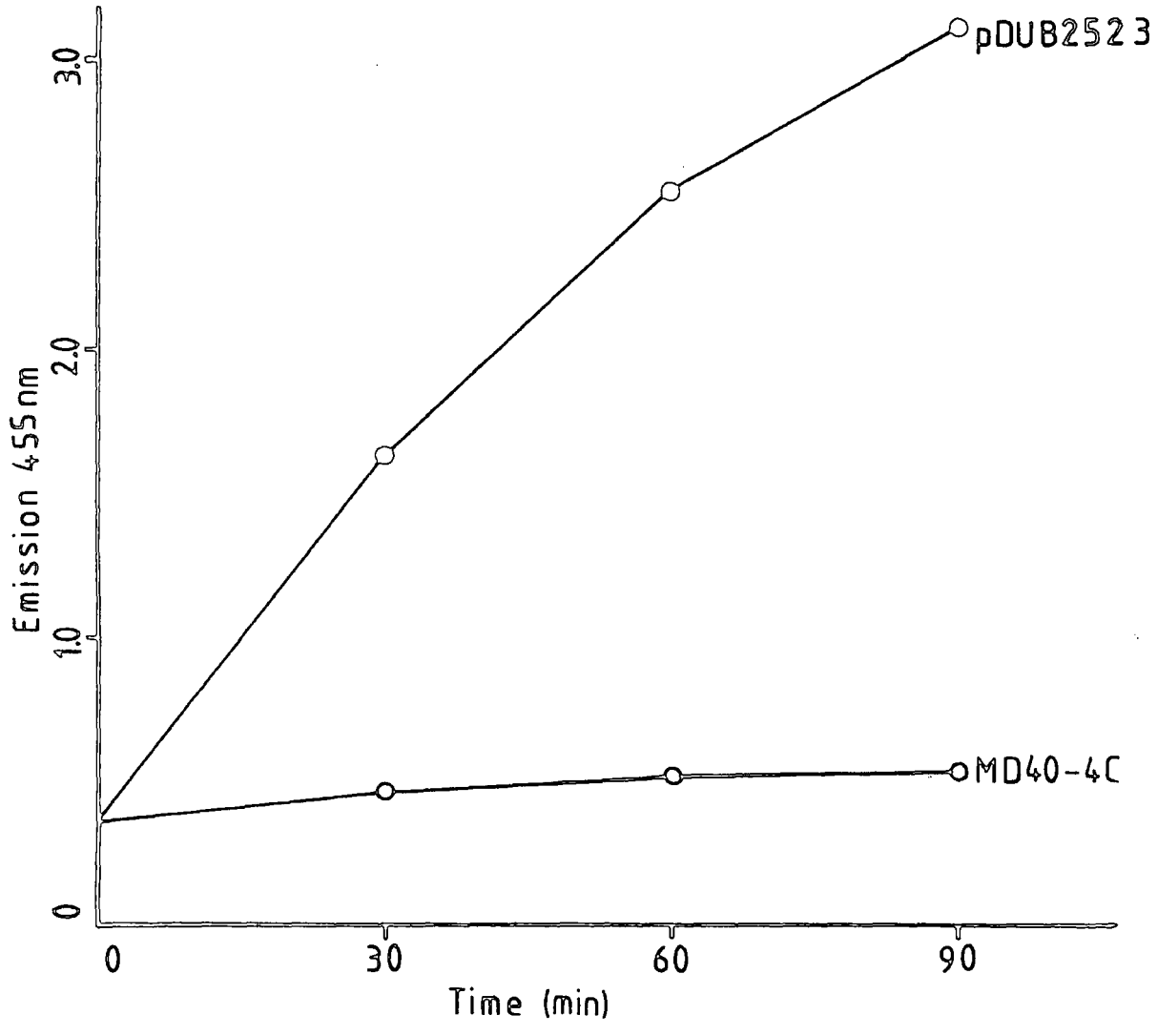

pDUB2523
MD40-4C
Emission 455nm
0
1.0
2.0
3.0
0
30
60
90
Time (min)

Table 5.2.19.1: Determination of carboxypeptidase Y specific activity in sphaeroplasts, cytoplasmic, pellet and vacuole fractions of pDUB2523 transformed cells. The method described in section 2.4.4 was used.

Conditions	CPY specific activity (nmol/min/mg protein)
Sphaeroplast lysate	76.59
Pellet fraction	38.13
Cytoplasmic fraction	79.95
Vacuole fraction	848.15

Table 5.2.19.2: β-glucuronidase recorded value obtained by dividing the absolute emmision value at 455nm by the gain setting. Incubation of the reaction mixtures was carried out for 90mins at 37°C.

Conditions	β-glucuronidase recorded value
Sphaeroplast lysate	3.30
Pellet fraction	2.57
Cytoplasmic fraction	3.38
Vacuole fraction	41.66

ble 5.2.19.3 that the differences in β-glucuronidase activity seen in table 5.2.19.2 are not due to differences in protein concentration, but are due to genuine differences in sub-cellular localization.

5.3 Discussion

The traditional reporter gene, especially with bacterial systems, is *lacZ* which codes for β-galactosidase. However, the first use of *lacZ* to study the secretory pathway in yeast proved that it was unsuitable (Emr, *et al.,* 1984). Fusion of a signal peptide to β-galactosidase allowed it to enter the lumen of the ER, but because of its size (125kD) it accumulated there and was not transported further. Subsequent experiments have used a natural yeast reporter invertase. Whilst valuable information has come from using invertase as a reporter, it has certain inherent disadvantages. It is heavily glycosylated, and western blots of cell extracts using anti-invertase antibody do not give clear bands due to its size heterogeneity. It is an endogenous enzyme found both as secreted and cytoplasmic forms. To avoid confusion of results when using invertase as a reporter, the host gene, *SUC2*, must be deleted. This means that for many experiments, tedious strain construction must be carried out before experimental work can begin. Thus a reporter gene with the advantages of β-galactosidase, and that is not normally found in yeast, would be ideal. The Gus gene appears to suit admirably. β-glucuronidase is a small protein (68.2kD), stable and has a wide range of synthetic substrates.

The sorting of vacuolar proteins, such as, CPY is signal dependent (Johnson, *et al.,* 1987; Valls, *et al.,* 1987). Therefore, sorting of β-glucuronidase to the yeast vacuole requires it to be fused to a vacuolar targeting signal such as the CPY prepro-

Table 5.2.19.3: β-glucuronidase recorded value for 0.012mg protein and specific activities of sphaeroplast lysate, pellet, cytoplasmic and vacuole fractions. The method described in table 5.2.19.2 was used.

Conditions	β-glucuronidase recorded value (0.012mg protein)	β-glucuronidase specific activity per 1mg protein
Sphaeroplasts lysate	0.043	3.58
Pellet fraction	0.036	3.0
Cytoplasmic fraction	0.05	4.16
Vacuole fraction	0.46	36.33

sequence. Results of sections 5.2.1, 5.2.2 and 5.2.3 show the successful isolation of the CPY prepro-fragment, a full length Gus gene and the construction of CPY-Gus gene fusion. The created plasmid, pDUB2505, when expressed in *S. cerevisiae* (MD40-4C) shows that Gus was expressed in yeast with high efficiency (Fig 5.2.5.1). Because of the vacuolar targeting signal fused to Gus, β-glucuronidase activity should have been located in the vacuole of the transformed cells. However, results of the cell fractionation experiments (Sections 5.2.6 and 5.2.10) show clearly that most of the β-glucuronidase activity was found in the cytoplasmic fraction rather than vacuole fractions. Some of the Gus activity was found in vacuolar fraction. This might be due to contamination of the vacuolar fraction with cytoplasmic fraction, or due to the sorting of the Gus product to the vacuole by the action of vacuolar sorting signal. β-glucuronidase activity was also found in both S100 and microsome fractions (Section 5.2.6) but Gus activity in the S100 is about 2 fold more than that in the microsomal fraction.

The above results could have one of several explanations. Firstly, the fusion is incorrect. Secondly, the prepro-fragment does not sort Gus to the vacuole. Thirdly, pDUB2505, is a multicopy plasmid based on pEMBLYe31. Expression of *PRC1* on a multicopy plasmid causes saturation of the vacuolar sorting pathway and secretion of proCPY (Stevens, *et al.*, 1986a,b). Over expression of CPY-Gus could also cause saturation of the pathway and even translocation into the ER, thus accounting for the cytoplasmic location. Lastly, Gus expression on pDUB2505, could be independent of CPY, as the coding region still has its own initiation codon.

When YCp50, containing CEN4 was substituted for pEMBLYe31 for expression of the CPY-Gus gene fusion, the expression of β-glucuronidase was much lower (Section 5.2.10, fig 5.2.10), however, the location was unchanged. Therefore, one can rule

out over-expression as an explanation of the results. Perhaps the translation of the majority of the Gus protein is initiated independently, but a small fraction of the Gus protein, that observed in the vacuole fractions and microsomes, might be due to the correct formation of a CPY-Gus hybrid protein.

To prevent this independent initiation the Gus initiation codon was removed by Exonuclease III/Mung bean nuclease treatment and replaced with a synthetic oligolinker bearing BamHI, BglII and BclI restriction sites. In order to confirm that the Gus start codon had been removed it was subjected to dideoxynucleotide sequencing. The results of this experiment (Fig 5.2.14) showed that Gus initiator was not deleted and the BclI restriction site encoded in the linker was not present. By analysing the Gus gene sequence (Jefferson, *et al.*, 1986) it was found that an internal BclI restriction site 90bp after the Gus start codon could be ligated to the 3′ BamHI site of the CPY prepro-fragment and maintain the correct reading frame.

The prepro-CPY-Deleted Gus gene fusion in pEMBLYe31 was transformed into MD40-4C (Section 5.2.18). Results indicate that the deleted Gus gene is functional and can be expressed in yeast when it is under the control of the CPY promoter. Cell fractionation results show the localization of Gus protein in the vacuole. β-glucuronidase specific activity in the vacuole is 11-13 fold greater than its activity in the sphaeroplasts or other cell fractions (Results of tables 5.2.19.2 and 5.2.19.3). Thus the conclusion of Johnson, *et al.*, (1987), who observed the targeting of yeast invertase to the vacuole when fused to the CPY prepro-fragment, that this signal is both necessary and sufficient for vacuolar targeting, is correct.

Independent confirmation that CPY-Gus is indeed routed through the vacuolar pathway can be made by the analysis of the CPY-Gus fusion in *sec* strains. The

prepro-fragment of CPY that is fused to Gus should still be processed as normal, unless some protein domain towards the C-terminal end of CPY is important for processing. Thus in *sec53* strains preproCPY-Gus should accumulate, in *sec18* strains proCPY-Gus should accumulate and mature CPY-Gus should accumulate in the vacuole. If the mature hybrid protein is glycosylated then differences between *sec18* (Core glycosylated) and *sec7* (Fully glycosylated) should also be apparent. these differences can readily be detected by the use of anti-Gus antibody. The N-terminal CPY domain fused to Gus in the hybrid protein is not recognised by the anti-CPY antibodies I have used.

The CPY-Gus fusion should be enormously useful for studing vacuolar sorting. Mutations that mislocalize the fusion to the periplasmic space will cause Gus activity to appear at the cell surface. Such mutants will give rise to blue-colonies on X-gluc plates. Wild type strains with the CPY-Gus fusion give white-colonies on X-gluc plates. A simple reversal of this procedure will enable genes from a gene bank to be isolated. Those that complement mutant strains will give rise to colonies that are white amongst the blue non-complemented colonies.

CHAPTER SIX

6. CONCLUSION

Yeast is a particularly attractive system for the study of protein transport because the pathways of protein modification and transport are well characterized (Schekman, 1985) and mutants defective in secretion and vacuolar targeting have been isolated (Novick, *et al.*, 1981; Bankaitis, *et al.*, 1986; Rothman and Stevens, 1986; Banta, *et al.*, 1988; Robinson, *et al.*, 1988). Therefore I have constructed a system to study the transport of proCPY from Golgi vesicles to vacuoles in an *in vitro* reconstituted cell free system. According to this system the accumulated proCPY in Golgi vesicles was purified as a part of microsomal fraction and mixed with purified vacuoles isolated from a CPY deficient strain that is PEP4 and capable of processing proCPY to a mature form. The transport of proCPY was determined enzymatically as only mature CPY is active. The system requires ATP and is stimulated by soluble factors (S100). The reconstitution was also temperature sensitive reflecting the *in vivo* temperature sensitivity of the *sec* donor strain use to accumulate proCPY in Golgi vesicles. These obseravtions are in agreement to those previously described by Haselbeck and Schekman (1986) for the transport of core glycosylated invertase from the ER to the Golgi complex using *sec18* strains.

The previously documented sensitivity of *sec7* thermoreversibility to glucose concentration (Novick, *et al.*, 1981; Scheckman, 1982) is also confirmed in my system. My results show transport of proCPY was only achieved in the reaction mixtures containing microsomes obtained from a *sec7* strain grown with 0.2% glucose. Such strains when grown with 2% glucose show an irreversible phenotype *in vivo*. This is also shown *in vitro*, where no transport was observed

There are several observations to support the conclusion that the *in vitro* maturation of CPY is due to correct transport of proCPY from microsomal membrane to vacuoles. 1. The addition of inhibitors of proteinases A and B have a negligible effect on the processing of proCPY. 2. Disrupting the membranes by TritonX-100 and the addition of proteinases inhibitors, inhibited the maturation. 3. The maturated CPY is sedimentable. 4. Analysis with western blots shows a clear band of mature CPY only in the sedimented fraction of the reaction mixtures containing microsomes isolated from *sec7* grown with 0.2% glucose.

For *in vivo* protein sorting to the vacuole in yeast, we are interested in the signals that are necessary for directing the products of heterologus genes to the vacuole. This was achieved by generating a CPY-Gus gene fusion encoding the promoter and prepro-sequence of CPY and the entire *E. coli* Gus gene (β-glucuronidase) that is not normally found in yeast. The results indicate that Gus was expressed in yeast with high efficiency, but sub-cellular localization shows that the Gus product was located in the cytoplasmic fraction due to the independent initiation of Gus gene expression from its own initiation codon. We found that deletion of the Gus gene 5' end including the start codon, does not inhibit Gus activity. This shortened Gus was fused to CPY and expressed in yeast under the control of the CPY promoter. Results of sub-cellular fractionation indicate that the Gus product was located in the vacuole. Thus the conclusion of Johnson, *et al.*, (1987) who observed the targeting of yeast invertase to the vacuole when fused to the CPY prepro-fragment, that this signal is both necessary and sufficient for vacuolar targeting is correct.

However, the observed activity of the shortened Gus in transformed yeast is about 1/5 of its normal activity and this can be explained as follow:- Firstly, when the shortened Gus was expressed in *E. coli* only 2/3 of its activit. Secondly, Gus

has two N-linked glycosylation sites, which have an inhibitory effect on Gus activity. This effect was shown when Gus was translocated to the ER of transformed tobacco (Iturriaga, *et al.*, 1989). Thus passage of Gus through the ER results in the almost complete inhibition of enzyme activity and it is difficult to determine accurately, by enzyme assay the true amount of Gus transported to the vacuole.

CHAPTER SEVEN

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