Characterisation of *Candida albicans* adhesion to epithelial cells

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Presented for the degree of Doctor of Philosophy in the
Faculty of Science, University of Glasgow.

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August 1995

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ACKNOWLEDGEMENTS

There are a number of people I wish to thank for helping me during the production of this thesis.

I am most grateful to my project supervisor Dr. L. J. Douglas for her help, patience, criticism and for providing such a stimulating project.

I must also thank the departmental heads, Professors A.C. Wardlaw, J.H. Freer and D.E.S. Stewart-Tull, for providing scientific and non-scientific assistance throughout my time at the University of Glasgow.

I am also indebted to Mr. A. Ellis and his team of technicians who assisted me with technical problems too numerous to mention.

I wish to thank Prof. H.G. Nimmo (Dept. of Biochemistry, University of Glasgow) for his expert assistance with HPLC and Drs. G. Curry, and M. Cusack (Dept. of Geology and Applied Geology) for their amino acid analysis and peptide sequencing expertise.

The support, encouragement and friendship extended to me by the remainder of the Dept. of Microbiology (staff and students past and present) helped me immensely and made my time in Glasgow one of the most rewarding times of my life.

Finally I wish to express my gratitude to my family and friends. They have suffered me being occasionally grumpy and often downright rude, for what seems like the last 50 years or so, during the production of this tome.

B.J.C.

DECLARATION

This thesis is the original work of the author.

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SUMMARY

The aims of this study were (i) to analyse an adhesin from *C. albicans* and (ii) to identify the epithelial cell molecule(s) to which the adhesin binds. Extracellular polymeric material (EP), composed mainly of mannoprotein, is thought to originate from the cell surface when yeasts are grown in medium containing a high concentration of galactose. Previous work has shown that this material contains a proteinaceous adhesin. More recently, a complete purification scheme for the yeast adhesin was devised (Tosh and Douglas, 1992) which made use of the findings from earlier studies on adhesion mechanisms. The purification protocol involves the stepwise treatment of EP with N-glycanase, papain, and dilute alkali followed by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen on inert silica beads. Adhesin purified in this way is devoid of carbohydrate, can inhibit adhesion of yeasts to buccal epithelial cells (BEC) by up to 80%, and consists of one major component as determined by reverse-phase fast protein liquid chromatography (FPLC). Purified adhesin was prepared in this study and it was found to inhibit adhesion of *C. albicans* GDH 2346 cells to BEC by up to 74.81%.

EP was subjected to some of the treatments previously found to have an effect on its adhesion-inhibition activity, prior to analysis by reverse-phase high performance liquid chromatography (RP-HPLC). Each of the treatments produced some alteration in the HPLC profile of EP and the combined treatment of EP with all the above reagents (N-glycanase, papain, and dilute alkali) resulted in the production of a number of protein fragments. The fragments appeared to be more hydrophobic than the parent molecule.

The purified adhesin was analysed further. Amino acid analysis showed that it was mainly composed of the amino acids Thr, Pro, Ala, Ser, Val, Gly, Asx and Glx, which made up around 80% of the total amino acids. No Cys was detected and there was very little Met, Phe and Arg. This composition is remarkably similar to a large number of microbial adhesins which would appear to indicate a relationship between the amino acid composition of lectin-like proteins and their function as carbohydrate binding molecules.

In previous studies, the visualisation of EP and purified adhesin on SDS-PAGE proved difficult. However, in this study, an alternative approach to protein visualisation was employed. Purified adhesin was biotinylated prior to SDS-PAGE and Western blot analysis. The adhesin was then probed for using ^{125}I-streptavidin and, after autoradiography, was seen as a strong band with a molecular mass of approximately 15.7 kDa. Its successful visualisation meant that partial amino acid sequencing could be attempted. The following sequence was obtained; Phe-Asp-Tyr-Glu-His-Val-Asp-X-Ala-Val. There was no complete match found in the protein database but the purified adhesin sequence contains an interesting motif, FDYE, that is found in a wide range of proteins from a variety of sources. The partial amino acid sequence obtained for the adhesin is most probably an internal sequence, produced as a result of enzymic cleavage by the action of papain.

The second aim of this study was to identify receptors for *C. albicans* on buccal epithelial cells. BEC glycolipids were prepared and a glycolipid chromatogram overlay system was employed. This system has been used to characterise a number of microbial adhesin-receptor relationships. Six strains in total were examined for their ability to bind to separated glycolipids. Five strains bound to glycolipids expressing H-blood group antigen as indicated by comparison with an identical chromatogram overlaid with biotinylated *Ulex europaeus* lectin. These strains also bound to an additional lipid from BEC which appeared to have a terminal N-acetyl-D-galactosamine residue, as determined by comparison with an identical chromatogram overlaid with N-acetyl-D-galactosamine-specific lectin from *Helix pomatia*. However, the yeast cells did not bind to control glycolipids with N-acetyl-D-galactosamine as a terminal sugar residue and so N-acetyl-D-galactosamine *per se* is unlikely to be the receptor. From the relative mobility of this *Candida* binding lipid and the control lipids, the yeast cells may be binding to blood-group A antigen, to which *Ulex europaeus* lectin cannot bind. This would suggest that fucose alone and not just the H antigen can act as a receptor for *C. albicans*. In a similar experiment, EP and purified adhesin from GDH 2346 were biotinylated and overlaid onto TLC plates of separated glycolipids and probed with

^{125}I-streptavidin prior to autoradiography. EP and purified adhesin were found to bind to H-blood group antigen but there was no binding to the additional lipid which was bound by the whole cells. The component on the *Candida* cell surface that binds to the additional receptor may not be released as part of EP or the ability of EP to bind to this antigen may be lost during the purification procedure. The binding of whole cells and EP from the sixth strain, GDH 2023, to glycolipids was also examined by the chromatogram overlay assay. Biotinylated whole cells, EP and biotinylated *Griffonia simplicifolia* lectin II (specific for N-acetyl-D-glucosamine) were overlaid onto TLC plates of separated lipids as previously described. By comparison of the resultant autoradiographs both EP and whole cells from this strain were seen to bind to glycolipids containing N-acetyl-D-glucosamine. These results support those of earlier investigators which suggested that strains GDH 2346, GRI 681, GRI 682, and MRL 3153 all share a common adhesion mechanism involving L-fucose-containing receptors but that strain GDH 2023 differs in that its adhesion mechanism involves GlcNAc-containing receptors. Use of the TLC overlay analysis system in the present study demonstrates unequivocally the identity of glycoconjugates as receptors and confirms that adhesin molecules on the yeast cell surface bind in a lectin-like manner to epithelial cell components containing L-fucose or GlcNAc.

ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
AII	Adhesion inhibition index
BEC	Buccal epithelial cells
BP	*Bauhinia purpurea* lectin
BSA	Bovine serum albumin
C3d	Complement conversion product
CDH	Ceramide dihexose
Cer	Ceramide
Ci	Curie
CMC	Chronic mucutaneous candidosis
CMH	Ceramide monohexose
CR3	C3 receptor
CTH	Ceramide trihexose
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EP	Extracullular polymeric material
5-FC	5-fluorocytosine
FN	Fibronectin
FORS	Forssman antigen
FPLC	Fast high pressure liquid chromatography
Fuc	L-Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GDH	Glasgow Dental Hospital
GL4	Globoside
GlcNAc	N-acetylglucosamine
Glu	D-Glucose
GM_1	Ganglioside M_1
GM_2	Ganglioside M_2
GRI	Glasgow Royal Infirmary
GSII	*Griffonia simplicifolia lectin* II
HIV	Human immunodeficiency virus
HP	*Helix pomatia* lectin
iC3b	Complement conversion product
Ig	Immunoglobulin (IgG, IgA, IgM, IgE, IgD-sub classes)

kDa	kilodaltans
Le^a	Lewis^a antigen
Le^b	Lewis^b antigen
M	Molar
mAB	Monoclonal antibody
MRL	Mycological Reference Laboratory
MWM	Molecular weight markers
NCYC	National Collection of Yeast Cultures
P	Probability value
PA	Purified adhesin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PIBMA	Polyisobutylmethacrylate
PMN	Polymorphonuclear leukocytes
RGD	Arginine-glycine-aspartic acid peptide
RP-HPLC	Reverse-phase high performance liquid chromatography
SAP	Secreted aspartyl proteinase
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sRBC	Sheep red blood cells
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
UE	*Ulex europaeus* lectin I
VEC	Vaginal epithelial cells
YNB	Yeast nitrogen base

AMINO ACID CODES

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

INTRODUCTION

1. THE GENUS *CANDIDA*

1.1 Historical Aspects

The earliest recorded descriptions of thrush as a clinical entity were made by Hippocrates in his "Epidemics" (circa 460-377 B.C.) when he described two cases of oral aphthae associated with underlying diseases (Adams, 1939). Conditions recognisable as oral and gastrointestinal candidosis were described by Rosen von Rosenstein (1771) in a paediatrics text.

The initial discovery of the organism causing thrush was not made until 1839 when Langenbeck described a fungus in buccal aphthae in a case of typhus. A great deal of confusion surrounded the taxonomic study of the thrush fungus for a century following Langenbeck's description. Gruby placed the fungus in the genus *Sporotriticum* in 1842, while Robin, in 1853, named it *Oidium albicans.* This was the first use of the name albicans which is derived from the Latin for "whitening". Quinquaud proposed the name *Syringospora robinii* in 1868 while Reess, in 1877, named the fungus *Saccharomyces albicans. Monilia albicans* was the name proposed by Zopf, in 1890. This name was retained for a number of decades and gave rise to the term "moniliasis" for infections due to the thrush fungus.

The taxonomy of the fungus was clarified by Berkhout in 1923 and she proposed the name *Candida* for the genus which was later adopted internationally. *Toga candida* is Latin for the white robe worn by "candidates" for the Senate. So in combination, *Candida* and *albicans* means "whitening white".

1.2 Ecological niche

Yeasts can be isolated from a wide variety of habitats including plants, insects and soil (Kreger van Rij, 1984). However, the yeast species acknowledged as principal agents of human candidosis have a comparatively restricted natural distribution, and have been discovered primarily in association with man and other

warm-blooded animals (Odds, 1988). *Candida* species are also frequently found in the hospital environment: in foods, in the air, and on a variety of fomites (Barnett *et al.*, 1983). They are commonly isolated from the mouth, gut and vagina of apparently healthy individuals. The majority of studies on yeast carriage have been conducted on subjects who are attending hospitals and clinics, and as *Candida* species are opportunistic pathogens, the results obtained may not reflect a truly normal population (Odds, 1988). *Candida albicans* is known to colonise humans more frequently than other *Candida* species. *C. glabrata* and *C. tropicalis* can be found in the normal flora of the oral cavity, gastro-intestinal tract and vagina, but less frequently than *C. albicans. C.krusei, C. guilliermondii* and *C. parapsilosis* are found more frequently as part of the skin flora. Colonisation rates increase dramatically for all species in patients receiving cytotoxic drugs that alter the gastrointestinal mucosa, and/or broad spectrum antibiotics (Odds, 1988).

2. BIOLOGY OF *CANDIDA*

2.1 Classification

Yeasts were defined by Lodder (1970) as fungi whose predominant morphological form is unicellular. This definition was refined by Kreger van Rij (1984) who described yeasts as unicellular fungi capable of reproducing by budding or fission.

The genus *Candida* as a whole comprises 196 species. Among these species, *C. albicans* isolates are consistently the most virulent. *C. tropicalis* is also pathogenic but is quantitatively less so than *C. albicans.* Less pathogenic are *C. parapsilosis, C. pseudotropicalis, C. glabrata. C. kefyr, C. guilliermondii* and *C. krusei.* Another two species, *C. lusitaniae* and *C. viswanathii,* are possibly to be regarded as pathogenic but only in the immunocompromised host (Odds, 1988). The species within the genus are separated primarily on the basis of their physiological properties. Each species has a characteristic pattern of assimilation reactions and relationships between species

may be inferred by comparisons of such patterns. *C. albicans* can be easily and quickly identified by using either the germ tube test (Taschjian *et al.*, 1960) or the test for chlamydospore production (Benham, 1931; Kligman, 1950; Dawson,1962).

2.2 Typing systems

Typing systems for the medically important fungi have not received the same attention as the systems used for pathogenic and epidemiological studies with bacteria and viruses (Merz, 1990). However both genotypic and phenotypic approaches are now being employed and the latest methodologies offer good to excellent discrimination between different species within the genus *Candida* (Odds, 1993). Typing systems based on genotypic differences have obvious advantages over phenotypic approaches, but problems of analysing the genetics of *Candida* species have been hampered by the absence of a sexual cycle and the lack of molecular tools (Merz, 1990).

Hasenclever and Mitchell (1961) were the first to demonstrate the existence of two antigenic types of *C. albicans* - serotypes A and B. The A serotype possesses the same group of antigens as serotype B, plus two extra antigens. The two types differ in their cell-wall structure. New approaches to biotyping include quantitation of relative growth in the presence of various inhibitors or substrates, typing of isoenzyme patterns (Lehmann *et al.*, 1989), and assessment of colour and morphologies of colonies on medium containing tetrazolium salts (Odds, 1993). A number of other methods of biotyping have been described including chromatographic assessment of yeast cell fatty acids (Brondz *et al.*, 1989) and whole cell protein electrophoresis (Vancanneyt *et al.*, 1991).

Genotypic identification and characterisation of species and strains within the genus *Candida* is currently an area of active research. Individual strains of *C. albicans* may now be typed by use of DNA restriction fragments in Southern blots probed with species-specific mid-repeat-sequence oligonucleotides (Odds, 1993). Random amplified polymorphic DNA profiles can also be used for providing

genotypic characters for taxonomic descriptions, for confirming the identities of stock isolates, for typing *Candida* species in epidemiological investigations, and for the rapid identification of pathogenic fungi (Lehmann *et al.*, 1992).

2.3 Genetics

Until relatively recently, the genetics of *Candida* species was little studied. However, in the last decade, progress has been rapid and a number of reviews on the subject have been published (Shepherd *et al.*, 1985; Whelan, 1987; Kurtz *et al.*, 1988; Magee *et al.*, 1988; Scherer and Magee, 1990; Ernst, 1990; and Lott *et al.*, 1992). Genetic analysis is complicated by the diploid nature of most of the species (not *C. glabrata*) and the absence of any known sexual cycle. The close similarity between *C. albicans* and the more often studied *Saccharomyces cerevisiae* has been useful in the isolation of *Candida* genes and the development of the *C. albicans* transformation system (Odds, 1988). The genetics of *C. albicans* is becoming increasingly important in researching virulence determinants, phenotypic variants and drug resistance.

When *C. albicans* is isolated it is normally diploid and naturally heterozygous (Whelan *et al.*, 1980). An important implication of this elevated ploidy is that it provides a significantly greater potential for genetic diversity than would be found in a haploid strain.

Candida genes cloned via their counterparts in *S. cerevisiae* range from those coding for actin (Mason *et al.*, 1987) and tubulin (Smith *et al.*, 1988) to those for amino acid biosynthesis (Rosenbluh *et al.*, 1985) and sugar utilisation (Magee *et al.*, 1988). Goshorn and Scherer (1989) were able to isolate several *Candida* biosynthetic genes by complementation. Mycophenolic acid-resistant auxotrophs were used to analyse prototrophic natural variants by spheroplast fusion. The fusion products were shown to be heterozygous for many of the parental chromosomes by molecular and genetic criteria. Rare fusion products expressed recessive parental markers. This approach will provide many new selectable markers for DNA transformation of

Candida species and makes possible the isolation of genes affecting functions not found in *S. cerevisiae* (Scherer and Magee, 1990).

The genetic map of *C. albicans* has been derived from experiments with only a few strains and although particular linkages seem to be widely conserved (Poulter and Hanrahan, 1983) the variability of the electrophoretic karyotype suggests that considerable differences may exist between strains (Scherer and Magee, 1990).

Phenotypic variation or switching is a phenomenon in *C. albicans* which involves a heritable change in colony morphology, cell shape or susceptibility to antifungal agents (Scherer and Magee, 1990). Slutsky *et al.* (1985,1987), working on the white-opaque switching system, demonstrated the reversibility of the switching process and obtained data on the frequency of the changes. They found that the range of colony phenotypes was limited for any given switching system and provided evidence for the role of ultraviolet radiation as a stimulator of the transition. There are potentially two genetic systems involved in any instance of phenotypic instability: the genes involved in the transition, and the genes involved in producing the alternate phenotype. This will complicate the study of the genetics of the switching mechanisms (Scherer and Magee, 1990). Phenotypic variations have been observed in strains isolated from patients with active *Candida* infections (Soll *et al.*, 1987). The possible role of these processes in pathogenesis is discussed later.

2.4 Morphogenesis

C. albicans can adopt different morphological forms: budding yeast cells or blastoconidia, pseudohyphae (elongated yeast cells which appear as filamentous cell chains) and true hyphae with septa. *C. albicans* can also form chlamydospores but only *in vitro*. These large, refractile, thick walled cells were proposed by Van der Walt (1967) as part of a sexual cycle of *C. albicans* but this theory is no longer credited (Odds, 1988).

The change from yeast to hyphal form is environmentally regulated and can be triggered *in vitro* in a number of ways including growth on nutrient poor plates, in

the presence of some amino acids (especially proline), at near neutral pH and above 33°C. None of these triggers acts alone under all conditions (Odds, 1988).

Interest in the ability of *C. albicans* to change its shape comes partly from the obvious significance of any morphogenetic process to developmental biology but mainly from the presumption that hypha formation is a process of pathological significance. The evidence, to date, comparing the relative pathogenicity of yeast and hyphal forms of *C. albicans* is inconclusive. The mycelial form is invariably seen in smears or scrapings within hours of inoculation into experimental animals supporting the link of morphogenesis and pathogenesis (Gresham and Whittle, 1961; Louria and Brayton, 1964; Balish and Philips, 1966). However, in the majority of established candidosis lesions, the fungus almost always produces yeasts and hyphae which suggests that neither form is pathologically inferior. The presence of only *C. albicans* yeast cells does not necessarily mean no infection, and similarly, the presence of hyphae in an individual smear or biopsy cannot be interpreted as of clinical significance (Odds, 1988). Other *Candida* species (and other fungi) can function as very effective pathogens without the ability to produce hyphae. There are reports of hyphal forms of *C. albicans* having an increased ability to adhere to human buccal epithelial cells (Kimura and Pearsall, 1978; Kimura and Pearsall, 1980). Sobel *et al.* (1984) found that a variant strain of *C. albicans*, incapable of hyphal formation at 37°C, was less likely to colonise the rat vagina than the wild-type strain. Rotrosen *et al.* (1985) demonstrated the ability of hyphal *C. albicans* to adhere to vascular endothelial cells to a greater extent than the yeast form. Other reports suggest that in experimental disseminated candidosis, yeast-form inocula rather than hyphal-form inocula produce higher mortality rates (Evans, 1980; Mardon *et al.*, 1975; Oblack *et al.* 1978).

The evidence for resistance to phagocytosis and intracellular killing of yeast and hyphal forms is equally contradictory. Both morphological forms of the fungus seem to have the ability to initiate and to sustain pathological responses in

mammalian hosts, but it is likely that one form may be better adapted than the other to survive in specific microniches *in vivo* (Odds, 1988).

3. CELL SURFACE OF *CANDIDA ALBICANS*

3.1 Cell-wall Composition

The cell-wall of *C. albicans* is composed primarily of mannoprotein, glucan, and chitin. The carbohydrate components comprise approximately 90% of the cell-wall (w/w) but there are also small quantities of protein (3-6%, w/w) and lipid (2%, w/w) (Shepherd and Gopal, 1991). On average, around two-thirds of the total carbohydrate is glucan and one third is mannan. Chitin is a minor constituent (0.6-2.7%) and its abundance in the wall varies with the growth form; mycelial walls contain roughly three times more chitin than yeast cell walls (Sentandreu *et al.*, 1991).

3.2 Glucans

Beta-glucans are the main structural components of the *C. albicans* cell-wall. There are three types of beta-glucan: an alkali-soluble polymer, a branched acid-soluble glucan containing mainly β1-6-linked glucose and a highly branched insoluble form with variable amounts of β1-3 and β1-6 linkages, and branch points (Sentandreu *et al.*, 1991). Studies using regenerating protoplasts have shown that radioactive glucose is incorporated into a β1,3-linked polymer (Gopal *et al.*, 1984b). Also, membrane preparations from *C. albicans* catalyse the synthesis of a linear polymer *in vitro* (Orlean, 1982).

3.3 Chitin

Chitin is a linear polymer of β1-4-linked N-acetyl glucosamine monomers. It is an important component of the cell wall, particularly at the septum between both hyphal cell units and blastospores (Sentandreu *et al.*, 1991). Chitin is thought to be localised

in the lower layers of the cell wall but is also present at bud scars (Sentandreu *et al.*, 1991). It is found in yeast forms, germ tubes, and hyphae, although the proportion is higher in hyphae (Chattaway *et al.*, 1968). Chitin and glucan provide rigidity and appear to be essential for cell division (Calderone and Braun, 1991). There is experimental evidence to suggest that glucans and chitin are covalently linked (Ruiz-Herrera *et al.*, 1994)

3.4 Mannoprotein

Because of their significance as antigenic determinants in *Candida* species, cell-wall mannoprotein structures have been studied far more intensively than have glucan structures (Odds, 1988). The general structural features of *C. albicans* mannoproteins, as well as their biosynthesis and export, are thought to be similar to those of *S. cerevisiae* (Sentandreu *et al.*, 1991). Protein accounts for approximately 10% of the total molecular weight of the *S. cerevisiae* mannoprotein molecule (Ballou, 1976). Mannoprotein is composed of mannose polysaccharides covalently linked to protein via serine, threonine and asparagine residues. Short polysaccharide chains are O-linked to the hydroxyl group of serine and threonine residues. Larger more complex polysaccharides are N-linked via two units of N-acetyl-D-glucosamine (N-acetylchitobiose) to asparagine residues on the protein (Calderone and Braun, 1991). The N-linked polymers are long linear chains of mannose units joined through α1-6 linkages to which either single mannose residues or polymers containing a variable number of mannose residues joined through α1-2 and occasionally terminal α1-3 linkages (Nelson, 1991). Both the N- and O-glycosylation processes are initiated in the endoplasmic reticulum (Ruiz-Herrera and Sentandreu, 1975; Zueco *et al.*, 1986).

There is some disagreement over the precise structures that function as specific immunodeterminants. Okubo *et al.* (1979) showed that the most heavily phosphorylated mannose polymers are the most serologically active in terms of *C. albicans* antigen factor 6, the component that distinguishes serotype A from serotype

B. Although it has long been known that *C. albicans* mannans are more heavily phosphorylated than those of *S. cerevisiae*, the significance of phosphate groups in mannan immunodeterminants has received relatively little attention (Odds, 1988). Casanova and Chaffin (1991) examined the phosphate containing proteins of the *C. albicans* cell surface. They demonstrated that not all glycoprotein components contained detectable phosphate, and at least one 19-20 kDa protein may be phosphorylated in the absence of carbohydrate.

3.5 Cell-wall organisation

Ultrastructural studies of the *C. albicans* cell wall have indicated a complex microarchitecture. The wall is of variable thickness and is composed of several layers that are revealed by differences in electron density (Calderone and Braun, 1991). The number of layers and their morphology are variable and may be related to the stage of growth, growth form, growth medium or fixation procedure (Poulain *et al.*, 1978). The inner layer adjacent to the plasma membrane is formed by a microfibrillar glucan network filled with amorphous material (possibly mannoprotein) together with other glucan polymers. Chitin is located at bud scars and is also distributed throughout the cell wall, but mostly in the inner layers (Sentandreu *et al.*, 1991). The overall structure of the wall and its chemical nature appear to be similar in both growth forms of the fungus. Hyphal walls do differ quantitatively from those of the yeast form; they are only about half as thick, they have three times the amount of chitin and they have a noticeably reduced amount of mannoprotein. The outer fibrillar layer is mannoprotein enriched, although mannoprotein is distributed throughout the cell wall. Synthesis of this fibrillar layer is promoted by growth in medium containing high concentrations of galactose (McCourtie and Douglas, 1984).

3.6 Plasma membrane

The plasma membrane of *C. albicans* is a typical eukaryotic phospholipid bilayer with frequent invaginations. The membrane is composed of large quantities of lipid and protein with some carbohydrate and little or no nucleic acid (Hubbard *et al.*, 1986). The levels of total lipids, sterols, and phospholipids are significantly higher in the mycelial form (log phase) of *C. albicans* than in the yeast form. Increased phospholipid levels in the mycelial form are due to higher levels of phosphatidylcholine, phosphatidylserine, and phosphatidylinositol. The changes in lipid composition are clearly demonstrated in altered thermotropic phase behaviour. Gel-to-liquid crystalline phase transitions are observed at 36 and 27°C for the lipids of the yeast and mycelial forms, respectively. These changes coincide with a higher uptake rate for L-proline and an increased sensitivity of the mycelial form for antifungal drugs (Goyal and Khuller, 1994). Study of the membrane is important as it is a prime target for attack by antifungal antibiotics. This will be expanded upon later.

3.7 Extracellular polymeric substances

Masler *et al.* (1966) reported the excretion of polysaccharide-protein complexes from *C. albicans* cells. These complexes were composed of 74-86% mannan, 21-31% glucose, 1-1.5% glucosamine and 11-14% protein, and were immunologically active. Mankowski (1968) reported that extracellular polymeric material was biologically active, having the ability to inhibit the growth of newborn mice and also kill them. Other biological activities attributed to this material include the release of histamine from mast cells (Sikl *et al.*, 1964), and reduction of the phagocytic ability of white cells (Trnovec *et al.*, 1978). Extracellular polymeric material inhibits adhesion of yeasts to neutrophils (Diamond *et al.*, 1980) and buccal epithelial cells (McCourtie and Douglas, 1985). This EP is thought to originate from a fibrillar layer on the yeast surface and its ability to inhibit adhesion suggests that it contains an adhesin.

4. CANDIDOSIS

Candida species are strictly opportunistic pathogens that rarely or never cause clinically important disease in a host with intact antimicrobial defences (Odds, 1988). Candidosis can occur in two forms: superficial and systemic candidosis. As the name implies, superficial candidosis means "on the surface" e.g. skin, nails and oral mucosa. Systemic infection can refer to disease as a result of entry of organisms into the bloodstream (*Candida* septicaemia) and it may involve several organs. Systemic disease can also appear as localised primary disease with only one organ affected.

4.1 Factors influencing infections

Gentles and LaTouche (1969) listed 39 disorders, diseases, debilities and iatrogenic factors that predispose individuals to various forms of candidosis.

4.1.1 Dietary factors

There is evidence to suggest that a diet rich in carbohydrate may promote oral colonisation of *Candida* (Samaranayake, 1986). Sucrose oral rinses are known to aggravate palatal candidosis in denture stomatitis sufferers (Olsen and Birkland, 1976) Also, experiments on primates suggest an increase in oral yeast concentration when they are fed a high sugar diet by mouth. The oral yeast concentration fell when the primates were fed via an intragastric tube (Bowen, 1974; Bowen and Cornick, 1970). Vitamin and iron deficiencies may also be a factor in susceptibility to candidosis (Jorizzo, 1982; Samaranayake, 1986).

4.1.2 Mechanical factors

Trauma, burns, local occlusions and maceration all predispose the host to candidosis. Since severely burned patients often receive intravenous hyperalimentation and extensive antibiotic treatment, it is possible that these other

predisposing factors, rather than the burn injury alone, increase their susceptibility (Gauto *et al.*, 1977; MacMillan *et al.*, 1972; Richards *et al.*, 1972). Occlusions such as wound dressings, nappies and even a wristwatch have all been shown to cause skin lesions due to *Candida* (Maibach and Kligman, 1962).

4.1.3 Medical factors

A number of surgical techniques and drug therapies predispose to candidosis. Transplantation surgery, such as heart-lung, kidney, bone marrow and pancreas transplantation, often has systemic candidosis as a complication (Odds, 1988; Gentry and Price, 1991). Intravascular catheters, especially those used for parenteral nutrition also present a risk of systemic candidosis especially when such devices are left *in situ* for long periods (Band and Maki, 1979). It is probable that the *Candida* species that colonise these catheters come from both exogenous and endogenous sources. Indwelling urinary catheters and heart valve prostheses are also strongly associated with *Candida* infections (Odds, 1988).

Drugs that predispose individuals to candidosis include antibiotics, corticosteroids, oral contraceptives and agents that induce neutropenia (Winner and Hurley, 1964; Odds, 1988). Yeasts are able to multiply more readily in situations where commensal bacteria have been eliminated (Odds, 1988). Corticosteroids and drugs that induce neutropenia have pronounced immunosuppressive effects. Oral contraceptives have been implicated as a predisposing factor for vaginal candidosis. This area of research has become controversial but there are indications that the oestrogen component of the combined-type pill has the greatest effect on candidal carriage (Jackson and Spain, 1968).

4.1.4 Natural factors

There are a number of diseases and disorders that are associated with candidosis including diabetes mellitis, other endocrine disorders, infection, immunopathological disorders, cystic fibrosis and various forms of cancer (Gentles

and La Touche, 1969). The mechanism by which diabetes increases host susceptibility to candidosis is not clear. It has been proposed that high tissue- and blood-glucose levels may favour the growth of *Candida* in diabetics. It has also been suggested that in diabetics, the conversion of glucose to sorbitol reduces the available concentration of NADPH and thus inhibits the oxidative killing mechanism of neutrophils (Wilson and Reeves, 1986).

Candidosis is also more common in infants, the elderly and in pregnancy (Odds, 1988). Infants undergoing treatment with antibiotics and intravenous catheterisation (especially premature babies) are clearly at risk from *Candida* infections (Winner and Hurley, 1964; Gentles and La Touche, 1969). Old age may not necessarily be a factor predisposing to candidosis in itself. However, other late onset disease and medical treatments used on the elderly may increase the risk of candidosis (Odds, 1988); for example, there is a clear association between oral candidosis and denture wearing.

Vaginal thrush is a common complaint and a number of factors are believed to favour its onset. Pregnancy is known to be associated with an increase in vaginal carriage of yeasts and there is a marked decrease in the proportion of women carriers post-partum (Singha and Balsdon, 1979). Hormonal changes may be an important factor during pregnancy (Mathur *et al.*, 1978). Sobel (1985) listed a number of factors that may influence the incidence of vaginal thrush in pregnancy: vaginal pH, vaginal glycogen concentration, and receptor states of the vaginal epithelium. The pH value of the vagina varies, being highest in the upper part and during menstruation, with an average of pH 4.5 (Tindall, 1987). King *et al.* (1980) reported much less adherence to *C. albicans* yeast cells at pH 4 (close to vaginal pH in pregnancy) than at pH 7.3. However, other workers have found that vaginal pH is not related to candidal carriage in pregnant or non-pregnant women (Drake *et al.*, 1980).

Among the many types of cancer that are known to predispose to candidosis, leukaemia and lymphomas are by far the most commonly associated with serious *Candida* infections. Anti-cancer therapy tends to be vigorous and many treatments

are known risk factors for candidosis e.g. cytotoxic and immunosuppressive drugs, and intravenous catheterisation.

4.2 Oral Candidosis

Candida species can cause a variety of diseases in the oral cavity of which thrush and denture stomatitis are most commonly seen. *Candida* can also cause oral leukoplakia and some other forms of oral pathology.

4.2.1 Oral Thrush - acute pseudomembranous candidosis

This disease is commonly seen in infancy, old age, and in the terminally ill (Finlay, 1986). It is also found in association with other disease states such as diabetes, cancer and AIDS. Oral thrush appears as white lesions on the buccal mucosae, tongue, throat, and gum linings. The lesions can form confluent plaques which give rise to the term "pseudomembranous". The *C. albicans* hyphae and yeast cells invade the tissue to the extent of the stratum corneum but do not penetrate beyond the stratum spinosum (Lehner, 1967). When the patches are scraped off they reveal a raw area of epithelium.

4.2.2 Denture stomatitis - chronic atrophic candidosis

This most common of *Candida* associated disease presents as chronic erythema and oedema of the portion of the palate that is in contact with dentures. The patient may experience slight soreness but it is frequently the associated angular cheilitis that is the presenting complaint. Angular cheilitis appears as encrusted erosive lesions at the corners of the mouth. Separate studies on the incidence of the disease have suggested that 24% (Nyquist, 1952), 50% (Jacobsen *et al.*, 1979) and 60% (Budtz-Jorgensen *et al.*, 1971) of denture wearers suffer from denture stomatitis. Yeasts are known to adhere well to the plastics used in dentures (McCourtie and Douglas, 1981; McCourtie *et al.*, 1986). There tends to be no tissue invasion in relation to denture stomatitis (Odds, 1988).

4.2.3 *Candida* leukoplakia - chronic hyperplastic candidosis

Oral leukoplakias occur when the surface of a chronically inflamed area becomes white and smooth. *Candida* leukoplakias occur most commonly on the cheeks and less commonly on the tongue. Smoking (and perhaps denture wearing) is associated with the disease (Odds, 1988). Tissue invasion, as in oral thrush, is limited to the upper epithelial layers (Mohamed, 1975). The condition may be related to iron and folate deficiencies (Jenkins *et al.*, 1977; Mackie *et al.*, 1978).

4.3 Vaginal thrush - vulvovaginal candidosis

Candida infections of the female genitalia are approximately ten times more common than infections of the penis (Odds, 1988). This is most likely due to the more favourable growth conditions to be found in the vagina. Vaginal secretions have been shown to promote the growth of *C. albicans* (Bisschop *et al.*, 1985). The secretions consist of tissue fluids, leukocyte and epithelial cell debris, electrolytes, proteins and lactic acid (McGroarty, 1993).

Acute pruritus and vaginal discharge are the usual presenting complaints. The classical sign of vaginal thrush is the presence of white plaques, analogous to those seen in oral thrush, on the vulvar, vaginal or cervical epithelium (Odds, 1988). However, these plaques are not always seen. It is more common to find intense erythema and oedema of the labia minora and lower third of the vagina with or without plaques and discharge (Monif, 1985). The disease is predominantly seen in women of child-bearing age and frequently in the absence of underlying factors that predispose to the illness. Two factors that are strongly associated with the onset of vaginal thrush are pregnancy and diabetes while other factors have not been shown convincingly to play any significant part (Ryley, 1986). The *Candida* species found in the vagina are believed to be derived mainly from the gastrointestinal tract; however, sexual transmission is a possible route (Thin *et al.*, 1977). As in oral candidosis, the fungi do not seem to penetrate deeper than the stratum corneum (Odds, 1988). Chronic, recurrent, vulvovaginal candidosis applies to women who have had at least

three or four clinically and mycologically proven episodes of vaginal candidosis within 12 months (Witkin *et al.*, 1983; 1986). Recurrent thrush is a complex and possibly multi-factorial condition. One or more defects in leukocyte anti-*Candida* defences possibly allow a number of viable *Candida* cells to remain in the vagina, despite the action of antifungal agents, resulting in relapse (Odds, 1988).

4.4 Chronic mucocutaneous Candidosis (CMC)

Around 90% of cases of CMC have their onset within the first 20 years of life (Odds, 1988). In most cases, oral thrush is the earliest symptom. Angular cheilitis and lip fissures may develop and the oesophagus may occasionally be involved (Shepherd, 1986). Skin lesions, when present, mainly appear on the face, ears, neck and shoulders. Many of the cases are related to endocrine disorders such as hypoparathyroidism, hypoadrenocorticism, and hypothyroidism. However, diabetes mellitis is only rarely seen related to CMC (Odds, 1988). Defects in immune function are also implicated as causes of this disease (Rosman, 1979). The fact that it is almost exclusively *C. albicans* that accounts for CMC is probably significant. Even in extreme cases, the fungus appears unable to break superficial defences and initiate disseminated infection (Odds, 1988).

4.5 Systemic Candidosis

There has been a dramatic rise in the incidence of invasive *Candida* infections since the 1950s. This has been attributed to such factors as the increasing application of cytotoxic chemotherapy to the management of malignancies (Lew, 1989), the prevalence of infections due to HIV, and the widespread use of newer and more powerful antibacterial agents (Banerjee *et al.*, 1991, Anaissie *et al.*, 1989).

Systemic candidosis may arise as localised disease with only one organ affected or as *Candida* septicaemia in which several organs are affected. Virtually all visceral forms of *Candida* infection result from haematogenous spread of yeasts with the exception of the digestive, respiratory, and urogenital tracts whose epithelial surfaces

may be directly invaded by exogenous yeasts (Odds, 1988). Fungal cells enter the bloodstream either by persorption from the gut or contamination via intravascular catheters (Salfelder *et al.*, 1977). Wound infection may also play a role especially in burns patients (Spebar and Pruitt, 1981). The drugs available for the treatment of systemic candidosis carry the risk of host toxicity (amphotericin B), drug resistance (flucytosine) or low efficacy (azole antifungals) (Odds, 1988).

4.5.1 Candidosis of the gastrointestinal tract

The gastrointestinal tract can be infected by *C. albicans* from its mucosal surfaces or via the bloodstream although the latter is considered rare (Trier and Bjorkman, 1984). Oesophageal candidosis is the most common form of gastro-intestinal candidosis. Less commonly seen is gastric candidosis and rarer still is intestinal candidosis (Odds, 1988). Diagnosis requires biopsy showing candidal tissue invasion as commensal fungi are commonly found with smears and cultures. The characteristic endoscopic appearance of oesophageal candidosis is of yellow-white plaques on an erythmatous background analogous to oral thrush lesions (Scott and Jenkins, 1982). Oesophageal candidosis is often seen in cancer patients (2.8-6.7%) and there is an even higher incidence among AIDS patients (9.4%) (Chandler, 1985). Gastric candidosis is usually the result of *Candida* invasion of existing gastric ulcers and operative wounds (Minoli *et al.*, 1982). Intestinal candidosis is extremely rare and the literature on primary intestinal candidosis is small (Odds, 1988). *Candida* enteritis is probably rare but is likely to be more of a problem in infants (Kozinn and Taschdjian, 1962).

4.5.2. Candidosis of the respiratory tract

Candidosis in the lower respiratory tract is common in cases of disseminated candidosis where the fungus reaches the lungs via the bloodstream. Primary bronchial or pulmonary candidosis arising via the airways is probably very rare (Odds, 1988). Again, diagnosis requires histopathological evidence from biopsy due to the

likelihood of commensal *Candida* isolation with sputum samples. There is a high risk of pulmonary candidosis among severely immunocompromised patients, especially those with haematological malignancies and neutropenia (Odds, 1988).

4.5.3 Candidosis of the urinary tract

Candiduria is commonly initiated by indwelling catheters and the removal of such devices is often sufficient to eliminate the problem (Schonebeck, 1972). Other predisposing factors include immunosuppression, diabetes, and urinary tract dysfunction (Fisher, 1993). Candidosis of the urinary bladder (*Candida* cystitis) is very unusual in patients below the age of 50 years and is often related to diabetes and catheterisation. Symptoms of this disease are similar to other forms of cystitis and treatment usually involves amphotericin B instillations (Paladino and Crass, 1982).

4.5.4 Renal candidosis

Diabetes tends to be the most common single factor seen in association with renal candidosis. The infection is often obstructive with fungal material collecting in the renal pelvis. Fever is seen in around one third of cases (Odds, 1988). The most likely route of infection is via the bloodstream. Ascending *Candida* urinary infections seems to be rare in humans and the fungus adheres poorly to bladder mucosa (Levison and Pitsakis, 1987).

4.5.5 *Candida* endocarditis

Although rare, *Candida* endocarditis has aroused considerable interest as it is extremely difficult to diagnose and treat (Odds, 1988). Its occurrence is related to the placement of central venous catheters with their tips close to or within the right atrium causing injury to the endocardium or vascular endothelium (Sanchez *et al.*, 1991). Platelets adhere to the exposed subendothelial tissue and cells attach to fibrin-platelet matrices or to fibronectin, a component which is exposed on traumatised heart valve tissue (Scheld *et al.*, 1981; 1985). Fungi can also adhere to the plastics

used in the catheters themselves (Klotz *et al.*, 1985). The disease is also seen in intravenous drug abusers, patients undergoing hyperalimentation or as a complication of prolonged intravenous antibiotic administration (Scheld *et al.*, 1983). The untreated disease will eventually lead to heart failure but even if discovered the mortality rate is high with 24% survival with antifungal treatment alone and 73% survival with resection and antifungals (Odds, 1988).

4.5.6. Miscellaneous other forms of systemic candidosis

A number of other anatomical sites can be infected by *Candida* species. These include the central nervous system, bones and joints, liver, spleen and pancreas. The brain is often involved in cases of disseminated candidosis but only rarely is the central nervous system the principle or sole focus of the disease (Walsh *et al.*, 1985). The disease can take two forms: meningitis and cerebral infections, usually in the form of brain abscesses.

Candida infections of the bones or joints can arise either by local inoculation (injury, surgery) or via the bloodstream. The disease can take the form of arthritis and osteomyelitis. Of infections arising from external sources, 40% are caused by *C. guilliermondii* or *C. parapsilosis*, both of which are common skin commensals. The majority of haematogenous bone and joint candidoses are caused by *C. albicans* and *C. tropicalis* and half of such cases have a recent history of abdominal surgery or malignancy (Odds, 1988).

Candida infections of the liver, spleen and pancreas are often seen in disseminated candidosis but again are only rarely present as primary foci of infection. Focal infections at these sites are most commonly found in cases of acute leukaemia and the causative agent is usually *C. albicans* (Tashjian *et al.*, 1984).

5. ANTIFUNGAL AGENTS

Due to the eukaryotic nature of fungi, the number of suitable targets for therapeutic attack is limited (Lyman and Walsh, 1992). There are a number of antifungal agents that are active against *Candida* species and they fall into three broad types: the polyenes, the azoles and flucytosine.

5.1 Polyene antifungals

There are four polyene antifungals that are routinely used in clinical practice: amphotericin B, candicidin, natamycin and nystatin. The polyenes are characterised by a macrolide ring that is closed by formation of an internal ester or lactone and contain a series of at least three conjugated double bonds (Hamilton-Miller, 1973). They are natural products of filamentous bacteria, usually *Streptomyces* species, and have their effects on eukaryotic cell membranes by altering their permeability. The membrane damaging action of polyenes is due to their high avidity for sterols especially ergosterol. Their selective toxicity for fungi occurs because fungal cell membranes contain ergosterol as the major sterol component whereas mammalian cell membranes contain cholesterol (Bolard, 1986).

Amphotericin B is the drug of choice for the treatment of systemic fungal infections (Midez *et al.*, 1989). Oral administration of the drug is only used in cases of gastrointestinal candidosis as the compound is not absorbed significantly from the gut. If parenterally administered, amphotericin B seems to be widely distributed around the body (Sugar, 1986). Amphotericin B binds to ergosterol which alters membrane permeability, causing leakage of sodium, potassium and hydrogen ions and eventually leads to cell death (Palacios and Serrano, 1978). It also binds to other sterols, such as cholesterol, which accounts for much of its toxicity (Medoff and Kobayashi, 1988). There are a number of side-effects associated with amphotericin B such as kidney damage, accompanied by uraemia, which is especially dangerous in premature neonates. Regular blood tests for signs of kidney damage are recommended (Baley *et al.*, 1984).

Nystatin is used commonly in the treatment of oral and vaginal candidosis as well as in cases of oesophageal candidosis (Odds, 1988). It is not absorbed from the gastrointestinal tract. Its use is restricted to topical treatment only due to its toxicity and insolubility (Kerridge and Nicholas, 1986).

Candicidin is another polyene antifungal that is too toxic for systemic chemotherapy but is of great use in vaginal candidosis. (Odds, 1988). Less impressive as an antifungal agent is Natamycin which has been used in vaginal candidosis (Odds, 1988).

5.2 Azole antifungals

A large number of azole antifungals have been developed for clinical use. The three main classes are topically active imidazole derivatives, orally active N-substituted imidazoles, and triazole derivatives. They are synthetic compounds with one or more 5-membered rings, where each ring has 2 (imidazoles) or 3 (triazoles) nitrogen atoms. Topical imidazoles cause alterations in the sterol composition of *Candida* membranes by their action on cytochrome P-450, and at high concentrations they will exert direct effects on the yeast cell membrane (Vanden Bossche, 1985). Azoles are also thought to inhibit the ATPase of fungal cell membranes. This effect has been shown with miconazole and ketoconazole (Irani and Truong, 1986). Although resistance to azoles is becoming more common (especially with fluconazole), it seems relatively rare with toxicity being the major problem associated with their use (Odds, 1988; Lyman and Walsh, 1992).

Imidazoles are one of the two major classes of antifungal azole derivatives. Among this group are clotrimazole and miconazole. They are both relatively water insoluble and are poorly absorbed by the gastrointestinal tract. Their use is limited to topical application (Lyman and Walsh, 1992). Ketoconazole on the other hand is the first successful, orally absorbable, antifungal azole. It is insoluble at neutral pH but dissolves in the presence of stomach acid. It can then gain access to the blood stream via the stomach mucosa. The most frequent side-effects are gastrointestinal

(Dismukes *et al.*, 1983) but some hepatotoxicity has been reported in 2-8% of patients (Lewis *et al.*, 1984).

The other major class of antifungal azole derivatives are the triazoles. The triazole ring is less nucleophilic and therefore more resistant to metabolic attack (Richardson *et al.*, 1990). In addition, the triazole ring may be more specific for fungal enzyme systems and have increased potency (Grant and Clissold, 1989).

Itraconazole is a water insoluble, lipophilic, triazole analogue that exhibits excellent activity against most human fungal pathogens. It is soluble in the gastric milieu, has a broader spectrum than ketoconazole, and is less toxic (Warnock, 1989). This drug appears to have low hepatotoxicity with the main side-effects being gastrointestinal disturbances, dizziness, headache, and leucopenia (Graybill, 1993).

Fluconazole is a water soluble, fluorine substituted, *bis*-triazole antifungal agent available for oral or intravenous use in the treatment of a number of localised and disseminated mycoses. It has comparable activity to that of ketoconazole: inhibition of fungal C-14 demethylase. However, it is significantly less potent against mammalian enzymes and hence is more specific to fungal cytochrome P450-mediated reactions (Shaw *et al.*, 1987). It acts by inhibiting synthesis of ergosterol, an essential sterol in fungal cell membranes. The drug is rapidly absorbed after oral dosing, and penetrates well into body fluids and tissues, including cerebrospinal fluid. Extensive clinical trials (reviewed by Zervos and Meunier, 1993) demonstrate its clinical efficacy in candidosis, including oropharyngeal, oesophageal, and disseminated forms. Other potential uses include prophylaxis of fungal infection in immunocompromised cancer patients and single-dose therapy of vaginal candidiasis. Fluconazole is generally well tolerated and is only rarely associated with serious adverse effects or laboratory test abnormalities (Zervos and Meunier, 1993). Gastrointestinal disruption and elevated hepatic transaminase activity are rare complications (Saag and Dismukes, 1988).

5.3 Flucytosine (5-fluorocytosine, 5-FC)

Flucytosine is a fluorinated pyrimidine with high activity against most *Candida* isolates *in vitro* (Odds, 1988). It was developed as a possible cancer therapy but was found to have no effect on tumour cells. However, it was found to have *in vitro* and *in vivo* antifungal activity (Berger and Duschinsky, 1962). It is absorbed well by the gut so it may be given orally to treat systemic yeast infections. Flucytosine is less toxic than amphotericin B but there is a problem of resistance to the drug (Kerridge and Nicholas, 1986). The most common side-effects (6% of patients) are gastrointestinal problems, but abnormal liver function (5% of patients) has also been reported (Benett, 1977). The most serious toxicity associated with this agent is dose dependent bone marrow suppression (Kaufman and Frame, 1977). Flucytosine has seen increasing usage as an adjunct to amphotericin B therapy. This combination was originally proposed due to the observation that amphotericin B potentiates the uptake of flucytosine by increasing fungal cell permeability (Medoff *et al.*, 1972). Two mechanisms of action have been proposed for flucytosine. These are the disruption of DNA synthesis and alteration of the amino acid pool by inhibition of RNA synthesis. Cytosine permease actively transports flucytosine into the yeast cell where it is converted (by cytosine deaminase) to 5-fluorouracil, which replaces uracil in the pyrimidine pool and thus disrupts protein synthesis. In addition, 5-fluorouracil can then be converted through several steps to 5-fluorodeoxyuridine monophosphate, a competitive inhibitor of thymidylate synthetase, and thus arrests DNA synthesis (Diasio *et al.*, 1978). Resistance to flucytosine can arise at three locations in the above pathway: cytosine permease, cytosine deaminase, and UMP-pyrophosphorylase with the most common mutation occuring in the UMP-pyrophosphorylase (Whelan and Kerridge, 1984).

5.4 Antifungal drug development

The discovery of antifungal agents that possess selective toxicity against the eukaryotic fungal cell remains an important scientific challenge. Ideally, a selectively toxic antifungal agent should be developed that interacts with a fungal target not found in other eukaryotic cells. New developments have included the modification of existing drug molecules to eliminate toxicity and improve activity, for instance, the development of the lipid formulations of the polyene antifungal agent amphotericin B. New triazoles with broad-spectrum antifungal activity, such as saperconazole, are also being assessed (Hay, 1994). The development of drugs that act as cell-wall biosynthesis inhibitors has been slower, although a number of molecules with activity against key enzymes have been found. The major targets are glucan synthesis, inhibited by the echinocandin lipopeptides and papulacandins; chitin synthesis, inhibited by the polyoxins and nikkomycins; and mannan, to which the pradimicins selectively bind (Debono and Gordee, 1994). Chitin synthase inhibitors have been studied through chemical modification of the polyoxins and nikkomycins but are limited because of unfavourable pharmacokinetics.

6. PATHOGENICITY OF *CANDIDA*

6.1 Pathogenicity of different species of *Candida*

Among the many species of *Candida, C. albicans* isolates are consistently the most virulent; *C. tropicalis* is also pathogenic but is quantitatively less so than *C. albicans* (Odds, 1988). Less pathogenic are *C. parapsilosis, C. glabrata. C. kefyr, C. guilliermondii* and *C. krusei.* Another two species, *C. lusitaniae* and *C. viswanathii,* are possibly to be regarded as pathogenic but only in the immunocompromised host (Odds, 1988). Within the species *C. albicans,* it is possible that serotype B is less pathogenic than serotype A (Martin and Lamb, 1982; Auger, *et al.*, 1983). The above conclusions are drawn from both animal experiments and epidemiological studies.

Species and strain differences in pathogenicity can be exploited as a basis for determining molecular virulence attributes in the pathogenesis of candidosis. However, difficulties in genetic manipulation of *C. albicans* have hampered direct analysis of genes that code for virulence determinants (Odds, 1988).

6.2 Host defence mechanisms

There are a number of non-specific host defence mechanisms that act to prevent candidal invasion. The epithelial surfaces of the skin and mucous membranes are a physical barrier to the organism. The skin is not often colonised by *C. albicans*. Epithelial cell turnover is an important form of defence in the gastrointestinal tract as well as in the mouth and vagina (Smith, 1985). Saliva contains components that have antimicrobial effects such as lactoferrin and lysozyme It also has a mechanical function in washing and diluting. Pollock *et al.*, (1984) have purified histidine-rich polypeptides from saliva and shown them to be potent inhibitors of *C. albicans* growth. Reduction in the flow of saliva or its constituents, whether due to disease or age of host, leads to increased levels of *C. albicans* in the oral cavity often resulting in overt oral candidosis (Hoffman and Haidaris, 1993).

Polymorphonuclear leukocytes (PMNs) and macrophages can ingest and kill *Candida* species. This is a crucial mechanism in the defence of the host against invasive, deep-seated candidosis (Odds, 1988). Patients with reduced PMNs are more vulnerable to disseminated *Candida* infection (e.g. diabetics, patients with leukaemia or cancer, or those undergoing chemotherapy) than comparable patients with normal PMN levels. Also, in patients with AIDS or CMC that have impaired lymphocyte immunity but whose phagocyte function is normal, visceral spread of *Candida* is highly unusual (Odds, 1988). *Candida* antigens can stimulate formation of antibodies in all immunoglobulin classes and are found commonly in the serum of healthy individuals (Cassone and Torosantucci, 1991). *Candida* preparations tested as vaccines have been shown to have a limited protective effect against intravenous

challenge with live *C. albicans* in mice and rabbits (Giger *et al.*, 1978; Hasenclever and Mitchel, 1963, Bistoni *et al.*, 1986).

C. albicans cell-wall polymers are released from the cell surface and can stimulate an immune response (Cassone and Torosantucci, 1991). The specific antigens of *Candida* are proteins and mannoproteins. Chitin and glucan are not considered to be antigenically important. Individuals that carry *C. albicans* in the gastrointestinal tract will have a consistent level of anti-*Candida* antibody (mostly IgG and IgA). These individuals will show a positive skin test when challenged with cell-wall antigen (Odds, 1988).

6.3 *Candida albicans* virulence factors

A number of factors have been proposed that enhance the virulence of *C. albicans*. These include toxins, enzymes, dimorphism, phenotypic variation, and the ability to adhere to host surfaces.

6.3.1 Toxins

The action of *C. albicans* extracts as toxins has been studied extensively. For example, Cutler *et al.* (1972) compared fractions of *C. albicans*, prepared by mechanical and chemical methods, for toxicity in animals. They found that cell-wall glycoprotein was lethal to mice (provided they were pretreated with actinomycin D) and was also pyrogenic in rabbits. The potency of this material was much less than that of bacterial endotoxin. Iwata (1979) described a molecule called "canditoxin" which is an acidic intracellular protein. It produces anaphylactic shock in mice, is cytotoxic for mammalian cell lines *in vitro* and is heat labile. Antibodies to canditoxin were also found to be protective against *C. albicans* challenge in mice. This molecule has only ever been isolated from one strain of *C. albicans*, from a patient with candidal meningitis. No other workers have managed to isolate a similar molecule in any strain. It is unlikely that a microbe in frequent commensal contact with man could

contain or secrete a potentially bioactive toxin and retain its status as an opportunistic pathogen (Odds, 1988).

6.3.2 Enzymes

C. albicans produces a number of enzymes that are thought to contribute to its virulence. One such enzyme, an aspartyl proteinase, degrades albumin, haemoglobin, immunoglobulin, epidermal keratin, collagen and extracellular matrix proteins (Payne and Ray, 1993). MacDonald and Odds (1983) examined differences in virulence for mice between a proteinase-secreting strain of *C. albicans* and a proteinase-deficient mutant. The mutant was considerably less lethal than the parent when inoculated intravenously into mice and lower counts of *C. albicans* were recovered from the organs of mice infected with the mutant.

Kwon Chung *et al.*, (1985) produced genetic evidence for the role of extracellular proteinase in virulence of *C. albicans*. The relationship between extracellular proteinase and virulence for mice was studied by using a set of three isolates that included a proteinase-producing parent, a proteinase-deficient mutant derived by nitrous acid treatment of the parent, and a spontaneous revertant obtained by mouse passage of the mutant. The morphological markers and the carbon assimilation pattern were identical in these isolates. The parental isolate produced a high level of proteinase *in vitro* and caused foetal infection (100%) within 21 days. The mutant produced no detectable enzyme *in vitro*, and all mice survived until day 22. Only 30% of the mice infected with the mutant died between days 23 and 30. The isolates recovered from the dead mice were found to be proteinase sufficient, indicating that the mice died after the organism in tissue had reverted. The reverted mutant produced proteinase *in vitro* at 44% the level of the parental isolate and induced fatal infection in 90% of the mice within 30 days. These findings indicate that proteinase activity is one of the virulence factors associated with *C. albicans*. The secreted aspartyl proteinases of *C. albicans* (products of the SAP genes) are thought to contribute to virulence through their effects on *Candida* adherence, invasion, and

pathogenicity. (Magee *et al.*, 1993). Tsushima *et al.*, (1994) showed that *C. albicans* aspartyl proteinase cleaves and inactivates human epidermal cysteine proteinase inhibitor, cystatin A. Fusek *et al.*, (1994) investigated the specificities of extracellular aspartyl proteinases from *C. albicans, C. tropicalis*, and *C. parapsilosis.* By using a library of synthetic substrates, and testing inhibition by pepstatin A, these workers demonstrated that the proteinases differed substantially in their specificities.

C. albicans also produces phospholipases which may act during infection. Barratt-Bee *et al.* (1985) found a correlation between phospholipase activity, mouse lethality and adherence to buccal epithelial cells. More recently, Prakobphol *et al.* (1994) suggested that *C. tropicalis* may adhere to lysophospholipids on host cells and that the adhesin involved may be a phospholipase. They demonstrated that adhesion to a lysophospholipid resulted in the formation of at least five breakdown products. These products suggested the presence of lysophospholipase-transacylase and lysophospholipase. The identity of the adhesin remains to be confirmed.

There are other enzymes produced by *C. albicans* such as plasmocoagulase, haemolysin, and neuraminidase but their role in pathogenesis is not proven (Odds, 1988).

6.3.3. Dimorphism

C. albicans is capable of growing as yeasts or as elongate hyphae. Hyphae can play a role in adherence and invasion of host surfaces. They also may have the ability to grow out of phagocytic cells, but there is no clear relationship between cellular morphology and pathogenic status of *C. albicans* (Odds, 1988). Kimura and Pearsall (1980) and King *et al.*(1980) demonstrated that hyphal forms of the yeast were more adherent to epithelial cells than yeast forms. Hyphal forms have been shown to express different cell-surface components to yeast-form cells. For example, hyphal forms bind fibrinogen, laminin, and C3d (complement conversion product) while yeast forms do not (Bouali *et al.*, 1986; Calderone *et al.*, 1988; Bouchara *et al.*, 1990).

In addition to hyphae, other morphological forms may be observed in growing cultures of *C. albicans*. Pseudohyphae are considered to be an aberrant form of division by budding. They appear as chains of elongated cells with constrictions. Chlamydospores may also be produced under certain growth conditions. These structures are large, thick-walled, ellipsoidal-shaped cells and may be seen in stationary phase cultures (Shannon, 1981).

6.3.4. Phenotypic variation

Many successful pathogenic organisms have developed the capacity to vary phenotype by spontaneously generating variants within infecting populations. This capacity enables the organism to evade the immune system of the host. Bacteria such as *Salmonella typhimurium* (Glasgow *et al.*, 1989) and *Neisseria gonorrhoea* (Swanson *et al*, 1987) have developed such switching systems. Variants are likely to be enriched when the host immune system targets the predominant antigenic state. Soll and colleagues have examined the capacity of *C. albicans* to modify the nature of some of its wall constituents. Switching produced altered antigenicity in a reversible fashion and also affected a number of putative virulence factors (Anderson *et al.*, 1989; Soll *et al.*, 1991; Soll, 1992). One of the first *C. albicans* switching systems examined in detail was the white-opaque transition (Slutsky *et al.*, 1987). An isolate from a bone marrow transplant patient was found to be able to switch from a white-smooth colony morphology to a flat-grey morphology. After subculture, a number of the new colonies appeared to be revertants. In addition, a number of other morphologies appeared.

Further work has identified a number of virulence factors that appear to be affected by the switching process. The transcription of the gene which codes for *C. albicans* acid proteinase, as well as the excretion of the enzyme itself, is under the regulation of switching in the white-opaque transition. Resistance to antifungal drugs may also be influenced by switching, as switch phenotypes exhibit different profiles of drug susceptibilities (Soll, 1992). Antigenic variability has been demonstrated for the

white-opaque transition and involves the expression of an opaque-specific surface antigen (Anderson *et al.*, 1989). In addition, cells of the white phenotype appear to be more resistant to the candidacidal activity of polymorphonuclear leukocytes, and are similarly more resistant to cell-free oxidants than are opaque cells (Kolotila and Diamond, 1990).

The ability of *C. albicans* to adhere may also be under the influence of the switching process. Kennedy *et al.* (1988) demonstrated that cells of the white phenotype were more adhesive than opaque cells to buccal epithelial cells. The white cells were also found to be less cohesive which may have been due to increased hydrophobicity. However, both phenotypes adhered equally well to plastic.

7. ADHESION OF *CANDIDA* TO HOST SURFACES

7.1 The importance of adhesion

The adhesion of *C. albicans* to host tissues and implanted biomaterial is seen as an early step in the establishment of disease (Rotrosen *et al.*, 1983). Colonisation of certain body sites is made difficult by the rapid passage of material and bathing actions of fluids over mucosal surfaces. Adhered cells are constantly removed by sloughing of epithelial cells. Strong evidence for adhesion as a determinant of colonisation or pathogenesis by *C. albicans* comes from studies showing that mutants with a reduced ability to adhere to a variety of surfaces are less pathogenic than the wild type strain (Fukayama and Calderone, 1991). Also strains isolated from active infections are more adherent and virulent than strains isolated from carriers (McCourtie and Douglas, 1984).

7.2 Mechanisms of adherence

Candida cells go through a number of steps before adhesion can occur: the cells must (i) approach the surface, (ii) overcome physicochemical forces at the

hydrodynamic layer, (iii) penetrate the liquid-solid interface, (iv) adsorb to the surface and finally, (v) attach via specific adhesin-receptor interaction (Kennedy *et al.*, 1992).

In general, cell surfaces have an overall negative charge. The colloid theory of Derjaguin and Landau (1941) and of Verwey and Overbeck (1948) (the DLVO theory) describes how adhesive interactions can occur between two negatively charged surfaces which should repel one another (Jones, 1977). The DLVO theory proposes that the forces of repulsion (electrostatic interaction in the overlapping double layers) and attraction (London-van der Waals forces) between two similarly charged surfaces are additive but vary independently with the distance separating the surfaces. This model is not sufficient to describe close range interactions that are regulated by adhesin-receptor binding. One role of fibrillar adhesins from *C. albicans* may be to make contact through the electrostatically charged layers. This effect may also be mediated by germ tubes which are known to be significantly more adherent than yeast cells to both epithelial cells and plastic (Kennedy *et al.*, 1992).

7.3 *In vitro* adhesion assays

In a study of adhesion, it is usually necessary to develop an experimental system that allows for the characterisation of both adhesin and receptor components by competitive inhibition. Liljemark and Gibbons (1973) first measured the attachment of *C. albicans* to rat buccal cells. A modification of their procedure was employed by Kimura and Pearsall (1980) and this has been adopted by a number of other laboratories (Douglas, 1987). Both radiolabelling protocols (King *et al.*, 1980) and visual determination (Douglas *et al.*, 1981) of yeast adherence have been used. Each method has its limitations but the visual method allows monitoring of adhesion to individual epithelial cells as well as yeast-to-yeast coadherence (Douglas, 1987).

7.4 Factors affecting *Candida* adhesion

Candida species can adhere to a wide variety of cell types. These include buccal, gastrointestinal and vaginal epithelial cells, corneocytes, vascular endothelial

cells, and platelet matrices. Among the synthetic materials to which *Candida* can adhere are PVC, acrylic and Teflon (Douglas, 1985).

7.4.1 Yeast Factors

The ability of different species of *Candida* to adhere to epithelial cells has been shown to parallel closely differences in pathogenicity (King *et al.*, 1980). *C. albicans* and *C. tropicalis* demonstrated significant adhesion to epithelial cells, while *C. parapsilosis* adhered only to a slight degree. *C. guilliermondi, C. krusei* and *C.kefyr* failed to interact with epithelial cells.

Temperature of growth is known to affect the adhesion of *Candida.* Yeasts harvested from cultures grown at 25-28°C have been shown to bind to epithelial cells in greater numbers than those isolated from cultures grown at 37°C (Segal *et al.*, 1982a).

Medium composition affects cell-surface composition and hence influences yeast adhesion. The adhesion of some strains of *C. albicans* is significantly increased after growth in defined media containing high concentrations of sugars (notably galactose) as the carbon source (Douglas *et al.* 1981; McCourtie and Douglas, 1984). Enhanced adhesion seems to be a result of the production of an additional fibrillar-floccular layer on the yeast cell surface.

Yeast cell-surface hydrophobicity may play an important role in regulating the adhesion to cells and plastics (Hazen, 1990). *C. tropicalis* is more hydrophobic than *C. albicans* and has been shown to adhere better to PVC, acrylic and Teflon (Rotrosen *et al.*, 1983; Minagi *et al.*, 1985). *C. albicans* can become more hydrophobic depending on growth conditions (Hazen and Hazen, 1988); for example growth at room temperature as compared with 37°C enhances its hydrophobicity.

Germ-tube formation has been demonstrated to increase the adherence of *C. albicans* to BEC. Kimura and Pearsall (1980) and King *et al.*(1980) demonstrated that hyphal forms of the yeast were more adherent to epithelial cells than yeast forms.

7.4.2 Environmental factors

The optimal pH value for adherence of yeasts is reported to be in the range of 5-8 (King *et al.*, 1980; Sobel *et al.*, 1981; Samaranayake and MacFarlane, 1981; Persi *et al.*, 1985). The optimum appears to be cell-type specific. For example, the adhesion of *C. albicans* to vaginal epithelial cells was shown to be greatest when yeasts were incubated with the cells at pH 5 in ambient air supplemented with 10% CO_2 (Persi *et al.*, 1985).

7.4.3 Epithelial cell factors

A wide range of cell types have been employed in the study of *Candida* adhesion. These include buccal, gastrointestinal and vaginal epithelial cells, corneocytes, vascular endothelial cells, and platelet matrices. Although some surface molecules are similar in all cell types, there are distinct differences in the expression of certain components between cells from different tissues, which is of relevance to tissue tropism of infections. It is also widely recognised that the cells from different individuals vary in receptor expression (Karlsson, 1989).

7.5 Adherence of *C. albicans* to buccal epithelial cells

A number of investigators have studied the adherence of *C. albicans* to buccal epithelial cells (BEC) (King *et al.*, 1980; Macura *et al.*, 1983; Douglas *et al.*, 1981; Brassart *et al.*, 1991). *C. albicans* was found to be most adherent species with *C. tropicalis* moderately adherent and *C. parapsilosis* only slightly adherent to BEC. There seems to be a clear relationship between ability to adhere to epithelial cells and capacity for colonisation and infection of mucosal surfaces. BEC are readily obtainable from human volunteers by gentle swabbing or scraping, for use in adhesion assays.

7.6 Adhesion of *C. albicans* to vaginal epithelial cells

Adhesion of *C. albicans* to vaginal epithelial cells (VEC) has also been frequently studied (King *et al.*, 1980; Lee and King, 1983; Sobel *et al.*, 1981; Segal *et al.*, 1982a). King *et al.* (1980) found that pH was not a factor in adhesion of *C. albicans* to VEC. Salt or divalent cations did not seem to influence adhesion either. In a later study (Lee and King, 1983), it was reported that *C. albicans* cell-wall fragments contained an adherence factor and that integrity of the yeast wall mannoprotein was important in adhesion.

The pH value of the vagina varies, being highest in the upper part and during menstruation, with an average of pH 4.5 (Tindall, 1987). The effect of pH on the adhesion of *C. albicans* to VEC *in vitro* has been examined. King *et al.* (1980) found much less adherence of *C. albicans* yeast cells at pH 4 than at pH 7.3. Similarly, Sobel *et al.*, (1981) reported that at pH 6, adhesion of *C. albicans* to VEC was enhanced when compared with pH 3-4.

Hormonal changes may also be an important factor in relation to *Candida* infections during pregnancy (Mathur *et al.*, 1978). Segal *et al.*, (1984) demonstrated that VEC from pregnant women were more receptive to *C. albicans* than VEC from women on oral contraceptives.

7.7 Adherence to endothelium and fibrin-platelet matrices

Lesions such as endophthalmitis, nephritis, and endocarditis arise as a result of adherence of *C. albicans* to endothelium or exposed subendothelial surfaces (Klotz, 1987). Fibrils on the yeast cell surface have been shown to be responsible for adhesion of *C. albicans* to renal endothelium (Barnes *et al.*, 1983). The mechanism of adherence to endothelium appears to differ from that for adherence to epithelium (Odds, 1988). Proteolytic treatment of fungi fail to reduce their attachment to endothelium whereas similar treatment is known to inhibit adhesion of *C. albicans* to VEC and BEC (Lee and King, 1983). Maisch and Calderone (1980) demonstrated that yeast surface mannan is an important determinant in the adhesion of *C. albicans*

to fibrin-platelet matrices formed *in vitro*. They also showed that other species of *Candida* did not adhere to the matrices to the same extent.

7.8 Adherence of *C. albicans* to acrylic and plastics

C. albicans adheres well to a variety of materials used in catheters such as polyvinyl chloride and also to denture acrylic. Urinary catheters, prosthetic heart valves, silicone voice prostheses, endotracheal tubes, and cerebrospinal fluid shunts are all strongly associated with *Candida* infections (Odds, 1988). The adhesion of *Candida* to denture acrylic has been studied by a number of groups (Samaranayake *et al.*, 1980 ; McCourtie and Douglas, 1981; Critchley and Douglas, 1985; Minagi *et al.*, 1985). Cell-surface hydrophobicity may be a key factor in this interaction. *C. tropicalis* is more hydrophobic than *C. albicans* and has been shown to adhere better to PVC, acrylic and Teflon (Rotrosen *et al.*, 1983; Minagi *et al.*, 1985). *C. albicans* can become more hydrophobic depending on growth conditions (Hazen and Hazen, 1988); for example, growth at room temperature as compared with 37°C enhances its hydrophobicity. The use of detergents in adhesion assays has confirmed the importance of hydrophobicity in yeast adhesion to plastics (Kennedy *et al.*, 1989). *Candida* species treated with saliva prior to adhesion assays with acrylic showed markedly lower adherence (McCourtie and Douglas, 1981; McCourtie *et al.*, 1986). This finding is reflected in patients with Sjogrens syndrome whose saliva flow is absent or minimal and who often succumb to chronic oral candidal infection (MacFarlane and Mason, 1974). In contrast, a recent study found that coating denture acrylic with whole saliva enhanced *C. albicans* adhesion (Vasilas *et al.*, 1992). *C. albicans* surface mannoproteins are likely to be involved in adherence to acrylic. Yeasts grown in 500mM galactose (the conditions under which the fibrillar surface material is most pronounced) adhere better than yeasts grown in 50mM glucose (McCourtie and Douglas, 1981,1985).

Tronchin *et al.* (1988) demonstrated that germ-tube specific components were involved in attachment of *C. albicans* to plastic surfaces. The components were

identified as mannoproteins with M_r values of 60 kDa and 68 kDa with two additional proteins with M_r > 200 kDa.

In a more recent study, Hawser and Douglas (1994) examined the ability of a number of *Candida* isolates to form biofilms on various catheter materials. They found that the non-*albicans* isolates were less able to produce biofilms than *C. albicans*. They were also able to demonstrate differences in the ability of *C. albicans* to form biofilms on the different catheter materials. Biofilm formation was slightly increased on latex or silicone elastomer, compared with polyvinyl chloride, but substantially decreased on polyurethane or 100% silicone.

7.9 Effects of commensal bacteria on adherence

Endogenous bacteria on mucosal surfaces can inhibit candidal adherence (Samaranayake and MacFarlane, 1982) or assist the fungus in its binding (Makrides and MacFarlane, 1983; Levison and Pitsakis, 1987). The fact that *E. coli* expressing type 1 pili can enhance adhesion is not surprising due to the amount of mannan on the surface of *C. albicans*. Sobel *et al.*, (1981) found that a *Lactobacillus* isolate significantly inhibited yeast adhesion to VEC. Lactobacilli are common vaginal commensals and due to their non-pathogenic nature have been proposed as useful probiotic agents (Bibel, 1988). Vulvovaginal candidosis following systemic antibiotic treatment is thought to result from the depletion of the protective flora (McGroarty, 1993). Whether *Lactobacillus* and *Candida* compete for the same receptor molecule on VECs remains to be determined.

7.10 The yeast adhesin

C. albicans may produce more than one adhesin (Ghannoum and Abu-Elteen, 1991). Most of the major cell-wall components have been implicated as adhesins (Kennedy *et al.*, 1992). Proteins on the surface of *C. albicans* with the ability to bind a variety of blood proteins may act as adhesins in certain disease states (Calderone, 1993).

Chitin has been suggested as a possible adhesion molecule (Lehrer *et al.*, 1983; Segal *et al.*, 1988) as have candidal lipids (Ghannoum *et al.*, 1986). However, the majority of reports describing lectin and carbohydrate blocking experiments indicate that the mannoprotein probably acts as the adhesion molecule (Kennedy *et al.*, 1992).

Evidence for the role of mannoprotein in the adhesion of *C. albicans* to epithelial glycosides has been obtained by a variety of methods (Douglas and McCourtie, 1983; McCourtie and Douglas, 1985; Sandin *et al.*, 1982). *In vitro* adhesion of *C. albicans* to epithelial cells was blocked by the addition of mannose to adhesion mixtures. Pretreatment of yeasts with mannose-specific lectin (concanavalin A) or tunicamycin (which inhibits mannoprotein synthesis) also inhibited adhesion. Tunicamycin was seen to inhibit the production of fibrillar material on the yeast cells (Douglas and McCourtie, 1983). Proteolytic enzymes (Sobel *et al.*, 1981) and reducing agents (Lee and King, 1983) such as β-mercaptoethanol and dithiothreitol (DTT) also caused a reduction in adhesion. McCourtie and Douglas (1985) and Critchley and Douglas (1987a) inhibited the adhesion of *C. albicans* to buccal epithelial cells (BEC) by pretreatment of the BEC with extracellular polymeric material (EP) derived from culture supernatants of the yeast grown in medium rich in galactose. This finding suggests that the EP (composed primarily of mannoprotein) contained an adhesin. The interaction seems to be strain-specific as EP from one strain (*C. albicans* GDH 2023) failed to inhibit the adhesion of a second strain (McCourtie and Douglas, 1985). Purification of the components of EP that inhibit adhesion has been pursued (Critchley and Douglas, 1987a). EP subjected to heat, chemicals, and enzymes was examined for blocking activity. Heat (60°C, 15min), DTT, mild acid, and the proteases chymotrypsin, bromelain, and pronase significantly destroyed the blocking ability of EP. However, periodate and α-mannosidase had no effect on this ability. This suggests that the protein part of the mannoprotein is important in mediating adherence in this mechanism. Interestingly, treatment of EP with the protease, papain, or dilute alkali appeared to enhance its ability to inhibit adhesion. When these

two treatments were combined, substantially greater inhibition of adhesion was observed.

Adhesion inhibition tests with sugars and lectins have suggested that a receptor containing L-fucose is required for most strains of *C. albicans* to bind to BEC (Critchley and Douglas, 1987b). More recently, a degradation protocol was developed which involved the sequential treatment of EP with N-glycanase, papain and dilute alkali to cleave the protein and carbohydrate portions of the mannoprotein (Tosh and Douglas, 1992). Fucoside-binding protein fragments were recovered by affinity chromatography with the trisaccharide determinant of H (type 2) blood group antigen which terminates in an L-fucose residue. This purified adhesin was devoid of carbohydrate and could inhibit adhesion of *C. albicans* to BEC roughly 220 times more effectively, on a protein weight basis, than EP.

Some *C. albicans* adhesins are only expressed on hyphae. Hyphal-form cells have been shown to express cell-surface components which are different from those of yeast-form cells. For example, hyphal forms bind fibrinogen, laminin, and C3d (C3 complement conversion product) while the yeast form does not (Bouali *et al.*, 1986; Calderone *et al.*, 1988; Bouchara *et al.*, 1990). Heidenreich and Dierich (1985) described the binding of antibody-coated sheep erythrocytes conjugated with C3d or iC3b to hyphal and yeast forms of *C. albicans*. Both types of binding could be partially inhibited by D-mannose and D-glucose. Hostetter *et al.* (1990) and Gilmore *et al.* (1988) have reported that expression of *C. albicans* iC3b receptor is enhanced in growth medium containing glucose.

In addition to components that mimic mammalian complement receptors, *C. albicans* posseses CR3-like proteins that can bind to fibrinogen, fibronectin and laminin. Calderone and others demonstrated that fibronectin adheres to *C. albicans* and *C. tropicalis in vitro* and that less pathogenic species of *Candida* do not bind the glycoprotein to the same extent (Scheld *et al.*, 1985; Skerl *et al.*, 1984). Experimental evidence for fibronectin as a receptor for *C. albicans* on epithelial cells was provided by Skerl *et al.* (1984). The binding of fibrinogen has also been

demonstrated for *C. albicans* (Bouali *et al.*, 1986, 1987; Tronchin *et al.*, 1987). Binding was found to be 12-fold higher for germ tubes than for yeast cells. Bouchara *et al.* (1990) reported the presence of laminin receptors on *C. albicans* germ tubes but yeast cells were found not to bind laminin. Binding was eliminated when germ tubes were heated or subjected to proteolysis by trypsin. Fibrinogen was also found to inhibit the binding of laminin by 57%. Two mannoproteins were identified as receptors of laminin, one of 68 kDa, the other 60-62 kDa. Similar proteins were found to have affinities for fibrinogen, laminin, and C3d binding protein by Tronchin *et al.* (1989). The mannoprotein of 60 kDa extracted from hyphae of *C. albicans* appears to recognise ligands that contain the amino acid sequence arginine-glycine-aspartic acid (RGD), which is found in iC3b, fibrinogen, laminin, and fibronectin. This mannoprotein also seems to bind to C3d which does not contain the RGD sequence. RGD-containing proteins are important constituents of the extracellular matrix of endothelial cells. Preincubation of endothelial cells with RGD-containing ligands blocks the adherence of *C. albicans* to these cells (Calderone and Braun, 1991). Indwelling catheters are rapidly coated with proteins such as fibronectin and this is likely to promote the adhesion of *C. albicans* .

Other investigators have proposed that candidal chitin may act as an adhesin. This minor constituent of the cell wall is involved primarily in budding and septum formation. Ultrastructural studies have shown that chitin is distributed throughout the cell wall as well as at bud scars and septa (Tronchin *et al.*, 1981). Degradation products of chitin block adherence of the organism to vaginal epithelial cells and protect mice from vaginal infection (Lehrer *et al.*, 1983; Segal *et al.*, 1988).

7.11 Glycosides as epithelial receptors for *C. albicans*

The plasma membrane of animal cells is composed of lipids and proteins both of which can be glycosylated. Carbohydrates on these lipids and proteins are essential binding sites for bacteria and viruses (Karlsson and Stromberg, 1987).

Research involving sugar inhibition tests with *C. albicans* has produced a variety of contradictory results. L-Fucose (Sobel *et al.*, 1981), amino sugars (Segal *et al.*, 1982a) and D-mannose (Sandin *et al.*, 1982) have all been reported to be major inhibitors of *C. albicans* adhesion and therefore the likely receptor determinant. More recently, Critchley and Douglas (1987b) demonstrated that a receptor containing L-fucose is required for most strains of the yeast. Although the addition of L-fucose to assay mixtures caused only partial inhibition of adhesion with sensitive strains, it is possible that the natural mucosal receptor is larger than L-fucose or that a particular stereochemical configuration is necessary. Brassart *et al.* (1991) showed that *C. albicans* adhesion to BEC *in vitro* was inhibited by the addition of Fuc α1-2Galβ-bearing complex carbohydrates. This structure is found on all blood group substances in the ABO system. Other studies also indicate a role for the Lewis^a antigen, which is found in the body fluids of non-secretors of blood group antigens (May *et al.*,1986, 1989; Tosh and Douglas, 1991). The Lewis antigens are adsorbed on to the surface of oral epithelial cells from saliva. In both studies, anti-Lewis^a antiserum had a significant inhibitory effect on yeast adhesion. From these results, it appears that the most likely candidates for *C. albicans* on BEC are fucose-containing glycosides such as the H antigen and the Lewis^a antigen (Tosh and Douglas, 1992). Support for this theory comes from a clinical survey, carried out by Burford-Mason *et al.* (1988), which found that blood group O and non-secretion of blood group antigens were separate and cumulative risk factors for oral candidal carriage in healthy subjects.

In studies of the role of glycosides as receptors for bacterial adhesion, most workers have adopted glycosphingolipids over glycoproteins for binding analysis (Karlsson and Stromberg, 1987). Glycosphingolipids only carry one polysaccharide chain and are easily extracted from tissues. Glycoproteins, on the other hand, may express two or more different glycoside moieties and, as a result, make the identification of receptors more difficult.

8. GLYCOSPHINGOLIPIDS

8.1 Glycosphingolipid structure and nomenclature

Glycosphingolipids are the glycosides of N-acylsphingosine, the trivial name for which is ceramide. There are a great number of chemically distinct glycosphingolipids due to structural variation in fatty acids, sphingosine and carbohydrate. Glycosphingolipids can be classified as neutral glycolipids, sulfatides (sulphate-containing) and gangliosides (sialic acid-containing) (Hakomori, 1993). They can also be classified according to the number of carbohydrate residues present (ceramide monohexose, ceramide dihexose etc.). An alternative classification depends on the backbone structure of the carbohydrate. Under this system, the globo series glycosphingolipids contain globotriaosylceramide (Galβ1-4 or 1-3Galβ1-4Glc-Cer). The lacto series contain lactotriaosylceramide (GlcNAcβ1-3Galβ1-4Glc-Cer). The ganglio series contain gangliotriaosyl ceramide (GlcNAcβ1-4Galβ1-4Glcβ1-1Cer) and the gala series contain galabiosyl ceramide (Galβ1-4Galβ1-1Cer) as the common structure. The lacto series show the most extensive substitutions with sialosyl, fucosyl, and α- and β- galactosyl moieties. They can also have extensions or branching by repeating N-acetyllactosamine units which can result in a great number of distinct molecular structures. The fucosylated species include the major blood group antigens.

8.2 Glycosphingolipids as receptors for micro-organisms

Glycosphingolipids have been reported to be cell-surface receptors for a number of bacteria. For example, uropathogenic *E. coli* specifically bind to a Galα1-4Gal sequence on urinary tract epithelial cells (Leffler *et al.*, 1982). Also *Pseudomonas aeruginosa* and *Pseudomonas cepacia* isolated from cystic fibrosis patients bind to glycosphingolipids containing GalNAcβ1-4Gal sequences (Krivan *et al.*, 1988a).

Saccharomyces cerevisiae (Horvath *et al.*, 1994; Sipos *et al.*, 1994) and *Candida deformans* (Mineki *et al.*, 1994) are known to contain some glycosphingolipid structures. Mineki *et al.* (1994) recently identified a glucocerebroside that contained a single glucose residue attached to ceramide, from the n-alkane-assimilating yeast *Candida deformans.* Ghannoum and colleagues found that various classes of lipid derived from *Candida* cell walls (including ceramide mono- and di-hexosides) were able to inhibit adherence of the yeast to BEC. They subsequently showed that total lipid extracts from BEC also blocked adherence (Ghannoum *et al.*, 1987). More recently, Jimenez-Lucho *et al.* (1990) proposed lactosylceramide as a receptor for *C. albicans*, *Cryptococcus neoformans* and other fungi. They found that ^{125}I-labelled fungi bound to lactosylceramide but not to other glycosphingolipids separated on TLC plates. Lipids expressing L-fucosyl residues were not used in this investigation. In a preliminary study, Yu *et al.* (1993) found that *C. albicans* bound to gangliosides GM_1 and asialo-GM_1.

9. BLOOD GROUP ANTIGENS AND SECRETOR STATUS

Blood group antigens may be defined as molecules formed by sequential addition of saccharides to the carbohydrate side chains of lipids and proteins on erythrocytes and certain epithelial cells (Kabat, 1973). The direct gene products of each of these blood group systems are the glycosyl transferases which catalyse the addition of specific sugar residues thus conferring specific blood group activity to the glycolipid or glycoprotein molecule (Green, 1989). The term "blood group secretor" refers to the ability of the secretory cells of an individual to produce blood group substance in their body fluids (Green, 1989). The A and B blood group antigens are major histocompatibility antigens and can cause graft and transplant rejections including heart, skin, and cornea (Joysey *et al.*, 1977; Herksho *et al.*, 1980). Substances very similar in structure to the ABH antigens are also widely distributed in Nature, being found in many species of animals, plants, and bacteria (Springer, 1956, 1965). In

man, various receptor properties including specific interactions with antibodies, viruses, toxins and pharmacological agents have been attributed to the ABH determinants (Green, 1989).

The type of polysaccharide chain to which the ABH specific sugars are added are of two main classes: type 1 chains have a terminal Galβ1-3GlcNAc sequence and type 2 chains have a terminal Galβ1-4GlcNAc (Rege *et al.*, 1963). The structures of the type 1 and 2 chains are shown in Fig. 1.

On the red blood cells, all the ABH active structures are synthesised on type 2 chains with up to 80% bound to protein and 20% existing as glycolipid (Green, 1989). Synthesis of the ABH structures is controlled by genes at three different loci i.e. ABO, H and Se loci. A total of four different glycosyltransferases are coded for by these genes. The H gene specifies a fucosyltransferase which adds fucose to galactose in an α1-2 linkage.

The A gene specifies an N-acetylgalactosaminyltransferase that attaches N-acetylgalactosamine in an α1-3 linkage (Morgan and Watkins, 1969). The B gene product is a galactosyltransferase which attaches galactose in an α1-3 linkage to the type 1 and type 2 precursor chains. The structures of the A and B chains are shown on Fig. 1. Both the A- and B- specific transferases can only add their respective sugars when the terminal galactose is substituted with fucose in the 2- position i.e. a type 1 or 2 H-active structure.

The genetics of the ABO blood groups are subject to Mendelian inheritance. One allele is passed by each parent with A and B co-dominant with each other and both being dominant over O. The O allele is recessive and must be in the homozygous form to be expressed in the phenotype. In rare hh individuals (Bombay phenotype), H active structures are not formed from the precursor so no fucose is added to the terminal galactose. This means that these individuals are unable to catalyse the addition of A-and B-specific sugars, although the appropriate gene specified transferase is present, because the H-active structure is required for enzyme activity (Green, 1989).

Control of the secretion of blood group antigens is under the influence of the Se gene. The exact mechanism by which the Se gene acts is not fully understood. Se and H genes are two distinct but closely related structural genes, each of which codes for a distinct α–2-L-fucosyl transferase. One enzyme reacts preferentially with type 1 chains, the other with type 2 chains. Se is dominant to se hence SeSe and Sese individuals are secretors of blood group antigens while sese individuals are non secretors.

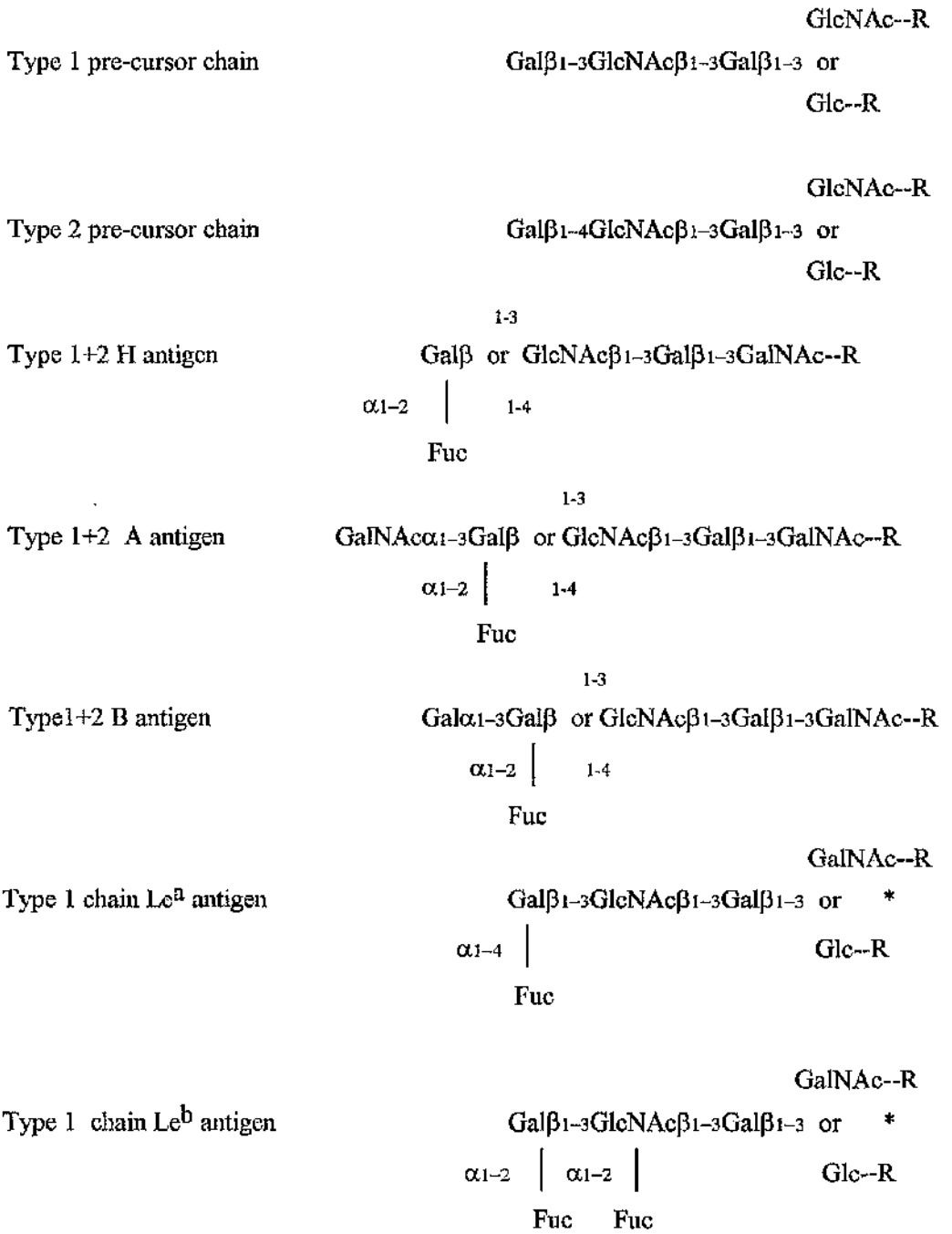
The Lewis blood group antigens are present in plasma and body secretions such as saliva. Those Lewis antigens in plasma consist of polysaccharide chains bound through D-glucose to glycosphingolipids (Marcus and Cass, 1969; Tilley *et al.*, 1975). In saliva, the Lewis antigens are identical to those of plasma but are bound via N-acetylglucosamine to peptides (Watkins and Morgan, 1959). Plasma Lewis antigens are inserted into red cell, platelet, and lymphocyte membranes via the lipid component. Non-secretors of ABH blood group antigens are Le^a and secretors are Le^b.

The Le gene produces another fucosyltransferase enzyme which adds another fucose residue to the type 1 H chain. When the H/Se gene product and the Le gene product add fucosyl residues, this will confer Le^b activity on to the type 1 chain. If an individual lacks the secretor gene, Le^a is the only product. The structures of Lewis antigens are shown in Fig. 1.

9.1 Blood group antigens in the oral mucosa

The expression of blood group antigens is related to cellular maturation. Blood group antigens A and B and their precursors are all expressed in different layers of the oral epithelium (Dabelsteen *et al.*, 1982). In blood group A or B individuals the H antigen is expressed in the deeper epithelial layers and the A or B antigen is expressed on the more superficial cells. The palatal epithelium appears to be less differentiated than the buccal epithelium and, as a consequence, the A or B antigens are present in higher amounts on buccal epithelium than on palatal epithelium. Conversely, H

Fig. 1. The A, B, H, and Lewis blood group structures.



GalNAc = N-acetyl-D-galactosamine, Gal = D-Galactose, Fuc = L-Fucose,
GlcNAc = N-acetyl-D-glucosamine, Glc = D-Glucose.

*Glc when R is sphingolipid and GalNac when R is protein.

antigens are at a higher concentrations on palatal cells than buccal epithelium (Vedtofte *et al.*, 1984).

OBJECT OF RESEARCH

The search for host cell receptors for *C. albicans* has, until recently, been limited to blocking studies using monosaccharides and/or lectins in adhesion-inhibition assays. A more recent investigation showed that *C. albicans* adhesion to BEC *in vitro* was significantly inhibited by the addition of Fucα1-2Galβ-bearing complex carbohydrates. This structure is found on all blood group substances in the ABO system. Blood group antigens are expressed on the carbohydrate side-chains of lipids and proteins on erythrocytes and certain epithelial cells. Two recent studies provided evidence for the role of lipids as receptors for *C. albicans*. One of the studies showed that *C. albicans* and other fungi bound specifically to lactosylceramide (a glycosphingolipid commonly found on epithelial cells), by employing a chromatogram overlay assay previously used in the characterisation of bacterial adhesion mechanisms. The other study employed a similar overlay system and demonstrated that *C. albicans* fimbriae bound to asialo-GM_1 and asialo-GM_2, but failed to bind to lactosylceramide.

A complete purification scheme for the yeast adhesin was recently devised which made use of the findings from earlier investigations of adhesion mechanisms. The purification protocol involved stepwise treatment of EP with N-glycanase, papain, and dilute alkali followed by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen. Adhesin purified in this way is devoid of carbohydrate, can inhibit adhesion of yeasts to BEC by up to 80%, and consists of one major component as determined by reverse-phase fast protein liquid chromatography (FPLC). The aims of this study were:

(i) to characterise further the purified adhesin isolated previously

(ii) to isolate and identify buccal epithelial cell glycolipid receptors for *C. albicans* using the chromatogram overlay assay system.

A more complete understanding of the molecules involved in the adhesion of *C. albicans* to epithelial cells may lead to the development of novel drug therapies and/or vaccines for this opportunistic pathogen.

MATERIALS AND METHODS

1. ORGANISMS

Six strains of *C. albicans* were used in this study. Four of the strains were isolated in Glasgow and are now deposited with the National Collection of Yeast Cultures (NCYC), Food Research Institute, Norwich, UK. *C. albicans* GDH 2346 (NCYC 1467) and GDH 2023 (NCYC 1468) were isolated at Glasgow Dental Hospital from patients with denture stomatitis; strains GRI 681 (NCYC 1472) and GRI 682 (NCYC 1473) were obtained from routine cervical smears taken from asymptomatic women at Glasgow Royal Infirmary. *C. albicans* MRL 3153 was from the Mycological Reference Laboratory at the London School of Hygiene and Tropical Medicine, London, UK. *C. albicans* "outbreak strain" was isolated from a patient at the London Hospital Medical College, London, UK, and was kindly supplied by Dr. J.P. Burnie. The organisms were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every two months, cultures were replaced by new ones freshly grown from freeze-dried stocks..

2. GROWTH CONDITIONS

2.1 For freeze-drying

For freeze-drying, yeasts were grown in yeast nitrogen base (Difco; YNB) containing 500mM sucrose. Batches of medium (50ml, in 250ml Erlenmeyer flasks) were inoculated with overnight yeast cultures (5ml) and incubated at 37^{O}C in an orbital shaker operating at 150 rpm. Cells were harvested after 24h (MSE bench centrifuge: 5min, 1200g) and washed in sterile 0.15M phosphate-buffered saline (pH 7.2; PBS). Yeast cells were suspended in a small volume of 2% (w/v) sterile skimmed milk and a drop of suspension was added to a sterile glass ampoule. Ampoules were frozen before being dried in a centrifugal freeze-dryer (Edwards High Vacuum Ltd., Sussex).

2.2 For adherence assays

Batches of medium (50ml, in 250ml Erlenmeyer flasks) containing YNB supplemented with 500mM galactose were inoculated from stock yeast cultures and

incubated overnight at 37°C in an orbital shaker operating at 150 rpm. Fresh medium (50ml) was inoculated with the overnight culture (5ml) and incubated at 37°C in an orbital shaker operating at 150 rpm for 24h to allow the yeast cells to reach stationary phase. Cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200g) and washed twice in 0.15M PBS (pH 7.2).

2.3 For isolation of extracellular polymeric material.

Yeasts were grown in YNB medium containing 500mM galactose. Batches of medium (500ml, in 2l Erlenmeyer flasks) were inoculated with 50ml of an overnight culture and incubated at 37°C in an orbital shaker operating at 150 rpm for 5 d.

3. ADHERENCE TO HUMAN BUCCAL EPITHELIAL CELLS

3.1 The yeast adherence assay

3.1.1 Collection and preparation of buccal epithelial cells

Buccal epithelial cells were collected from a single donor by gently swabbing the inside of the cheeks with a sterile swab. The swab was agitated in PBS (10ml) in a universal bottle. Epithelial cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200g) and washed twice in PBS to remove loosely-bound microorganisms. After the second wash, epithelial cells were standardised using an improved Neubauer haemocytometer to a concentration of $1x10^5$ cells ml^{-1} in PBS. Buccal cells were always collected between 8.30 am and 10.30 am to reduce variability.

3.1.2 Preparation of yeast cells

After the second wash of the yeasts in PBS, yeast suspensions were standardised in PBS using an improved Neubaur haemocytometer to the stated concentration for each experiment.

3.1.3 Adherence assay

The method used was similar to that described by Douglas *et al.* (1981). Epithelial and yeast cell suspensions were vortexed using a whirlimixer before being used in adherence assays. Standardised suspensions of buccal cells ($1x10^5$ cells ml^{-1} in PBS; 0.1ml) and yeasts (0.1ml, at the concentration stated for each experiment) were mixed in small screw-capped bottles and incubated at 37^oC, with gentle shaking, for 45min. After incubation, 3ml of PBS was added to each bottle to stop any further attachment. The epithelial cells were collected on polycarbonate filters (12μm pore size; 25mm diameter; Nuclepore Corp., Pleasanton, CA) and washed with PBS (25ml) to remove unattached yeasts. The washed filters were placed on labelled slides and air-dried. Epithelial cells on the filters were fixed with absolute alcohol-acetone (1:1) for 5 sec and stained using the Gram procedure. After drying, filters were mounted under coverslips using DPX (BDH Chemicals Ltd.). The numbers of adherent yeasts on each of 100 epithelial cells were counted on every filter. Triplicate filters were prepared for each assay. All adherence values quoted represent mean figures from 3 independent assays.

3.2 Use of inhibitors to block *in vitro* adherence

3.2.1 Extracellular polymeric material (EP)

Epithelial cell suspensions (1ml, containing $1x10^5$ cells ml^{-1}) were centrifuged in an MSE bench centrifuge at 1200g for 5 min and the supernatants were discarded. Crude EP samples (usually $10mgml^{-1}$; 1ml unless stated otherwise) were added to the cell pellets and the cells were mixed using a whirlimixer. Epithelial cells were incubated with the EP solution for 30 min at 37^oC. After this treatment, epithelial cells were recovered by centrifugation, resuspended in PBS (1ml) and used in adherence assays.

3.2.2 RGD peptide

Yeast cell suspensions (1ml, containing $1x10^7$ cells ml^{-1}) were centrifuged in an MSE bench centrifuge at 1200g for 5 min and the supernatants were discarded. RGD (arginine-glycine-aspartic acid) peptide solutions (either $0.5mgml^{-1}$ in PBS or $0.5mg\ ml^{-1}$

in 50 mM Tris-HCl pH 7.3 containing 0.02% CaCl and 0.02% MgCl) were added to the cell pellets. The yeast cells were resuspended and incubated with the RGD solutions for 30 min at 37^{O}C in an orbital shaker operating at 150 rpm. After this treatment, yeast cells were recovered by centrifugation, resuspended in PBS (1ml) and used in adherence assays.

4. ISOLATION AND PURIFICATION OF THE YEAST ADHESIN

4.1 Preparation of extracellular polymeric material (EP)

4.1.1 Isolation of EP from culture supernatants of *Candida albicans*

EP from strains GDH 2346 and GDH 2023 was prepared by freeze-drying dialysed culture supernatants using a minor modification of a method previously described (Tosh and Douglas, 1992). Batches of medium (500ml in 2l Erlenmeyer flasks) containing yeast nitrogen base (Difco) and 500mM galactose were inoculated with overnight yeast cultures (50ml) and incubated at 37^{O}C for 5 d in an orbital shaker operating at 150 rpm. Yeasts were removed by centrifugation and the culture supernatant fluid was ultrafiltered in an Amicon DC2 Hollow Fibre Concentration System using a 3.5K filter and then dialysed at 4^{O}C for 3d against 5 changes (5l each) of distilled water. The retentate (crude EP) was then freeze-dried.

4.1.2 Biochemical analysis of EP

Carbohydrate was estimated by the method of Dubois *et al.* (1956) using mannose as a standard (see Appendix I.A). Protein was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard (see Appendix I.B).

4.2 Isolation of purified adhesin

EP (100mg) was dissolved in 1ml of 0.55M Na_2HPO_4 (pH 8.6). N-Glycanase (Genzyme; 250 Units ml^{-1}; 0.096ml) was added and the mixture was incubated for 24h at 37^{O}C. The reaction was terminated by the addition of Sodium citrate buffer (0.1M, 0.04ml, pH 4.4). Potassium phosphate buffer (0.01M, pH6.2) was used to adjust the EP

concentration to 50 $mgml^{-1}$. Papain (0.1 $mgml^{-1}$; 0.16ml) was added and the solution was incubated for 30 min at 25°C. The protease inhibitor α_2-Macroglobulin (2 $mgml^{-1}$, 0.5ml) was employed to terminate the reaction. NaOH (0.366M; 1ml) was added and the mixture was incubated for 24h at 30°C. 1 ml of HCl was used to neutralise the NaOH.

4.3 Extraction of purified adhesin by affinity chromatography

Synsorbs (Chembiomed Ltd., Edmonton, Alberta, Canada) are specific affinity adsorbents for the isolation and purification or removal of biologically important molecules. They are composed of synthetic carbohydrate structures of known specific composition covalently linked to an inert, insoluble matrix. Adsorbed material can be removed in a highly purified form and the adsorbent regenerated by elution with high or low pH buffers.

Synsorb H-2 (0.5g) was washed twice in PBS (2ml) and allowed to settle. The buffer was removed to just above the surface of the synsorb bed and EP digest was applied. The digest and the Synsorb were gently rotated for 3h at 4°C. Unbound material was eluted with 5ml aliquots of PBS until the A_{280} returned to 0.0. Bound material was then eluted by the addition of 2 ml aliquots of 1.5% NH_4OH in sterile saline for 15 min at 4°C until the A_{280} returned to 0.0. Bound samples were freeze-dried and desalted on a Sephadex G-25 (5ml) desalting column. The adsorbent was regenerated with PBS and then stored at 4°C in PBS containing 0.02% NaN_3 as a bacteriostat.

4.4 Adhesion inhibition activity of the purified adhesin

Epithelial cell suspensions (10^5 cells ml^{-1} in PBS; 1ml) were centrifuged in a bench centrifuge at 5,000 x g for 5 min, and the pellets were resuspended in PBS or purified adhesin solution (6.375 μg protein ml^{-1}). After incubation at 37°C for 30 min with gentle shaking, the buccal cells were recovered by centrifugation and resuspended in PBS (1ml) for use in the adhesion assay, performed as described above (3.1.3). All adhesion-inhibition values quoted represent mean figures derived from three independent assays, each carried out in triplicate. Epithelial cells were obtained from the buccal mucosa of a

single, healthy, male, blood group A donor and were collected at the same time of day to reduce variability

4.5 Adhesion inhibition index

The adhesion inhibition index (AII) is a measure of the relative efficiency with which PA inhibits yeast adhesion compared with crude EP, on a protein weight basis. Preparations of crude EP, at a concentration of 10 mg ml^{-1} routinely inhibit adhesion by 45 to 50%. The AII = W_{EP} /W_{PA} where W_{EP} is the weight of EP protein required to produce 50% inhibition of adhesion and W_{PA} is the weight of PA required to inhibit adhesion by 50%.

4.6 High Performance Liquid Chromatography of EP and purified adhesin

High Performance Liquid Chromatography (HPLC) was performed using an LKB Bromma HPLC machine with a TosoHaas C18 TSK-Gel reversed phase column. Samples (EP, EP digests and purified adhesin) were prepared in 0.1% trifluoroacetic acid (TFA). Details are given in the text and figure legends for individual experiments. Samples were filtered through a 0.2μm filter prior to loading and 200μl of sample was loaded at a time. The machine was run with a flow rate of 30 mlh^{-1} using an acetonitrile gradient of 0-100% in 0.1% TFA. Absorbance was read at 208nm and was recorded on a chart feeder.

4.7 Amino acid analysis of purified adhesin

Amino acid analysis was kindly performed by Drs. Gordon Curry and Maggie Cusack from the Department of Geology and Applied Geology, University of Glasgow, using Applied Biosystems 420-H amino acid analyser with an automatic hydrolysis head. Purified material (1μg dissolved in 50μl of 0.1% TFA) was loaded.

4.8 Amino acid sequencing of purified adhesin

Amino acid sequencing was kindly performed by Sandra Miller in the Department of Geology and Applied Geology, University of Glasgow. Purified adhesin was run on a polyacrylamide gel using the procedure of Schagger and Von Jagow (1987) and then transferred onto ProBlott (Applied Biosystems). The ProBlott was cut to include the band of the adhesin The position of the band was estimated using an autoradiograph of a gel blot of biotinylated adhesin from the same purified batch. The material on the ProBlott was then sequenced in an Applied Biosystems 477A Protein Sequencer.

5. LABELLING OF *C. ALBICANS* WHOLE CELLS, EP, AND PURIFIED ADHESIN

5.1 Iodination of EP and yeasts

Iodogen (Pierce; 1mg) was dissolved in 25ml of chloroform and 1ml aliquots were dispensed into clean glass universals. The chloroform was allowed to evaporate to dryness at R.T. under nitrogen to leave an Iodogen coated universal. The coated universal was rinsed with PBS prior to reaction to remove unbound flakes of Iodogen. EP (100 mg) was dissolved in 1 ml of PBS and then added to the universal. $Na^{125}I$ (1mCi) was added, followed by NaI (final concentration 0.25mM) to act as a carrier for safe handling. The universal was then placed in a lead container for the duration of the iodination. The reaction was allowed to proceed for 15 min with occasional shaking. The solution was then transferred to narrow bore dialysis tubing and dialysed for 24 h against PBS.

Yeast cells were iodinated using a modification of the method of Jimenez-Lucho *et al.* (1990). Washed yeast cells were adjusted to a concentration of $2x10^8$ cells ml^{-1}. To one of the Iodogen coated universals was added 0.5 ml of fungal suspension. $Na^{125}I$ (1mCi) was added, followed by NaI (final concentration 0.25mM) to act as a carrier for safe handling. The universal was then placed in a lead container for the duration of the iodination. The reaction was allowed to proceed for 15 min with occasional shaking.

After incubation, unreacted $Na^{125}I$ was removed by three centrifugal washes (MSE bench centrifuge: 5min, 1200g) in 0.15M phosphate-buffered saline (pH 7.2; PBS). The final pellet was resuspended in 10 ml of Tris-BSA buffer (0.05M Tris-hydrochloride [pH7.8] containing 0.15M sodium chloride and 1% BSA), and then used in TLC overlay analysis.

5.2 Biotinylation of yeasts, EP, and purified adhesin.

Yeasts were biotinylated by a modification of the method of Casanova *et al.* (1992) using a biotinylation kit from Amersham, UK. Washed cells were adjusted to $2x10^8$ organisms ml^{-1} in 0.05M sodium borate buffer, pH8.6. Biotinylation reagent (40µl) was added to 1 ml of cell suspension and incubated for 1h at room temperature with constant agitation. The cells were washed twice in 0.05 M Tris-hydrochloride (pH 7.8) containing 0.15 M sodium chloride and 1% BSA (Tris-BSA) and adjusted to 10^8 cells ml^{-1} in Tris-BSA. EP and purified adhesin were biotinylated using the Amersham biotinylation kit according to the manufacturer's protocol. EP (10mg) or adhesin (200µg) was dissolved in 1ml of 0.05M sodium borate buffer, pH8.6. Biotinylation reagent (40µl) was added and the mixture was incubated for 1h at room temperature with constant agitation. Unreacted biotinylation reagent was removed by desalting on a Sephadex G-25 (5ml) desalting column and the EP and adhesin were freeze-dried.

6. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

6.1 Gel Preparation

Two SDS-PAGE systems were employed in this study: the procedures of Laemmli (1970) and of Schagger and Von Jagow (1987).

Plates were prepared by first washing them in detergent to remove dirt and grease; they were then wiped clean with paper tissue dipped in absolute alcohol. Plastic spacers (1.5mm) were placed between the plates which were taped together along three sides and baked for 30 min at $80^{O}C$ to seal the tape.

Gels for the Laemmli procedure were prepared as follows. The separating gel solution containing 12.5% acrylamide, 1.5% Tris-HCl (pH 8.9), 0.4% SDS, 0.025% Temed and 0.8% ammonium persulphate, was mixed thoroughly and poured into the gel plates to about 4cm from the top (1cm for small plates). Absolute alcohol was used to overlay the separating gel, which was left for 20 min to polymerise. The absolute alcohol was then poured off and the gel was washed with distilled water. Stacking gel solution containing 4.5% acrylamide, 0.5M Tris-HCl (pH 6.8), 0.4% SDS, 0.025% Temed, and 0.08% ammonium persulphate was poured on top of the stacking gel. A sample comb was inserted into the stacking gel solution and any remaining spaces were filled. After polymerisation the comb was removed and the gel plate was dipped in running buffer to prevent drying out. Tris-glycine running buffer (pH 8.3) containing 0.1% SDS was used to fill the lower electrode tank. The sealing tape was removed from the bottom of the gel plates, and the plates were inserted into the top electrode tank. The upper tank was filled with running buffer. Sample buffer was prepared by combining 20% SDS (w/v; 4ml), glycerol (2.4 ml), 2-mercaptoethanol (0.4 ml), Brilliant Blue G (2.0 ml; Sigma) and tris-HCl (1M; pH 6.8; 1 ml) and water (10.2 ml). Proteins or biotinylated proteins were treated with solubilising buffer and then carefully pipetted into the sample wells. Gels were run at a constant current (20 mA per gel) for approximately 3.5h. When the tracking dye reached the end of the gel the power was switched off.

Gels for the procedure of Schagger and Von Jagow (1987) were prepared as follows. The separating gel solution contained 16.5% acrylamide, 12.1% Tris-HCl (pH 8.45), 0.1% SDS, 0.033% Temed, and 0.033% ammonium persulphate. The stacking gel solution contained 4% acrylamide, 9.01% Tris-HCl (pH 8.45), 0.1% SDS, 0.033% Temed, and 0.033% and 0.033% ammonium persulphate. The cathode buffer (pH 8.2) was prepared by combining tris (12.11 g), tricine (17.92 g), SDS (1.0 g) and 1 L of water. The anode buffer (pH 8.9) was prepared by combining tris (121.1 g) with water to a final volume of 5 L. Sample buffer was prepared by combining 20% SDS (w/v; 4ml), glycerol (2.4 ml), 2-mercaptoethanol (0.4 ml), Brilliant Blue G (2.0 ml) and tris-HCl (1M; pH 6.8; 1 ml) and water (10.2 ml). Samples and markers were treated with

solubilising buffer. Gels were run at 20 mA to allow samples to completely enter the stacking gel then the current was increased to 30mA until the marker dye was 1cm from the bottom of the gel plate.

6.2 SDS-PAGE and Western blot analysis of the *C. albicans* adhesin.

SDS-PAGE was performed in a minigel apparatus built by University of Glasgow Workshop based on the Bio-Rad Mini-Protean II electrophoresis system. The procedure of Schagger and Von Jagow (1987) was employed as described in section 6.1 (above). Samples (biotinylated PA) and molecular weight markers (MW-SDS-17S, Sigma) were electrophoresed for 3-4 hours at a constant current of 100 V. The gel was cut using a clean plastic ruler and the markers were stained with Brilliant Blue G (Sigma). The biotinylated PA lanes were transferred onto nitrocellulose (Hybond C, Amersham) for Western blot analysis which was performed according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). The transfer was performed at 0.13A (50V) for 1h, 0.08A (30V) for 18h then 0.13A (50V) for 1h. The nitrocellulose was transferred to a plastic box with lid and immersed in blocking solution (3% (w/v) skimmed milk containing 0.2% Tween 20 in PBS) overnight at 4°C. This solution was poured off and replaced with 10 ml of fresh blocking solution to which 5μCi of ^{125}I-streptavidin (Amersham) was added. The box was then closed and incubated with gentle shaking for 2h. The nitrocellulose was removed from the box, washed extensively in distilled water to remove unbound ^{125}I-streptavidin, allowed to air-dry, then exposed for 2-5 days to blue sensitive X-ray film (Genetic Research Instrumentation Ltd. Dunmow, Essex, UK) with intensifying screens.

6.3 Staining of gels

6.3.1 Coomassie blue stain (Weber and Osborne, 1969)

Destaining solution was prepared by combining methanol (50 ml), glacial acetic acid (75 ml) and made up to a final volume of 1 L with water. Staining solution was prepared by combining Brilliant Blue R (125 mg; Sigma) with 500 ml of destaining

solution. The staining solution was filtered before use. Gels were carefully removed from the plates and immersed in staining solution for 30 min. Gels were destained by several changes of destain solution then washed several times in distilled water for 30 min. The gels were then sealed in vacuum bags.

6.3.2 Silver staining (Oakley *et al.*, 1980)

Gels were carefully removed from the plates then pre-fixed for 30 min in 10% acetic acid, 30% methanol. Gluteraldehyde (10%) was used was used as a fixative for a further 30 min. After fixation, gels were rinsed overnight in a large volume of distilled water. Gels were then given a final rinse in distilled water for 30 min then placed in dithiothreitol ($5mgml^{-1}$) for 30 min. This solution was poured off and 0.1% silver nitrate (freshly prepared) was added, without rinsing, for a further 30 min. Gels were rinsed twice in distilled water and then twice in developer containing 37% formaldehyde (50ml) in 100ml of 3% Na_2CO_3, before being left in a third volume of developer until the desired level of staining was reached. Development was stopped by the addition of 2.3M citric acid (5ml) and the gels were rinsed for 10 min in distilled water. The gels were then sealed in vacuum bags.

6.3.3 Western blotting

Western blotting was performed according to the method of Towbin and Gordan (1984) in a Bio-rad 'Transblot' transfer apparatus. The procedure was used for the visualisation of biotinylated proteins. The electroblotting transfer buffer was made up as follows. Tris (7.2g) and glycine (33.4g) were dissolved in 3 litres of 20% methanol in distilled water (pH8). Hybond C (Amersham) was cut to the size of the gel and two sheets of Whatman filter paper were cut to the size of the gel cassette. The gel, the Highbond C, the filter paper and two Scotch brite pads were soaked in transfer buffer for 2 min. A Scotch brite pad was placed on one leaf of the gel cassette. One of the filter papers was placed on top followed by the gel. The Highbond C was carefully placed on the gel and air bubbles removed by gently rubbing the Highbond C with a clean glass rod.

The second filter paper sheet was laid on top followed by the second Scotch brite pad. The cassette was closed and secured then inserted into the Bio-rad blot apparatus so that the Highbond C was on the anode side of the tank. The tank was filled with transfer buffer and the transfer was run at 0.13A (50V) for 1h, 0.08A (30V) for 18h then 0.13A (50V) for 1h. The Highbond C was removed from the cassette, placed in a shallow plastic container and immersed in blocking solution (3% (w/v) skimmed milk containing 0.2% Tween 20 in PBS) to reduce the non-specific attachment of protein. This solution was poured off and replaced with 10ml of fresh blocking solution. 5μCi of ^{125}I-Streptavidin (Amersham) was added to the container which was then sealed and incubated with shaking for 2h. The Highbond C was removed from the box and washed in distilled water to remove unbound ^{125}I-Streptavidin, allowed to air-dry, then exposed for 2-5 days to blue sensitive X-ray film (Genetic Research Instrumentation Ltd. Dunmow, Essex, UK) with intensifying screens.

7. THIN-LAYER-CHROMATOGRAM OVERLAY ASSAY

7.1 Preparation of Glycosphingolipids.

Neutral glycolipid TLC standards were purchased from Calbiochem. These glycolipids comprised galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman antigen (FORS). Human buccal epithelial cells (BEC) were obtained by gently rubbing the inside cheek area of a healthy, male, blood group A donor with sterile cotton swabs. The cells were washed three times in PBS then stored frozen until required. A total lipid extract of BEC was obtained by an adaptation of the methods of Magnani *et al.* (1980) and Esselman *et al.* (1972). Cells collected over 10 days were thawed, washed in PBS, pooled and then weighed. The wet weight of cells ranged from 307-574 mg. The cells were resuspended methanol (7 ml) and homogenised with a pestle and mortar. Chloroform (14 ml) was added and the mixture homogenised again. The material was filtered with a Buchner funnel using an aspirator and a coarse-grade solvent-washed filter paper. The residue

was re-extracted with 10 ml of chloroform-methanol (2:1). The combined filtrates were evaporated to dryness and weighed. The yield of lipid extracted in this way ranged from 4.0-9.3 mg. This material contained the total lipid extract from BEC. Sheep red blood cell (sRBC) glycolipids were a gift from Chris Brotherston (University of Glasgow) and were prepared by a similar procedure.

7.2 Chromatogram overlay assay.

The chromatogram overlay assay was performed by a modification of the methods of Jimenez-Lucho *et al.* (1990) and Magnani *et al.* (1980). Glycolipids were chromatographed on aluminium-backed silica gel thin-layer plates (E. Merck AG, Darmstadt, Germany) and developed with chloroform-methanol-water (65:25:4) unless otherwise stated. The plates were dipped in a solution of polyisobutylmethacrylate (0.1%) in hexane, air dried, soaked for 1h in Tris-BSA buffer (0.05M Tris-hydrochloride [pH7.8] containing 0.15M sodium chloride and 1% BSA), and overlaid for 2h at room temperature with labelled ligand. Among the labelled ligands used were ^{125}I-EP, ^{125}I-yeast cells, biotin labelled yeasts, biotin-EP, biotin-adhesin or biotin-labelled lectins. The concentration of each ligand and details of the blocking procedures employed are given in the text and figure legends for the individual experiments. The plates were gently washed in distilled water to remove unbound material, soaked for 1h in Tris-BSA then overlaid with 5μCi ^{125}I-streptavidin (Amersham) when a biotinylated ligand was to be detected. When a radioiodinated ligand was used, the plates were just dried and autoradiographed. The ^{125}I-streptavidin plates were then washed, dried, and exposed for 2-5 days to blue sensitive X-ray film (Genetic Research Instrumentation Ltd. Dunmow, Essex, UK) with intensifying screens.

7.3 Lectins

The following lectins were used in this study:

Lectin	*Sugar Specificity	Source
Bauhinia purpurea	Gal(β1-3)GalNAc, GalNAc and Gal	Camel's foot tree
Griffonia simplicifolia II	GlcNAc	Bandeiraca
Helix pomatia	GalNAc	Edible snail
Triticum vulgaris	GlcNAc	Wheat germ
Ulex europaeus I	Fuc	Gorse
Vicia villosa	GalNAc	Hairy vetch

* Fuc = L-Fucose
Gal = D-Galactose
GalNAc = N-acetyl-D-galactosamine
Glc = D-Glucose
GlcNAc = N-acetyl-D-glucosamine

All lectins were purchased from Sigma, Poole, UK, with the exception of biotinylated *Griffonia simplicifolia* lectin II was purchased from Vector Laboratories, Peterborough, UK.

8. STATISTICAL ANALYSIS

Student's t test was used to detect significant differences between adherence values and the results are presented as p-values. Standard errors of the mean are quoted for all adherence assay results.

RESULTS

1. ADHESION OF *CANDIDA ALBICANS* TO EPITHELIAL CELLS

1.1 Effect of EP on yeast adhesion *in vitro*

The adhesion of some strains of *C. albicans* to buccal epithelial cells (BEC) is substantially increased after growth in defined media containing certain sugars (notably galactose) as the carbon source. This increase in adhesive ability seems to be a result of the production of an additional fibrillar-floccular layer on the yeast cell surface (Douglas, 1987). These surface fibrils have been seen to mediate attachment of *C. albicans* to epithelial cells *in vivo* (Lee and King, 1983). Prolonged incubation of *C. albicans* in medium containing galactose causes release of surface fibrils, and extracellular polymeric material (EP) can be isolated from culture supernatants. This EP appears to contain the yeast adhesin as it can inhibit the *in-vitro* adhesion of *C. albicans* to epithelial cells. McCourtie and Douglas (1985) isolated EP by acetone precipitation, Critchley and Douglas (1987a) used prolonged dialysis and Tosh (1991) employed dialysis followed by ultrafiltration. Throughout this study, EP was produced by a method very similar to that of Tosh (1991). The molecular weight exclusion limit of the ultrafiltration membrane employed in this study was 3.5K in place of the 5K filter used by Tosh. The compositional results are shown in Table 1.1 alongside results obtained in previous studies (McCourtie and Douglas, 1985; Critchley and Douglas, 1987a; Tosh, 1991). The protein content of EP from strain GDH 2346 prepared in this study was 8.3% and the carbohydrate content was 80.9%. These values are similar to those obtained by Tosh, 1991 (7.2% and 79.2% for protein and carbohydrate, respectively). However, the protein content of EP from a different strain (GDH 2023) prepared in this study was 5.5% and the carbohydrate content was 84.4% which differ slightly from the values obtained by Tosh (10.5% and 72.4% for protein and carbohydrate respectively). The different protein and carbohydrate compositions for EP produced in each study are most likely due to the different methods of preparation. In the following adhesion-inhibition experiments, yeasts were grown to stationary phase at

Table 1.1 Composition of EP produced in this and previous studies

Strain	Component	Percentage composition of EP in study of:			
		BJC[1]	FDT[2]	IAC + LJD[3]	JMcC+ LJD[4]
GDH 2346	protein	8.3 ± 0.6	7.2 ± 0.3	10.0 ± 0.7	6.9 ± 0.2
	carbohydrate	80.9 ± 2.2	79.2 ± 1.7	70.0 ± 2.6	78.8 ± 2.6
GDH 2023	protein	5.5 ± 0.4	10.5 ± 0.3	N.D.	N.D.
	carbohydrate	84.4 ± 1.8	72.4 ± 0.8	N.D.	N.D.

[1] This study (mean ± SEM)
[2] Tosh, 1991 (mean ± SEM)
[3] Critchley and Douglas, 1987a (mean ± SEM)
[4] McCourtie and Douglas, 1985 (mean ± SD)

In each study, *C. albicans* was grown for 5 days in yeast nitrogen base medium containing galactose (500mM) as the carbon source. EP was isolated from culture supernatants in each study by a different method. McCourtie and Douglas (1985) used acetone precipitation, Critchley and Douglas (1987a) used prolonged dialysis and Tosh (1991) employed dialysis followed by ultrafiltration. Throughout this study, EP was produced by a method very similar to that of Tosh (1991). The molecular weight exclusion limit of the ultrafiltration membrane employed in this study was 3.5K in place of the 5K filter used by Tosh. In all studies, carbohydrate was estimated by the method of Dubois et al. (1956) using mannose as a standard and protein was determined using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The results in this study represent the means of three independent assays performed in triplicate.

37°C with shaking in yeast nitrogen base (Difco) containing 500mM galactose as a carbon source. *C. albicans* grows exclusively in the yeast phase under these conditions.

1.1.1 Effect of EP concentration on yeast adhesion

EP from two *C. albicans* strains (GDH 2346 and GDH 2023) was prepared and used to pretreat buccal epithelial cells (BEC) prior to adhesion assays with cells from the homologous strains. The concentrations of yeast cells used were $5x10^7$ and $1x10^8$ cells ml^{-1} for strains GDH 2346 and GDH 2023 respectively. BEC were collected on the day of the experiment and after washing in PBS, were adjusted to a concentration of $1x10^5$ cells ml^{-1}. Pre-incubation of BEC with increasing concentrations (2-20 mg ml^{-1}) of EP from strain GDH 2346 inhibited yeasts from the homologous strain by nearly 60% (Fig.1.1). This result is consistent with the findings of Critchley and Douglas (1987a). In a recent study by Brassart *et al.* (1991) similar results were obtained. They found that EP could inhibit the adhesion of *C. albicans* cells of the homologous strain by 50%. Pre-incubation of BEC with increasing concentrations (2-20 mg ml^{-1}) of EP from GDH 2023 inhibited yeasts from the homologous strain by just over 40% (Fig. 1.1). Jenq *et al.* (1994) isolated EP with adhesion-inhibition activity of 42%. This level of inhibition is similar to that for the EP from GDH 2023 prepared in this study. Relatively high values for yeast adhesion were seen in these adhesion assays. The average number of adherent yeasts on untreated BEC was 19.82 and 24.85 for strains GDH 2346 and 2023 respectively. The different shape of the curves for the two strains in Fig. 1.1 is most likely due to the different yeast : BEC ratio employed.

1.1.2 Effect of yeast cell concentration on adhesion inhibition by EP

The relatively high values obtained for yeast adhesion in the previous experiment prompted an investigation into the effect of yeast concentration on adhesion inhibition. EP from strain GDH 2346 was used to pretreat BEC prior to incubation with cells from the homologous strain at two different concentrations ($1x10^7$ and $5x10^7$

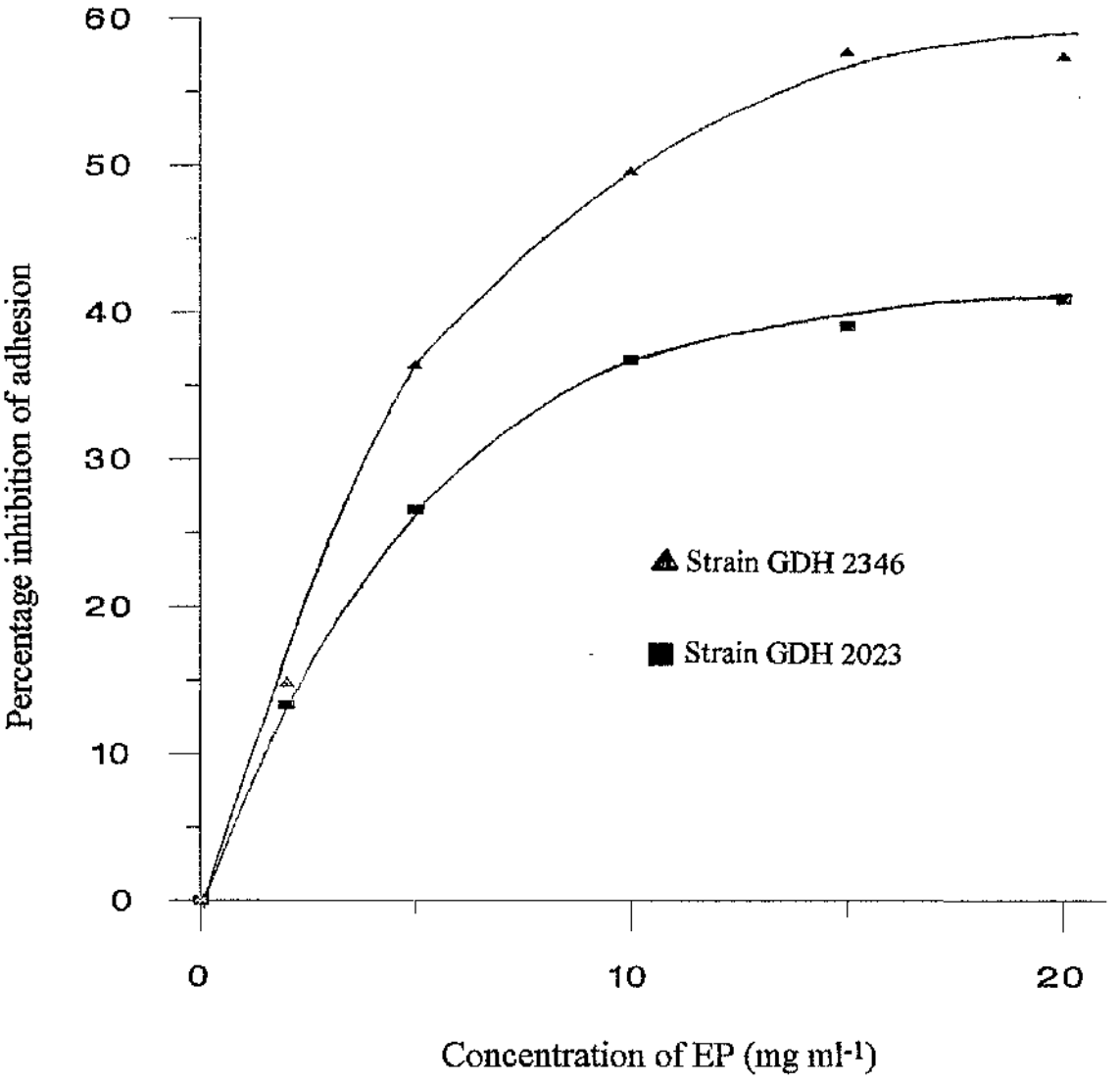


Fig. 1.1 Effect of EP concentration on the adhesion of *C. albicans* to BEC.

EP was isolated from culture supernatants of *C. albicans* strains GDH 2346 and GDH 2023 as described in Materials and Methods. Buccal cells were pretreated with EP solutions (2-20mg ml^{-1} in PBS) at 37°C for 30 min, and were then used in adhesion assays with the homologous strain of yeast. The concentrations of yeast cells used were $5x10^7$ and $1x10^8$ cells ml^{-1} for strains GDH 2346 and GDH 2023 respectively. The concentration of BEC was 1 $x10^5$. Results shown represent the mean values of three assays performed in triplicate.

yeasts ml^{-1}). The results are shown in Table 1.2. There was no significant difference between the adhesion-inhibition values for the two concentrations of yeasts. Yeasts at a concentration of $1x10^7$ cells ml^{-1} were inhibited by 49.1% while yeasts at $5x10^7$ cells ml^{-1} were inhibited by 48.5%, when compared to PBS treated controls. Interestingly, with a five-fold increase in the number of yeasts there was only a 65% increase in the number of adherent yeasts per 100 BEC. A yeast cell concentration of $1x10^7$ was adopted for all subsequent adhesion-inhibition assays.

1.1.3 Distribution of bound yeasts among BEC

The number of yeasts attached to individual BEC can vary greatly. A large number of BEC appear to have no yeasts attached while some have their entire surface covered in yeasts. This suggests that different BEC express different numbers of yeast receptors. Previous work by Sandin *et al.* (1987) examined this phenomenon. They performed adhesion assays with *Candida* and BEC in the ratio 10:1 and found that 41% of BEC had no yeasts attached. They also found that only 12% of BEC had 9 or more attached yeasts but that 52% of the total yeasts were attached to this 12%. In this study, a similar experiment was performed but with higher yeast : BEC ratios (Table 1.3). When the yeast : BEC ratio was 50:1, 33.7% of BEC had no yeasts attached and only 13.9% of BEC had 9 or more attached yeasts. Around 46% of the total yeasts were attached to this 13.9%. When the yeast : BEC ratio was increased to 250:1, only 21.1% of BEC had no yeasts attached but 32.9% of BEC had 9 or more attached yeasts.

1.1.4 Effect of papain degradation on EP

A number of proteases have been examined for their effects on EP. All proteases tested destroyed the adhesion-inhibition activity of EP except for papain which was shown to enhance this activity (Critchley and Douglas, 1987a). This effect was attributed to the possible production of peptide fragments that could more easily bind to BEC receptors and hence block adhesion. Papain is a cysteine protease i.e. it

Table 1.2 Effect of yeast cell concentration on adhesion inhibition by EP.

BEC treatment	Yeast conc. (cells ml^{-1})	Yeast: BEC ratio	No. of adherent yeasts per 100 BEC (± SEM)	Adhesion inhibition (%)
EP	$1x10^7$	100:1	1016.7 ± 104	49.1
PBS	$1x10^7$	100:1	1996.0 ± 139	-----
EP	$5x10^7$	500:1	1701.0 ± 117	48.5
PBS	$5x10^7$	500:1	3300.0 ± 178	-----

EP from strain GDH 2346 was used to pretreat BEC (at 37°C for 30 min) prior to incubation with cells from the homologous strain at two different concentrations ($1x10^7$ and $5x10^7$ yeasts ml^{-1})and the concentration of BEC was $1x10^5$ cells ml^{-1}. Results shown represent the mean values of three assays performed in triplicate.

Table 1.3 Distribution of bound yeasts among BEC

	Yeasts bound per 100 BEC ± SEM	Percentage of BEC with 9 or more bound yeasts ± SEM	Percentage of total yeasts on BEC with 9 or more bound yeasts ± SEM	Percentage of BEC with no bound yeasts ± SEM	Maximum No. of yeasts found on a single BEC
Yeast : BEC, 50:1 ($1x10^7$ yeasts ml^{-1})	352.7 ± 57.6	13.9 ± 3.7	46.22 ± 4.6	33.7 ± 4.7	34
Yeast: BEC, 250:1 ($5x10^7$yeasts ml^{-1})	728.3 ± 101.4	32.9 ± 3.8	65.43 ± 6.3	21.1 ± 3.4	60
Yeast : BEC, 10 : 1 ($2x10^6$ yeasts ml^{-1})*	270-300	12	52	41	32

*Data of Sandin *et al.* (1987)

Adhesion-inhibition assays performed with *C. albicans* GDH 2346 cells at two different concentrations ($1x10^7$ and $5x10^7$ yeasts ml^{-1}) and BEC at $2x10^5$ cells ml^{-1}. Results shown represent the mean values of three assays performed in triplicate.

Table 1.4 Effect of papain on the ability of EP from *C. albicans* GDH 2346 to inhibit adherence of the homologous yeast strain to buccal epithelial cells

Pretreatment of BEC	Mean adherence[1]	% inhibition	P[2]
Control (no EP)[3]	540.9 ± 34.8	-	-
Papain-treated EP[4] (non- reducing)	223.1 ± 19.5	58.8%	<0.001
Papain-treated EP[5] (reducing)	546.1 ± 34.6	0.0%	NS

[1] Mean number of adherent yeasts per 100 epithelial cells ± standard error (SEM).

[2] Probability values relative to adhesion of PBS control; NS, not significant.

[3] BEC were incubated in PBS at 37°C for 30 min prior to being used in adhesion assays. The results represent the means of three independent assays performed in triplicate.

[4] EP (20 mg) was dissolved in 1 ml of 0.01 M potassium phosphate buffer (pH 6.2). To the EP solution was added 0.016 ml of papain (0.1 mg ml^{-1} in 0.01 M potassium phosphate buffer (pH 6.2) and the mixture was incubated at 25°C for 30 min. The reaction was stopped by the addition of 0.05 ml of α_2-macroglobulin (2 mg ml^{-1}). BEC ($1x10^5$ cells) were then incubated at 37°C for 30 min in the solution of EP. The BEC were then washed and used in adhesion assays.

[5] EP (20 mg) was dissolved in 1 ml of 0.01 M potassium phosphate buffer (pH 6.2) containing 5 mM cysteine and 2 mM EDTA. The EP solution was then treated exactly as in [4] above.

has an essential cysteine residue at its active site that must be maintained in the reduced form for activity (Butler, 1989). However, papain was previously used to digest EP under non-reducing conditions (Critchley and Douglas, 1987a; Tosh and Douglas, 1992). In this study, EP was treated with papain under both reducing and non-reducing conditions. EP treated with the enzyme under non-reducing conditions retained its ability to inhibit adhesion (58.75%) whereas EP treated with papain under reducing conditions was found to have lost this activity (Table 1.4). There was no significant difference between the adhesion values obtained for BEC pretreated with this papain-digested EP and untreated BEC. These results suggest that when EP is treated with papain under reducing conditions, its adhesion-inhibition activity is destroyed due to extensive damage to the protein part of the molecule.

2. HPLC ANALYSIS OF EP

Previous work by Douglas and co-workers has shown that the ability of EP to inhibit adhesion can be enhanced or reduced by the action of various enzymes and chemicals. In this study reversed-phase high performance liquid chromatography (RP-HPLC) was used to analyse EP and to monitor the effects of the various treatments on EP. RP-HPLC is commonly used to resolve and prepare products of proteolysis for further analysis. The columns used for this system contain a hydrophobic stationary phase such as octyl silica (C8) or octadecyl silica (C18). Proteins and peptides are applied under acidic conditions, usually in the presence of an ion-pairing agent such as trifluoroacetic acid (TFA) which also acts to maintain protein solubility throughout the HPLC run. The majority of peptides adsorb to the stationary phase under these conditions and are subsequently eluted by decreasing the polarity of the mobile phase. This is achieved by the slow introduction of a water-miscible organic solvent. The most commonly used solvent system for RP-HPLC is a gradient of acetonitrile in a weak aqueous solution of TFA. Eluted proteins and peptides are detected by U.V.

absorbance. At a wavelength of 280nm, only proteins and peptides that contain aromatic residues will be detected. However, at wavelengths of between 200-230nm, the peptide bond absorbs light and this range is usually very sensitive.

The solvent gradient used was 0-100% acetonitrile and TFA was at a constant 0.1% throughout all HPLC runs. The percentage values stated for HPLC runs (% acetonitrile) refer to the total solvent mixture derived from pump B i.e. the percentage of acetonitrile in the column at a particular time.

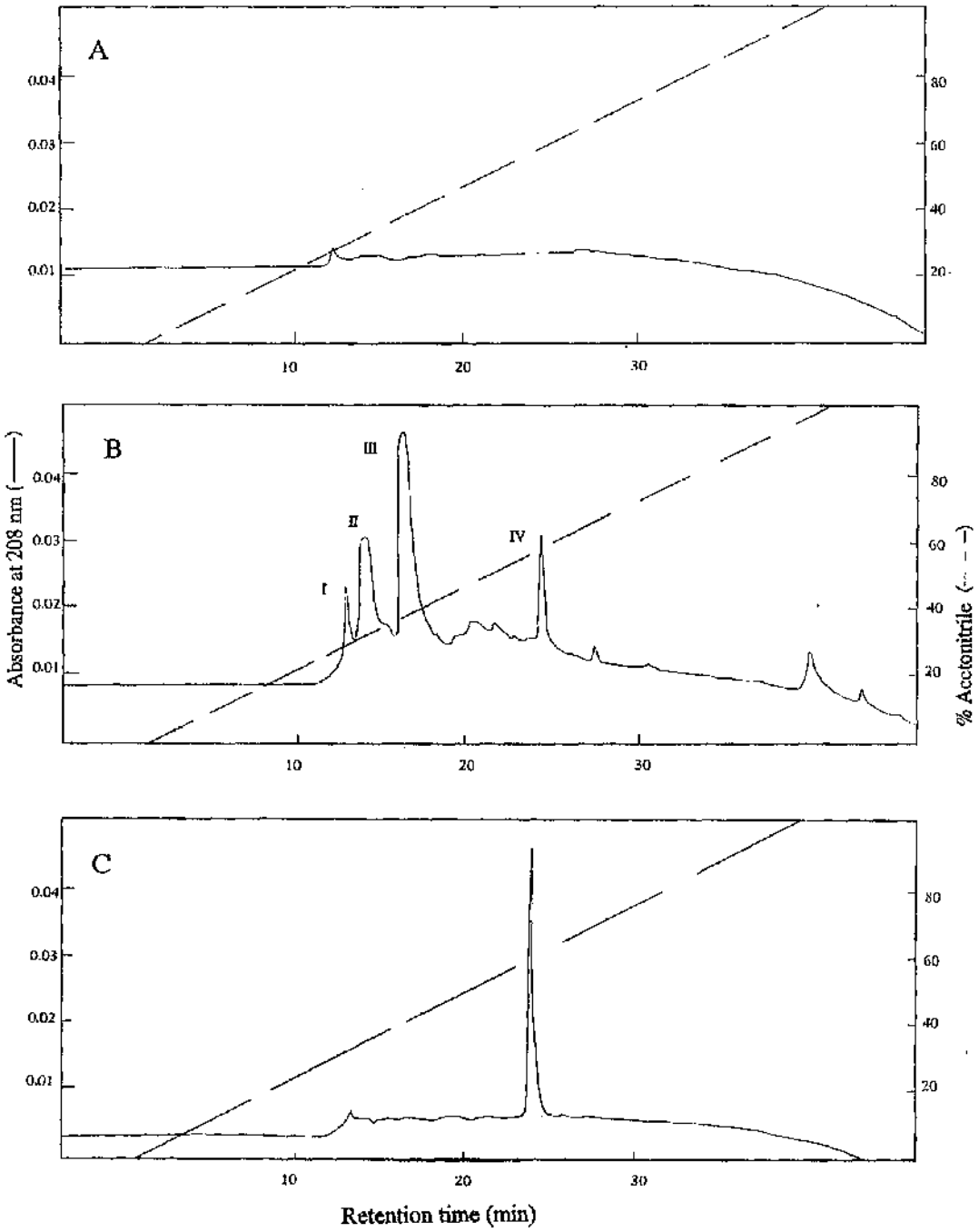
2.1 Blank HPLC run

A typical blank HPLC run is shown in Fig. 2.1 A. After a stable baseline reading was obtained the acetonitrile gradient was started. From 0-30% acetonitrile the baseline remained steady. At 30% acetonitrile a small peak was observed. There followed a slight rise in absorbance until 65% acetonitrile was reached. The absorbance then fell gradually with increasing acetonitrile and absorbance was still falling after 100% acetonitrile was reached before returning to the original baseline level.

2.2 HPLC profile of EP from strain (GDH 2346).

A typical HPLC run of EP from strain GDH 2346 is shown in Fig. 2.1 B. As in the blank run, the baseline was stable until 30% acetonitrile when there was a peak (peak I). This peak was larger than the peak observed at the same point on the blank run. A second larger peak (peak II) was observed between 33-35% acetonitrile. The largest peak (peak III) was eluted at between 39-42% acetonitrile. A number of smaller and less distinct peaks were observed until at 59% acetonitrile a fine sharp peak (peak IV) was seen. Further peaks appeared at 68%, 75%, 98% and 100% acetonitrile. The remainder of the run gave a similar profile to the blank run. These results indicated that there are a large number of distinct proteins present in EP from strain GDH 2346, with varying degrees of hydrophobicity. Of particular interest was the fine sharp peak seen at 59% acetonitrile. This component was relatively hydrophobic and appeared to make up a considerable proportion of the total composition of EP.

Fig. 2.1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Absorbance at 208 nm (——)
% Acetonitrile (- - -)
Retention time (min)

Fig. 2.1 A Blank HPLC run.

High Performance Liquid Chromatography (HPLC) was performed using an LKB Bromma HPLC machine with a TosoHaas C18 TSK-Gel reversed phase column. The HPLC pumps were flushed with HPLC grade water then HPLC grade acetonitrile (both degassed) at 5 ml min^{-1}. The column was attached and flushed with HPLC grade water then HPLC grade acetonitrile at 0.5ml min^{-1}. Absorbance was read at 208nm and was recorded on a chart feeder. The absorbance was monitored while the column was primed with 0.1% TFA-HPLC grade water, and once a stable baseline was achieved, a blank run was started. This consisted of loading a 200µl sample of HPLC grade water-TFA, to check for injection effects, and also to monitor the effect of the acetonitrile gradient on absorbance. After a stable baseline reading was obtained the acetonitrile gradient was started. The gradient used was 0-100% acetonitrile and TFA was at a constant 0.1% throughout all HPLC runs. The percentage values stated for HPLC runs (% acetonotrile) refer to the total solvent mixture derived from pump B, i.e. the percentage of acetonitrile in the column at a particular time.

Fig. 2.1 B EP (GDH 2346) HPLC profile.

After a stable baseline was achieved EP from strain GDH 2346 (20mg) was dissolved in 0.1% TFA (400µl) in HPLC grade water. The entire solution was then filtered through a 0.2 µm filter and 200 µl was loaded on to the column.

Fig. 2.1 C Insulin HPLC profile

Insulin was used as a regular check on protein detection sensitivity and column efficiency. Insulin was dissolved in 0.1% TFA at a final concentration of 0.1 mg ml^{-1}. After a stable baseline was achieved, 200 µl of insulin solution was loaded on to the column.

2.3 HPLC profile of Insulin

The manufacturers of the column recommended the use of insulin as a regular check on protein detection sensitivity and column efficiency. Insulin normally eluted in the region of 60% acetonitrile (Fig. 2.1 C) with the rest of the trace resembling the blank HPLC run.

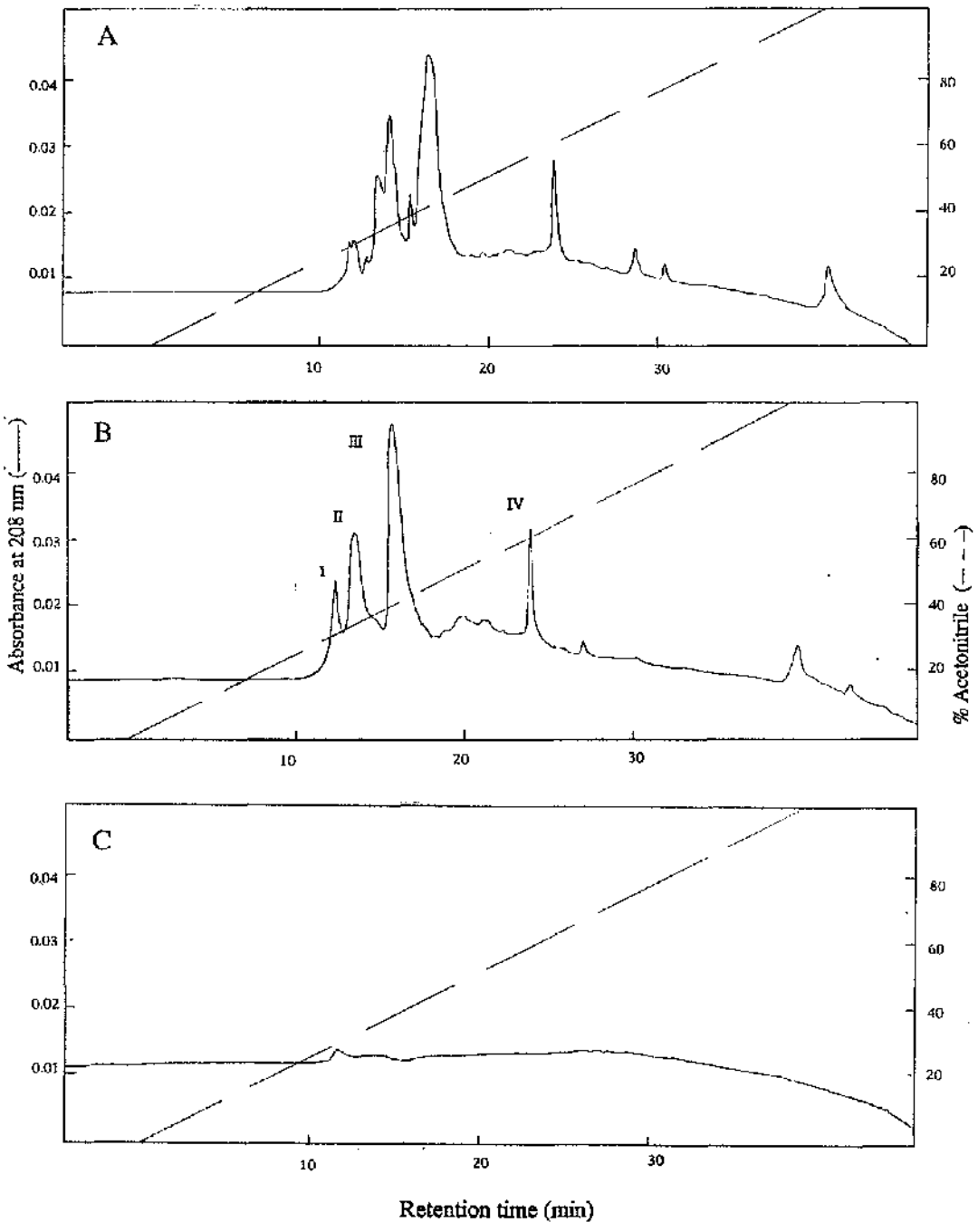
2.4 HPLC profile of EP treated with N-glycanase

The enzyme N-glycanase cleaves the N-glycosidic linkage between N-acetyl-D-glucosamine and asparagine on the protein backbone of glycoproteins (Tarentino *et al.*, 1985). This reaction does not affect the protein part of the molecule. Previous work by Tosh and Douglas (1992) had shown that N-glycanase treatment of EP enhanced the adhesion inhibition activity of EP. The HPLC trace of N-glycanase treated EP (Fig. 2.2 A) was not greatly different from the trace of EP incubated under the same conditions but in the absence of N-glycanase (Fig. 2.2 B). Peak I (30% acetonitrile, Fig.2.2B) appeared split in two and there was a new peak at 32% acetonitrile. Peak II (33-35% acetonitrile) was also seen to be split with a small peak at 34% acetonitrile and a larger peak at 36% acetonitrile. A new small fine peak was observed at 38% acetonitrile. Peak III (39-42% acetonitrile) and Peak IV (59% acetonitrile) appeared unchanged. A control HPLC run with N-glycanase alone produced a trace that was not visibly different from that of the blank run (Fig. 2.2 C).

2.5 HPLC profile of NaOH treated EP

Mild alkali was previously found to enhance the ability of EP to inhibit adhesion of *C. albicans* to BEC (Critchley and Douglas, 1987a). The optimum conditions for this effect were determined by Tosh (1991). He found that 0.1M NaOH produced the maximal inhibitory effect. Base-labile oligosaccharides linked to the protein through serine or threonine residues are removed by the action of NaOH (Ballou, 1976). NaOH treatment had a profound effect on the HPLC trace of EP when

Fig. 2.2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Absorbance at 208 nm (——)
% Acetonitrile (— – —)
Retention time (min)

Fig. 2.2 HPLC analysis of EP treated with N-glycanase

A. EP (10mg) was dissolved in 400µl of 0.55M Na_2HPO_4 (pH8.6). N-glycanase was added (2.4 units, 9.6µl) and the mixture was incubated for 24h at 37°C.

B. EP (10mg) was dissolved in 400µl of 0.55M Na_2HPO_4 (pH8.6) and incubated for 24h at 37°C.

C. N-glycanase (2.4 units, 9.6µl) was added to 400µl of Na_2HPO_4 (pH8.6, 0.55M) and the mixture was incubated for 24h at 37°C.

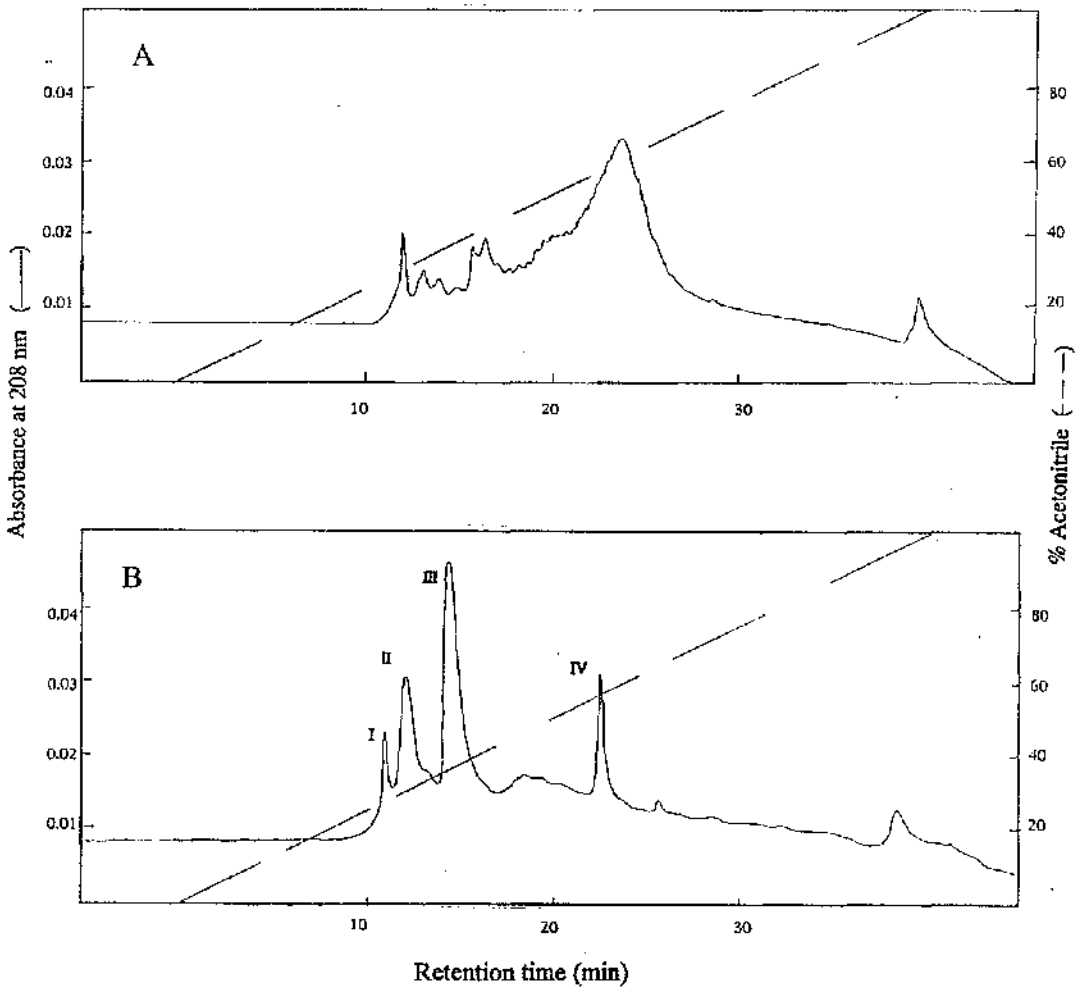
Each reaction mixture was filtered through a 0.2µm filter and loaded on to the column prepared as described in the legend to Fig. 2.1A.

compared with a trace of EP incubated under the same conditions but with PBS in place of NaOH (Fig 2.3, A and B). Peak I (30% acetonitrile) appeared unchanged but Peaks II and III (33-35% and 39-42% acetonitrile) were largely gone, replaced with five small peaks at 33, 35, 37, 39, and 41% acetonitrile. After this point there was a large broad peak ranging from 47-70% acetonitrile with its summit at 60% acetonitrile. One further peak was seen at 100% acetonitrile. These results indicate that a number of changes have occurred to the mannoproteins present in EP after treatment with NaOH. In general, there seems to be a shift from a relative abundance of hydrophilic proteins to more hydrophobic proteins. This change in the proportion of hydrophilic components could be consistent with the removal of polysaccharide chains from the protein backbones of the glycoproteins. This could result in the exposure of hydrophobic regions of the protein that were previously unavailable for interaction with the hydrophobic column packing. There could also be some protein degradation due to the action of NaOH. The effect of NaOH treatment on the component normally eluted at 59% acetonitrile could not be determined as the broad peak between 47-70% acetonitrile obscures this part of the trace.

2.6 HPLC profile of EP treated with papain

Previous work by Critchley and Douglas (1987a) showed that a number of proteases significantly decreased the ability of EP to inhibit adhesion. However, the protease papain enhanced this ability. This increased inhibition was attributed to the production of peptide fragments which bound more easily to epithelial cell receptors. The HPLC trace of papain-treated EP (Fig. 2.4 A) was considerably different from the trace of EP incubated under the same conditions but in the absence of papain (Fig. 2.4 B). The EP mannoproteins appeared greatly fragmented with a large number (19) of sharp peaks ranging from 30%-67% acetonitrile which supports the theory of peptide production. Most of the fragments produced were of relatively high hydrophobicity compared to the trace of EP not exposed to papain. Of particular note was the fine sharp peak seen at 67% acetonitrile. A control HPLC run with papain and α_2-

Fig. 2.3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Absorbance at 208 nm (——)
% Acetonitrile (– – –)
Retention time (min)

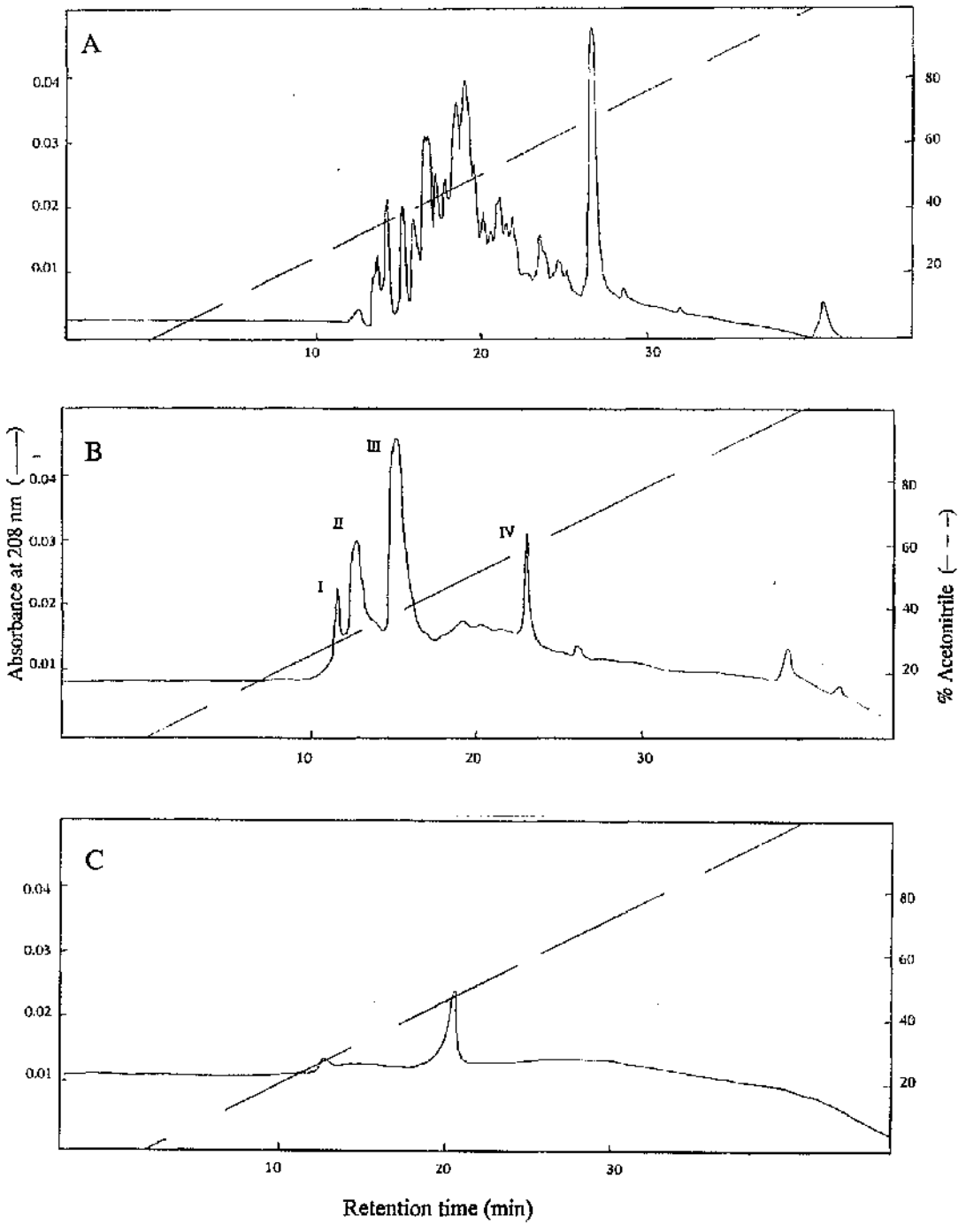
Fig. 2.3 HPLC analysis of NaOH-treated EP

A. EP (10mg) was dissolved in 400µl of NaOH (0.1M) and the mixture was incubated for 24h at 30°C.

B. EP (10mg) was dissolved in 400µl of PBS and the mixture was incubated for 24h at 30°C.

Each reaction mixture was filtered through a 0.2µm filter and loaded on to the column prepared as described in the legend to Fig. 2.1A.

Fig. 2.4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Absorbance at 208 nm (——)
% Acetonitrile (– – –)
Retention time (min)

Fig. 2.4 HPLC analysis of papain-treated EP

A. EP (10mg) was dissolved in 400µl potassium phosphate buffer (0.01M, pH6.2) containing papain (40µg). The mixture was incubated for 30 min at 25°C. After incubation, α_2-macroglobulin (2 mg ml^{-1}; 0.2ml) was added to terminate the reaction.

B. EP (10mg) was dissolved in 400µl potassium phosphate buffer (0.01M, pH6.2) and incubated for 30 min at 25°C.

C. Papain (40µg) was incubated for 30 min at 25°C in 400µl potassium phosphate buffer (0.01M, pH6.2). After incubation, α_2-macroglobulin (2 mg ml^{-1}, 0.2ml) was added.

Each reaction mixture was filtered through a 0.2µm filter and loaded on to the column prepared as described in the legend to Fig. 2.1A.

macroglobulin produced a trace that had a small peak in the region of 46-48% acetonitrile with the remainder of the run not visibly different from that of a blank run (Fig. 2.4 C). The peak is likely to consist of a papain-α_2-macroglobulin complex. The corresponding region on the EP, papain and α_2-macroglobulin trace shows no sign of this complex due to the presence of large amounts of EP digestion products.

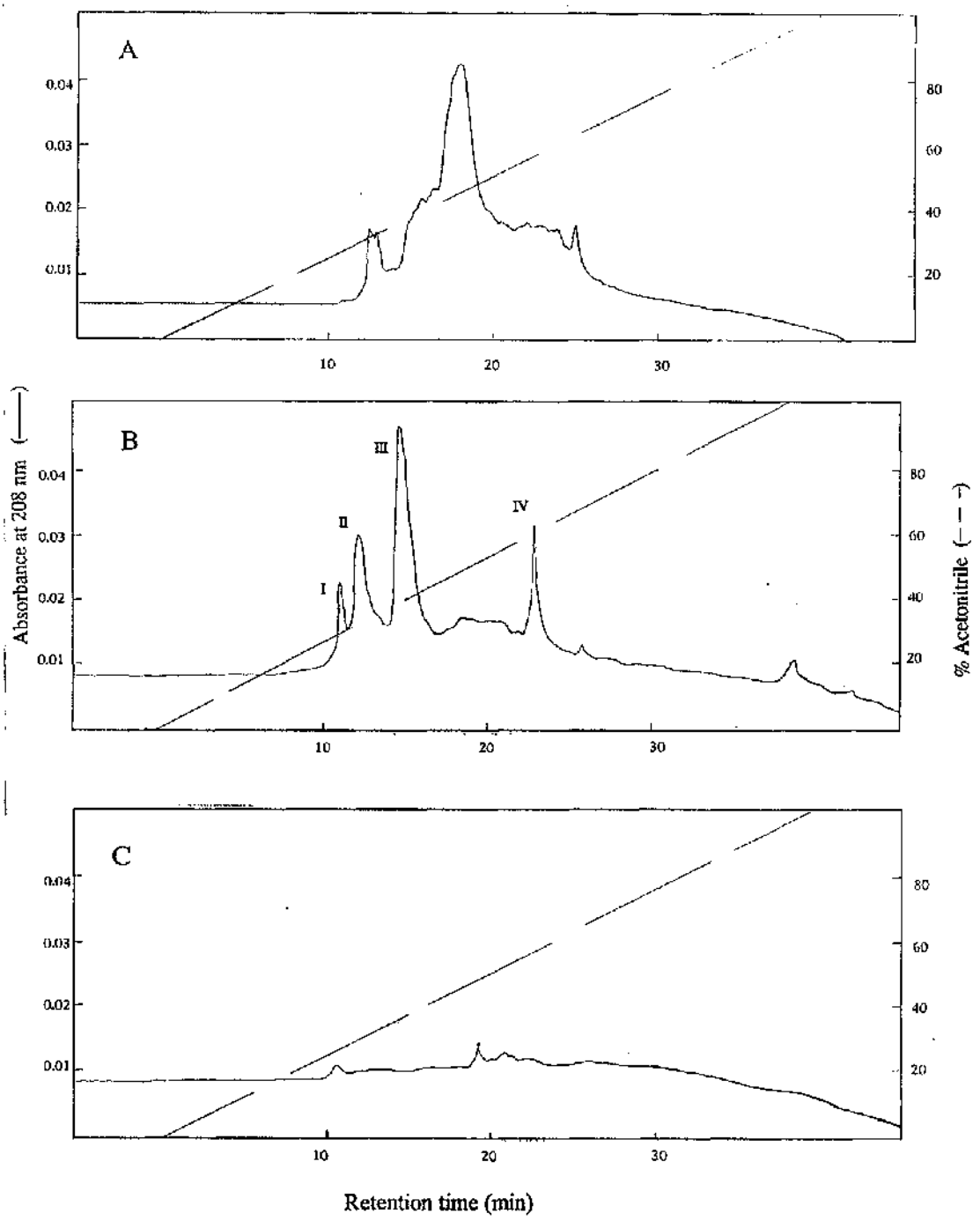
2.7 HPLC profile of EP treated with N-glycanase, papain, and mild alkali

A number of differences were seen between this HPLC trace (Fig. 2.5 A) and that of EP incubated under the same conditions but in the absence of N-glycanase, papain, and NaOH (Fig. 2.5 B). Peaks I and II (30% and 33-35% acetonitrile) were replaced by a double peak at 32-34% acetonitrile followed by a trough between 35-37% acetonitrile. There then followed a sharp rise in O.D. until 43% acetonitrile. A large broad peak was seen at 46%. After 50%, there were a number of small less distinct peaks, until at 64%, a small fine peak was observed. Tosh and Douglas (1992) showed that the combined treatment of EP with N-glycanase, papain and mild alkali produced an EP digest that was able to inhibit adherence by a far greater extent than untreated EP. A control HPLC run with N-glycanase, papain, α_2-macroglobulin and NaOH produced a trace that was slightly different from that of a blank run (Fig. 2.5 C). A small peak was seen at around 48% acetonitrile and some smaller, less defined peaks were eluted soon after. These peaks indicate that NaOH had an effect on the possible papain and α_2-macroglobulin complex that had been seen in a previous experiment (Fig. 2.4 C). The corresponding region on the EP, N-glycanase, papain, α_2-macroglobulin, NaOH trace shows no sign of this complex due to the presence of large amounts of EP digestion products.

3. THE *CANDIDA ALBICANS* ADHESIN

Partial purification of a *C. albicans* adhesin from EP was described by Critchley and Douglas (1987a). EP, composed mainly of mannoprotein, is thought to originate

Fig. 2.5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Absorbance at 208 nm (——)
% Acetonitrile (— — —)
Retention time (min)

Fig. 2.5 EP treated with N-glycanase, papain, and mild alkali

A. EP (100mg) was dissolved in 1ml of 0.55M Na_2HPO_4 (pH 8.6). N-glycanase (250 Units ml^{-1}; 0.096ml) was added and the mixture was incubated for 24h at 37°C. Sodium citrate buffer (0.1M, 0.04ml, pH 4.4) was added to terminate the reaction. Potassium phosphate buffer (0.01M, pH6.2) containing papain (0.232 mg ml^{-1}) was added (0.864 ml) and the solution was incubated for 30 min at 25°C. α_2-macroglobulin (2 mg ml^{-1}, 0.5ml) was added to terminate the reaction. NaOH (1ml) was added and the mixture was incubated for 24h at 30°C. The reaction mixture was neutralised by the addition of 1 ml of HCl (0.366M).

B. EP (100mg) was dissolved in 1ml of 0.55M Na_2HPO_4 (pH 8.6). PBS (0.096ml) was added and the mixture was incubated for 24h at 37°C. Sodium citrate buffer (0.1M, 0.04ml, pH 4.4) was then added followed by 0.864 ml potassium phosphate buffer (0.01M, pH6.2). The solution was incubated for 30 min at 25°C and then PBS (0.5ml) was then added. An additional 1ml of PBS was added and the mixture was incubated for 24h at 30°C. A final 1 ml of PBS was then added.

C. N-glycanase (250 Units ml^{-1}; 0.096ml) was added to 1ml of 0.55M Na_2HPO_4 (pH 8.6). and the mixture was incubated for 24h at 37°C. Sodium citrate buffer (0.1M, 0.04ml, pH 4.4) was then added. Potassium phosphate buffer (0.01M, pH6.2) containing papain (0.232 mg ml^{-1}) was added (0.864 ml) and the solution was incubated for 30 min at 25°C. The activity of papain was terminated by the addition of α_2-macroglobulin (2 mg ml^{-1}, 0.5ml). NaOH (0.366M, 1ml) was added and the mixture was incubated for 24h at 30°C. 1 ml of HCl (0.366M) was added to neutralise the NaOH.

Each reaction mixture was filtered through a 0.2µm filter and loaded on to the column prepared as described in the legend to Fig. 2.1A.

from the cell surface when yeasts are grown in medium containing a high concentration of galactose. The protein portion of the mannoprotein was more important than the carbohydrate moiety in mediating yeast attachment to buccal epithelial cells (BEC). When lectins and sugars were included in adhesion-inhibition assays, most strains tested were primarily inhibited by L-fucose and the L-fucose-specific lectin from *Lotus tetragonolobus* (Critchley and Douglas, 1987b). One other strain tested was inhibited by N-acetylglucosamine and wheatgerm agglutinin (N-acetylglucosamine specific). These results suggested that the protein portion of yeast cell-surface mannoproteins may bind in a lectin-like manner to glycosides containing L-fucose or N-acetylglucosamine expressed on epithelial cell-surface glycoconjugates. L-Fucose is found on epithelial cell surfaces in the form of blood group antigens on glycoproteins or glycolipids.

A complete purification scheme for the yeast adhesin was recently devised (Tosh and Douglas, 1992) which made use of the findings from the earlier studies on adhesion mechanisms. The purification protocol involves the stepwise treatment of EP with N-glycanase, papain, and dilute alkali followed by Synsorb H-2 affinity adsorption (Fig. 3.1). The affinity adsorbent expresses the trisaccharide determinant of the H (type 2) blood group antigen which terminates in an L-fucose residue. Similar affinity adsorbents have been used in the purification of lectins (Hosomi *et al.*, 1993) and anti-blood group antibodies (Rydberg *et al.*, 1992). Synsorbs have also been used to purify L-fucose-binding adhesins from *Aeromonas hydrophila* (Quinn *et al.*, 1993, 1994a) and *Staphylococcus aureus* (Saadi *et al.*, 1994). Tosh and Douglas (1992) incorporated H-2 affinity adsorbent as a final purification step to isolate those components from EP digests that have an affinity for H-2 blood group substance. The resultant protein fraction was able to inhibit the adhesion of yeasts from the homologous strain by up to 80%. The material was devoid of carbohydrate but could not be visualised on conventional SDS-PAGE (Tosh, 1991). It was finally visualised by reverse-phase FPLC as a single sharp peak (Tosh and Douglas, 1992).

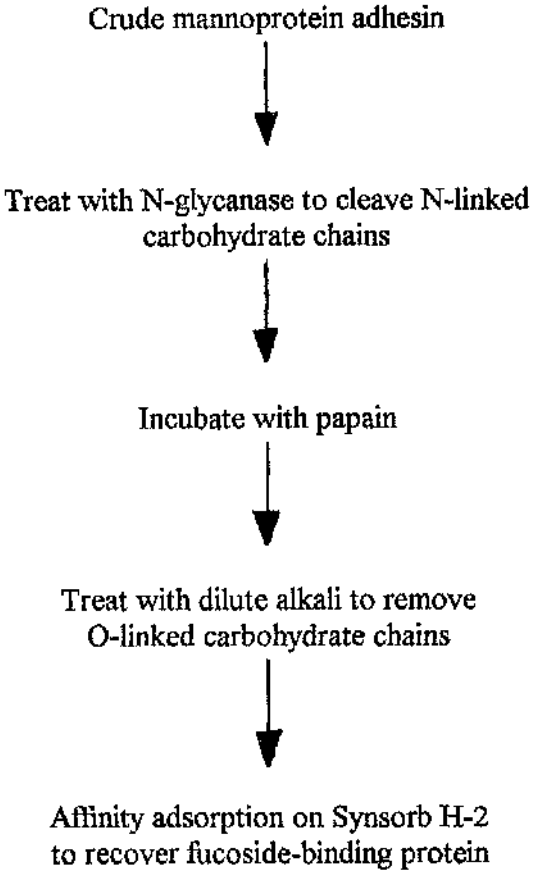


Fig. 3.1 Complete protocol for the purification of yeast adhesin

3.1 Purification of the yeast adhesin

EP was produced by a method very similar to that of Tosh (1991). The molecular weight exclusion limit of the ultrafiltration membrane employed in this study was 3.5K in place of the 5K filter used by Tosh. The combined treatment of EP with N-glycanase, papain, and NaOH followed by the Synsorb H-2 affinity chromatography step is the basis of the purification protocol for the yeast adhesin developed by Tosh and Douglas (1992) and was used throughout this study. EP digest was applied to PBS-washed Synsorb H-2 (0.5g). The digest and the Synsorb were gently rotated for 3h at 4°C. Unbound material was eluted with 5ml aliquots of PBS until the A_{280} returned to 0.0. Bound material was then eluted by the addition of 2 ml aliquots of 1.5% NH_4OH in sterile saline for 15 min at 4°C until the A_{280} returned to 0.0. A typical elution profile is shown in Fig. 3.2.1. Bound samples were freeze-dried and desalted on a Sephadex G-25 (5ml) desalting column. A typical desalting profile is shown in Fig. 3.2.2. The adsorbent was then regenerated with PBS and stored at 4°C in PBS containing 0.02% NaN_3 as a bacteriostat.

3.2 Adhesion-inhibition activity of the purified adhesin

To ensure that the purified adhesin prepared in this study was of the same activity as that obtained previously, BEC were treated with the new material prior to adhesion assays with *C. albicans* GDH 2346. This material inhibited adhesion by up to 74.8% (Table 3.1.1) and the adhesion inhibition index value (AII), which is a measure of the ability of EP or its derivatives to block adhesion, was calculated to be 178. These results are comparable with those obtained by Tosh and Douglas (1992).

3.3 Biochemical analysis of the purified adhesin

Protein and carbohydrate analyses were performed on the purified adhesin (Table 3.1.2). A value for the protein content was obtained using the procedure of Lowry *et al.* (1951). However, the adhesin was found to be devoid of carbohydrate

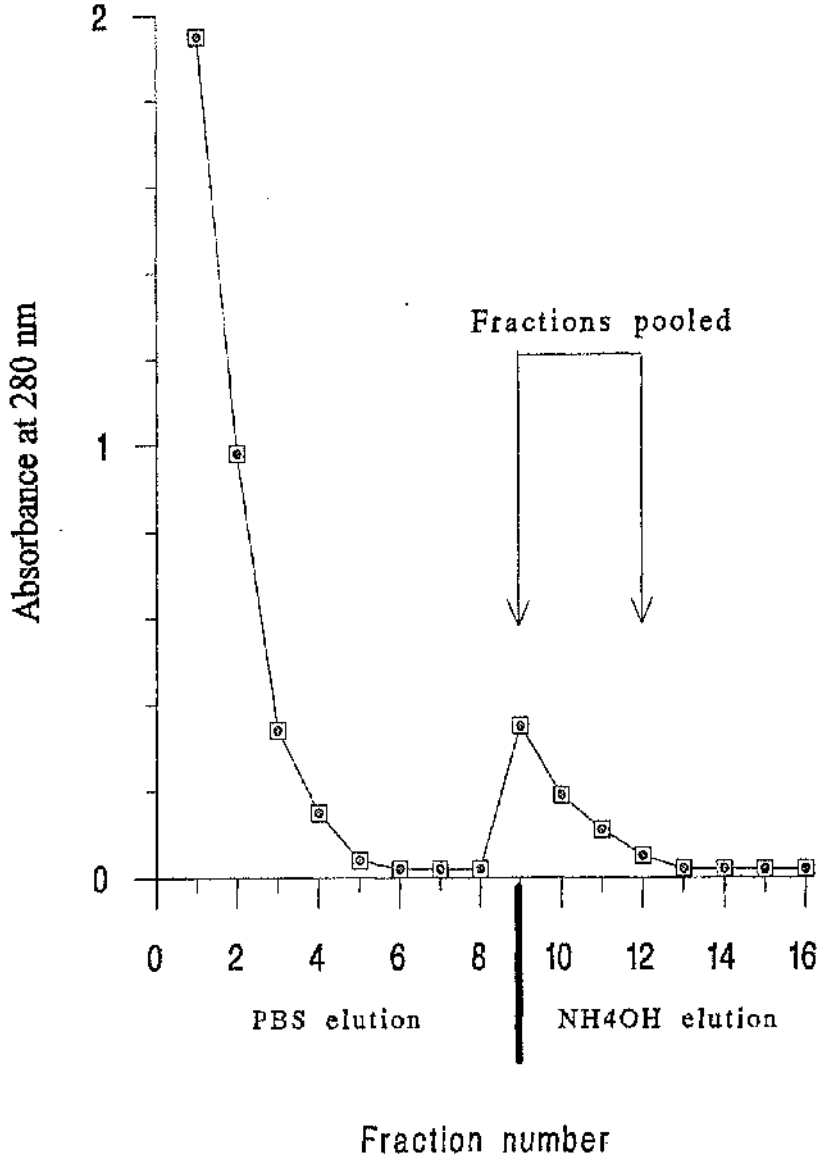


Fig. 3.2.1 EP components eluted from Synsorb H-2 affinity adsorbent

Synsorb H-2 (0.5g) was washed twice in PBS (5ml) and allowed to settle. The buffer was removed to just above the surface of the synsorb bed. EP digest was applied. The mixture was gently rotated for 3h at 4°C. Unbound material was eluted with 5ml aliquots of PBS until the A_{280} returned to 0.0. Bound material was then eluted by the addition of 2 ml aliquots of 1.5% NH_4OH in sterile saline for 15 min at 4°C until the A_{280} returned to 0.0.

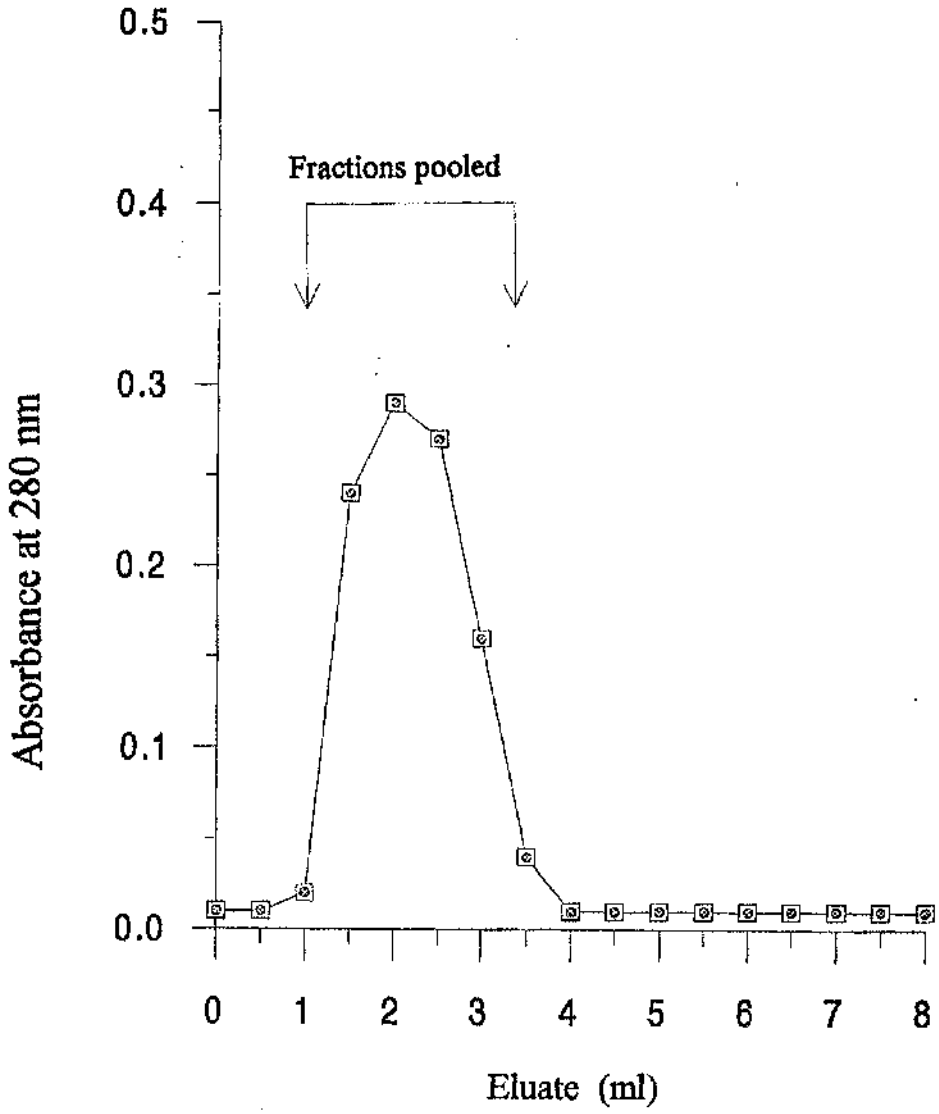


Fig. 3.2.2 Material eluted from Sephadex G-25 desalting column

Fractions containing Synsorb H-2 bound material were pooled, freeze-dried and then desalted on a Sephadex G-25 (5ml) desalting column. The fractions containing purified adhesin were pooled and freeze-dried.

Table 3.1.1 Effect of EP and purified adhesin on the adhesion of *C. albicans* GDH 2346 to buccal epithelial cells

[1]BEC treatment	[2]Mean adherence	% inhibition	[3]P	[4]AII
adhesin treated	254.11 ± 16.4	74.81	<0.001	178
EP treated	536.62 ± 22.98	46.74	<0.001	1
not treated (PBS)	1008.67 ± 18.23	-	-	-

[1] Buccal epithelial cells were incubated at 37°C for 30 min in a solution of purified adhesin (6.38 μg ml^{-1}), EP (10 mg ml^{-1}) or PBS.

[2] The results represent the mean number of adherent yeasts per 100 epithelial cells ± the standard error (SEM) for three independent assays performed in triplicate.

[3] Probability values relative to adhesion of PBS control.

[4] Adherence inhibition index (AII) calculated relative to untreated EP.

Table 3.1.2 **Biochemical composition of EP and purified adhesin from strain GDH 2346**

Component	Content of		AII[1]	Protein:carbohydrate ratio
	protein ($\mu g\ ml^{-1}$)	carbohydrate ($\mu g\ ml^{-1}$)		
EP	830	8090	1	1:9.75
purified adhesin	6.4	N.D.	178	1:0

[1] Adherence inhibition index (AII) calculated relative to untreated EP.

N.D. - Not detected

when the procedure of Dubois et al. (1956) was employed. This is consistent with the findings of Tosh and Douglas (1992).

3.4 HPLC analysis of the purified adhesin

Tosh and Douglas (1992) obtained a single peak for the adhesin on fast high pressure liquid chromatography (FPLC). This indicated that the adhesin was a single component. Attempts to repeat this result on HPLC proved unsuccessful. The traces obtained for purified adhesin were not remarkably different from the HPLC blank runs shown in section 2.1, even when as much as 50 μg of protein was loaded.

3.5 Amino acid analysis of the purified adhesin

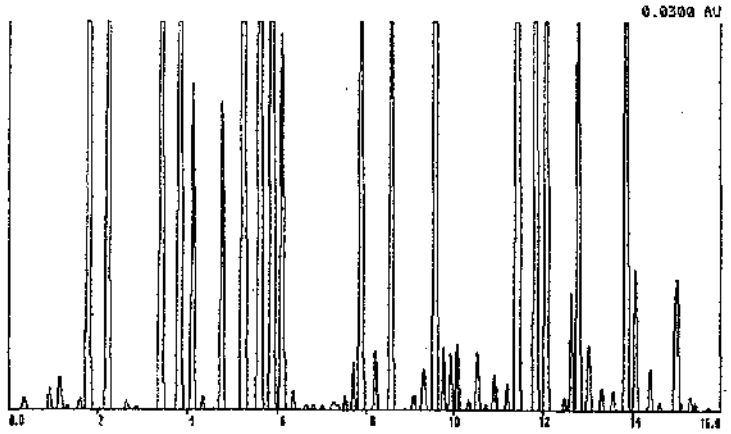
Amino acid analysis was performed on the purified adhesin by Dr. M. Cusack in the Dept. of Geology and Applied Geology, University of Glasgow. A sample of purified material was prepared by the normal procedure. A total of 18 μg of material was used for amino acid analysis. The results obtained are shown on Fig. 3.3. A blank sample that had undergone the entire purification protocol was used to adjust the background on the sample run. The purified adhesin was mainly composed of the amino acids Thr, Pro, Ala, Ser, Val, Gly, Asx and Glx, which made up around 80% of the total amino acids. There was no Cys detected and very little Met, Phe and Arg. This composition is remarkably similar to a large number of microbial adhesins (Table 3.2).

3.6 Analysis of purified adhesin by SDS-PAGE

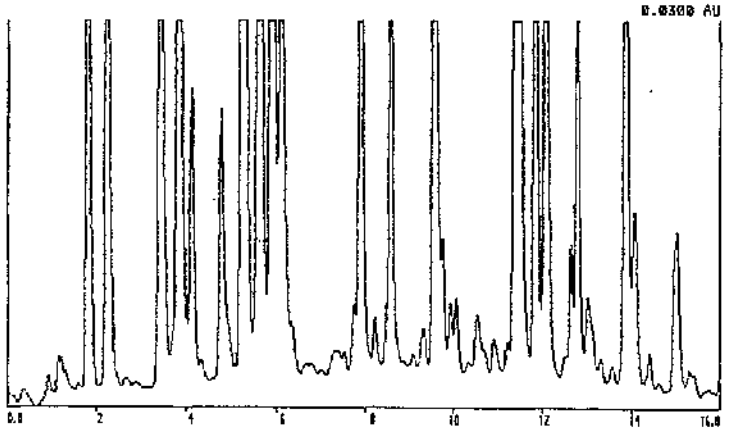
Two SDS-PAGE systems were employed in this study: the procedures of Laemmli (1970) and of Schagger and Von Jagow (1987). Initial attempts to visualise the purified adhesin employed the Laemmli procedure, using both Coomassie blue staining (Weber and Osborne, 1969) and silver staining (Oakley *et al.*, 1980) to visualise separated proteins after electrophoresis. As was found previously by Tosh (1991), purified adhesin could not be visualised in this way. The second SDS-PAGE system, devised by Schagger and Von Jagow (1987), was then employed in an

A. Adhesin sample

Enhanced data



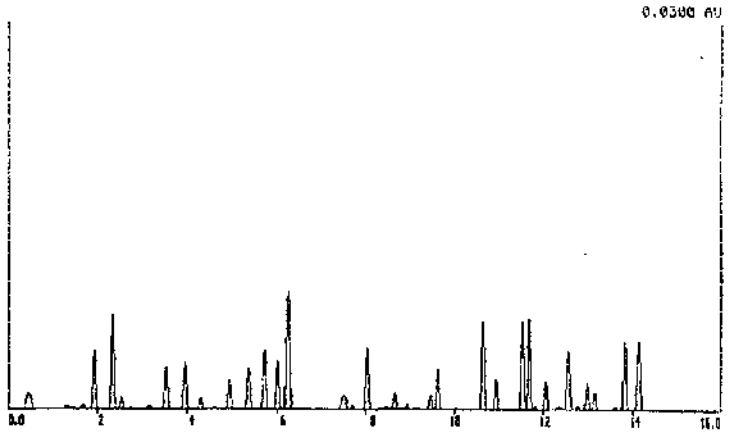
Raw data



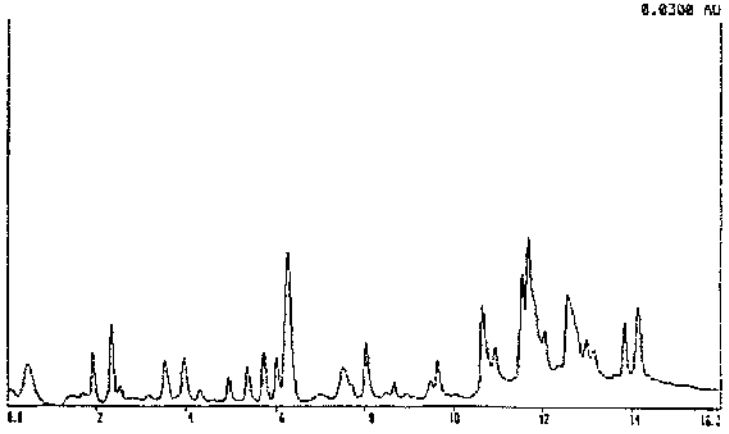
Retention time (min)

B. Background sample

Enhanced data



Raw data



Retention time (min)

Fig. 3.3 Amino acid analysis of the purified adhesin

Amino acid	Retention time (min)	pmol in original sample.	pmol in background sample	Adjusted pmol in sample
Asx	1.74	847.60	---	847.60
Glx	2.21	801.86	62.82	644.81
Ser	3.40	799.10	---	799.10
Gly	3.38	861.65	33.40	778.15
His	4.11	229.66	---	229.66
Arg	4.76	185.32	---	185.52
Thr	5.26	1761.02	27.75	1691.645
Ala	5.62	1188.81	32.07	1108.635
Pro	5.89	1319.72	---	1319.72
Tyr	8.57	280.04	9.32	256.74
Val	9.56	802.71	---	785.01
Met	9.94	47.67	---	47.67
Cys	10.90	21.50	54.71	-115.275
Ile	11.80	412.61	50.99	285.135
Leu	12.00	472.93	---	472.93
Phe	12.76	223.22	27.93	153.395
Lys	13.84	349.86	---	349.86

Purified adhesin was prepared by treatment of EP from strain GDH 2346 with N-glycanase, papain, and mild alkali followed by H-2 affinity chromatography as described in Materials and Methods. After determining the protein content of the material, 18 µg was used for amino acid analysis. The material was dissolved in 50 µl of 0.1% TFA and the entire sample was loaded (A). A blank sample that had undergone the entire purification protocol was used to adjust the background on the sample run (B). An equivalent amount of blank sample was dissolved in 50 µl of 0.1 % TFA and 20 µl was loaded. The pmol values for the original sample were adjusted by subtracting 2.5 times the pmol values for the blank sample. Prior to sample runs, a blank sample is analysed to assess the level of background. Raw data refers to sample runs that have not been adjusted for background and Enhanced data refers to background adjusted data.

Table 3.2 Comparison of the amino acid composition of the *C. albicans* adhesin with other microbial adhesins

Amino acid	Percentage composition in: *C. albicans* adhesin (this study)	*C. albicans* adhesin (Yu *et al.*, 1994)	*E. coli* Dr adhesin (Kist *et al.*, 1990)	*Ps. cepacia* PC7 adhesin Sajjan and Forstner (1992)
Asx	8.5	10.4	16.9	8.4
Glx	6.5	10.3	7.4	12.1
Ser	8.0	7.3	3.4	9.3
Gly	7.8	8.9	12.1	15.7
His	2.3	1.9	N.D.	1.8
Arg	1.9	4.8	4.1	4.3
Thr	17.0	6.0	12.8	6.3
Ala	11.1	7.6	6.8	10.6
Pro	13.3	3.5	4.1	4.5
Tyr	2.6	3.2	3.4	3.3
Val	7.9	7.0	8.1	5.6
Met	0.5	1.0	N.D.	3.0
Cys	0.0	N.D.	0.7	N.D.
Ile	2.9	6.0	3.4	2.7
Leu	4.8	8.6	7.4	6.8
Phe	1.5	4.1	4.1	2.5
Lys	3.5	7.5	5.4	3.1

N.D. -No data

attempt to visualise purified adhesin. This system is able to resolve proteins and peptides in the range 2.5 kDa-17.0 kDa due to the incorporation of tricine in place of glycine in the running buffer. Coomassie blue staining and silver staining were again both employed but the purified adhesin could not be visualised.

3.6.1 Chemical and enzymic degradation of ^{125}I-EP monitored by SDS-PAGE

In this series of experiments, EP was labelled with ^{125}I and then subjected to the purification protocol for the yeast adhesin. Samples were removed after each step of the procedure to monitor the effects of the various chemicals and enzymes on EP. Five samples were taken in total and were run together on SDS-PAGE using the procedure of Schagger and Von Jagow (1987). This gel system is able to resolve proteins and peptides in the range 2.5 kDa-17.0 kDa. The samples comprised:

1. Untreated EP
2. N-glycanase-treated EP
3. N-glycanase-, and papain- treated EP
4. N-glycanase-, papain-, and NaOH- treated EP
5. N-glycanase-, papain-, and NaOH- treated EP (bound to Synsorb H-2)

The lanes with molecular weight standards were cut off and stained with Coomassie blue. The remainder of the gel was sealed in a plastic bag and exposed to X-ray film. The autoradiogram is shown in Fig. 3.4. Small proteins and peptides within the range 2.5 kDa-17.0 kDa should appear as distinct bands but proteins of higher molecular weight are likely to be bunched at the top of the separating gel. Untreated EP (Lane 1) appeared as three distinct bands. Two strong bands were seen at the very top of the gel, one a short way into the separating gel, the other at the junction of the separating gel and the stacking gel. The third band, which was less intense than the

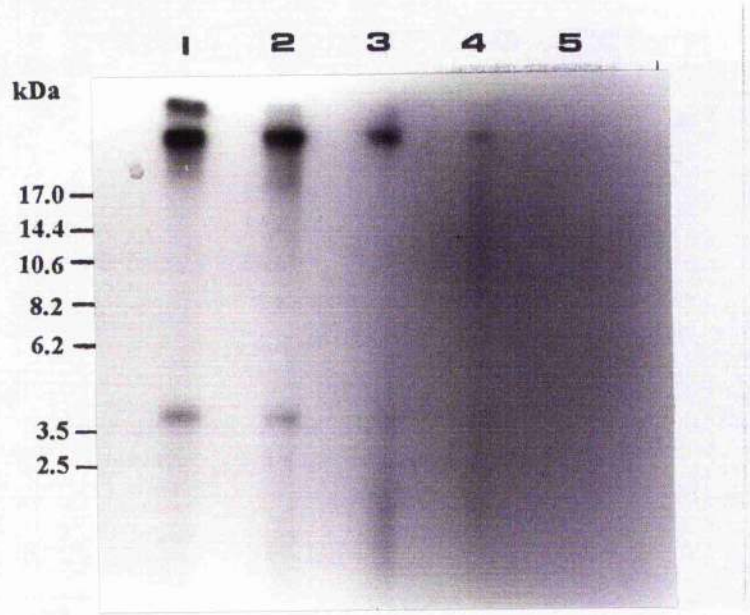


Fig. 3.4 SDS-PAGE of ^{125}I-EP from GDH 2346 before and after chemical and enzymic degradation

EP (produced as described in Materials and Methods, 100mg) was iodinated and then subjected to the purification protocol for the yeast adhesin as described in Materials and Methods. Samples were removed after each step of the protocol to monitor the effects of the various chemicals and enzymes on EP. Five samples were taken in total and were run together on SDS-PAGE using the procedure of Schagger and Von Jagow (1987) as described in Sigma Technical Bulletin No. MWM-100. The first sample was 100µl of iodinated EP, concentrated by freeze-drying, and resuspended in sample buffer. The volume of each subsequent sample was increased in an attempt to equalise the weight of labelled material. The entire Synsorb bound fraction was concentrated by freeze-drying and loaded on the gel. Bands were visualised by autoradiography

Lane designations

1. Untreated EP
2. N-glycanase-treated EP
3. N-glycanase- and papain-treated EP
4. N-glycanase-, papain-, and NaOH-treated EP
5. N-glycanase-, papain-, and NaOH-treated EP (bound to Synsorb H-2)

other two, was seen in the region of the 3.5 kDa marker. The N-glycanase-treated EP (Lane 2) appeared as only two distinct bands. The band that had been seen in Lane 1, at the junction of the separating gel and the stacking gel, was absent but the remainder of Lane 2 was similar to Lane 1. The lane containing N-glycanase- and papain-treated EP (Lane 3) showed only one clear band a short way into the separating gel. There was also a diffuse streak of ^{125}I-labelled material visible in the region of the 2.5 kDa marker. The lane containing N-glycanase-, papain-, and NaOH-treated EP (Lane 4) had only one band a short way into the separating gel. There was also a diffuse streak of ^{125}I-labelled material the entire length of the gel. No ^{125}I-labelled material was visible in the lane containing N-glycanase-, papain-, and NaOH-treated EP (bound to Synsorb H-2) (Lane5). This could have been a result of insufficient ^{125}I-labelled material at this point in the degradation. Another possibility is that the iodination may have damaged the adhesin molecules and prevented their binding to the Synsorb matrix.

3.6.2 Analysis of biotinylated EP by SDS-PAGE and western blotting

An alternative labelling approach was employed in this series of experiments. EP was biotinylated using a commercial kit from Amersham. Biotinylated EP and biotinylated molecular weight standards (SDS-6B, Sigma) were separated using the Laemmli procedure and a mini gel apparatus. After electrophoresis, the separated proteins were transferred to nitrocellulose and biotinylated material was detected by probing with ^{125}I-streptavidin followed by autoradiography (Fig. 3.5). Four bands were visible in the biotin-EP lanes. An intense band was visible in the region of the 97.4 kDa marker and a broad diffuse band was seen in the region of the 39.8 kDa marker. Two further bands were seen in the region between the 20.1 and 14.3 kDa markers but were relatively faint. Any other bands present were obscured by the streak of biotinylated material that ran the length of the gel.

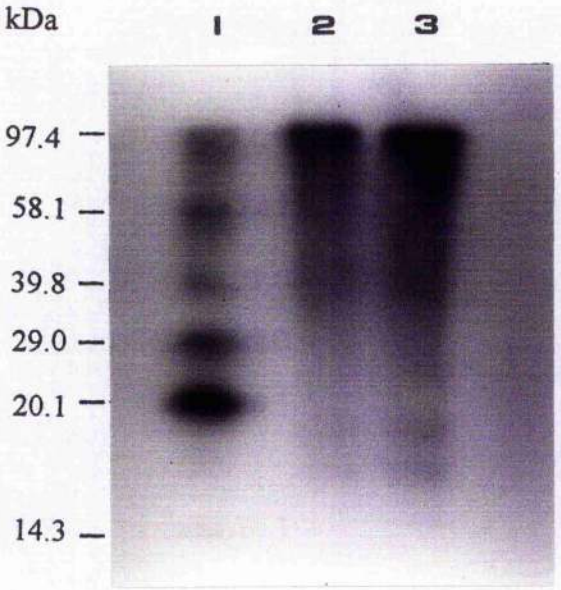


Fig. 3.5 SDS-PAGE and western blotting of biotinylated EP.

Biotinylation of EP (produced as described in Materials and Methods) was performed using a commercial kit from Amersham (following the manufacturers protocol). 10mg of EP was dissolved in 1ml of 0.05M sodium borate buffer, pH 8.6. 40µl of biotinylation reagent was added and the tube was incubated for 1h at room temperature with constant agitation. The unreacted biotinylation reagent was removed by desalting on G-25 Sephadex and the EP was freeze-dried. The biotin-EP was resuspended in 100µl of sample buffer and 25µl was loaded in each well. Biotinylated EP and biotinylated SDS molecular weight standards (SDS-6B, Sigma) were separated using the procedure of Laemmli (1970) and a mini gel apparatus. The stacking gel and the separating gel were 4.5% and 12.5% respectively. After electrophoresis, the separated proteins were transferred on to nitrocellulose (Hybond C, Amersham) according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). Biotinylated material was detected by probing with ^{125}I-streptavidin (30 µCi/µg: 5 µCi in 10ml reaction volume) followed by autoradiography. Lane 1, biotinylated SDS-6B , lanes 2 and 3, biotin-EP. Bands were visualised by autoradiography

3.6.3 Chemical and enzymic degradation of biotinylated EP monitored by SDS-PAGE

In this series of experiments, EP was biotinylated and then subjected to the purification protocol for the yeast adhesin. Samples were removed after each step of the procedure to monitor the effects of the various chemicals and enzymes on EP and using a mini gel to monitor the degradation (Fig. 3.6). The number of samples taken during the degradation was reduced to three and they comprised:

1. N-glycanase- and papain-treated EP
2. N-glycanase-, papain-, and NaOH-treated EP
3. N-glycanase-, papain-, and NaOH-treated EP (bound to Synsorb H-2)
4. Biotinylated SDS-6B markers

The results obtained in this experiment were poor possibly due to insufficient biotinylation of the EP. This seems unlikely as the degree of biotinylation seemed adequate in the previous experiment (3.6.2). A more likely explanation is that the degradation treatments had an adverse effect on the protein-bound biotin. The biotin may have been cleaved from the proteins or the ability of the bound biotin to bind to ^{125}I-streptavidin may have been affected.

3.6.4 Analysis of biotinylated purified adhesin by SDS-PAGE and western blotting

The previous experiment suggested the possibility that purified adhesin could be visualised by biotinylation prior to SDS-PAGE, western blotting and probing with ^{125}I-streptavidin. Purified adhesin was biotinylated prior to loading on a tricine mini SDS-PAGE gel. Samples (biotinylated PA) and molecular weight markers (MW-SDS-17S, Sigma) were subjected to electrophoresis for 3-4 hours at a constant current of 100 V. The gel was cut and the markers were stained with Brilliant Blue G (Sigma). The biotinylated PA lanes were transferred onto nitrocellulose (Hybond C,

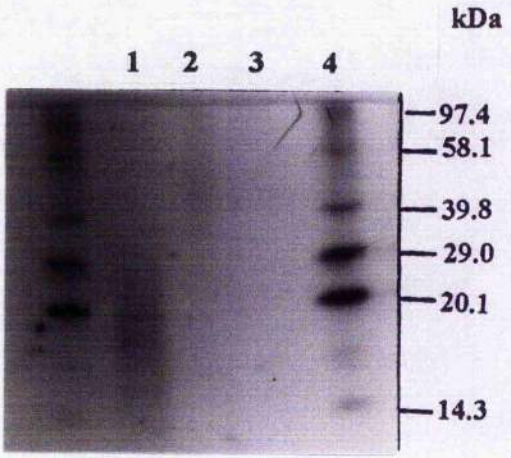


Fig. 3.6 SDS-PAGE and western blotting of biotinylated EP after chemical and enzymic degradation.

EP (produced as described in Materials and Methods) was biotinylated using a commercial kit from Amersham as described in the legend to Fig. 3.5 and then subjected to the purification protocol for the yeast adhesin. Three samples were taken during the degradation. The first sample was 100µl of N-glycanase- and papain-treated EP, concentrated by freeze-drying, and resuspended in sample buffer. The volume of each subsequent sample was adjusted to account for the dilution at each step. The entire Synsorb bound fraction was concentrated by freeze-drying and loaded on the gel. Proteins were separated using the procedure of Laemmli (1970) and a mini gel apparatus. The stacking gel and the separating gel were 4.5% and 12.5% acrylamide respectively. Proteins were then transferred on to nitrocellulose (Hybond C, Amersham) according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). Biotinylated protein was then visualised by probing with ^{125}I-streptavidin (30 µCi/µg; 5 µCi; in 10ml reaction volume) followed by autoradiography.

Lane designations

1. N-glycanase and papain treated EP
2. N-glycanase, papain, and NaOH treated EP
3. N-glycanase, papain, and NaOH treated EP (bound to Synsorb H-2)
4. Biotinylated SDS-6B molecular weight markers

Amersham) for Western blot analysis which was performed according to the method of Towbin and Gordan (1984) in a Trans-Blot Electrophoretic Transfer apparatus (Bio-Rad). The protein was then visualised by probing with ^{125}I-streptavidin followed by autoradiography. The purified adhesin was seen as a strong band with a molecular weight of approximately 15.7 kDa with two possible breakdown products also visible (Fig. 3.7). This approximate molecular weight and compositional analysis was used to produce an estimate of the number of amino acids in the PA molecule. The pmol value of each amino acid was multiplied by the molecular mass of each amino acid (subtracted by 18 to account for the loss of an H_2O molecule at each peptide bond). These figures were summed and the total was divided by the total pmols detected in the amino acid analysis. This gave an average amino acid molecular mass for the adhesin molecule. When 15.7 kDa was divided by this average the number of amino acid residues in the PA molecule was approximately 152. This was the first time that purified adhesin had been seen by SDS-PAGE and allowed an estimate of molecular weight to be made. The successful visualisation of the purified adhesin meant that partial amino acid sequencing could be attempted.

3.7 Amino acid sequencing of the purified adhesin

Amino acid sequencing was performed by Sandra Miller in the Dept. of Geology and Applied Geology, University of Glasgow. Purified adhesin was run on a tricine polyacrylamide gel as described previously and then transferred onto ProBlott (Applied Biosystems). The ProBlott was cut to include the band of the adhesin seen in a blot of an identical gel of biotinylated adhesin from the same purified batch. The material on the ProBlott was then sequenced in an Applied Biosystems 477A Protein Sequencer. The amounts of each amino acid detected after each sequencing cycle is shown in Table 3.3. The sequence determined initially was :-

Ser Arg Asp Leu Ile Ile Gln Phe Lys Asn (Amino acid sequence 1)

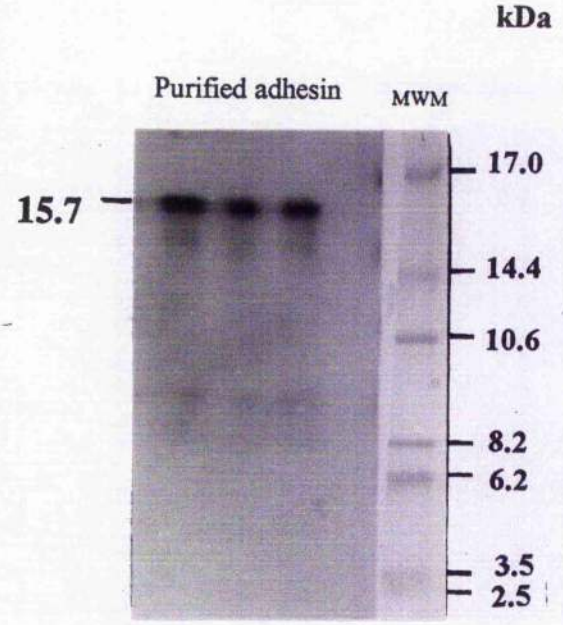


Fig. 3.7 SDS-PAGE and western blotting of biotinylated purified adhesin.

Biotinylation of purified adhesin was performed using a commercial kit from Amersham (following the manufacturers protocol). 200 μg of adhesin (produced as described in Materials and Methods) was dissolved in 1ml of 0.05M sodium borate buffer, pH 8.6. 40μl of biotinylation reagent was added and the tube was incubated for 1h at room temperature with constant agitation. The unreacted biotinylation reagent was removed by desalting on G-25 Sephadex and the adhesin was freeze-dried. The procedure of Schagger and Von Jagow (1987) was employed as described in Sigma Technical Bulletin No. MWM-100. The biotin-adhesin was resuspended in 100μl of sample buffer and 25μl was loaded in each well of a tricine mini SDS-PAGE gel. Samples (biotinylated PA, lanes 1,2 and 3) and molecular weight markers (MW-SDS-17S, Sigma, lane 4) were subjected to electrophoresis for 3-4 hours at a constant current of 100 V. The gel was cut and the markers were stained with Brilliant Blue G (Sigma). The biotinylated PA lanes were transferred onto nitro-cellulose (Hybond C, Amersham) according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). The protein was then visualised by probing with ^{125}I-streptavidin (30 μCi/μg; 5 μCi; in 10ml reaction volume) followed by autoradiography.

Table 3.3 Sequence analysis

	AMINO ACID SEQUENCE					
	1		2		3	
Amino acid No.	Amino acid	pMol	Amino acid	pMol	Amino acid	pMol
1	Ser	385.26	Ser	385.26	Phe	181.97
2	Arg	31.73	Asp	404.86	Asp	404.86
3	Asp	451.28	Asp	451.28	Tyr	38.63
4	Leu	876.52	Leu	876.52	Glu	83.25
5	Ile	62.11	Ser	265.73	His	21.19
6	Ile	88.07	Phe	683.92	Val	96.97
7	Gln	66.21	Lys	372.58	Asp	106.96
8	Phe	641.32	Phe	641.32	X	N.D.*
9	Lys	423.62	Lys	423.62	Ala	117.92
10	Asn	283.79	Asn	283.79	Val	139.79

Amino acid sequencing was performed by Sandra Miller in the Dept. of Geology and Applied Geology, University of Glasgow. Purified adhesin was run on a tricine polyacrylamide gel as described in the legend of Fig. 3.7. and then transferred onto ProBlott (Applied Biosystems) according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). The ProBlott was cut to include the band of the adhesin seen in a blot of an identical gel of biotinylated adhesin (as described in the legend of Fig. 3.7) from the same purified batch. The material on the ProBlott was then sequenced in an Applied Biosystems 477A Protein Sequencer.

* This amino acid could not be determined with any certainty but could be narrowed down to any one of the following:

Amino acid	pMol
Leu	108.79
Lys	115.20
Pro	108.61
Gly	95.73
Ala	95.46

When this sequence was run through peptide database using the FASTA program developed by Pearson and Lipman (1988), the closest homology was an outer capsid protein VP4 haemagglutinin from human Rotavirus serotype 4. The sequence had 75% identity in an eight amino acid overlap (Table 3.4). However, when the sequence data from the run was re-examined by Dr. Phil Jackson of Applied Biosystems, he determined that there were two distinct sequences present. The computer system attached to the sequencer had miss-read the data due to a high amount of background in the sample. The two most prominent sequences seen after re-examination of the data were :-

Ser Asp Asp Leu Ser Phe Lys Phe Lys Asn (Amino acid sequence 2)

Phe Asp Tyr Glu His Val Asp X Ala Val (Amino acid sequence 3)

The amino acid at position 8 in Amino acid sequence 3 could not be determined with any certainty but could be narrowed down to any one of the following: Leu, Lys, Pro, Gly, or Ala.

When Amino acid sequence 2 was run through the data base it had 100% homology with the first 10 amino acids at the N-terminus of the anti H (O) binding lectin from *Ulex europaeus* (Table 3.4). This astonishing finding prompted a careful examination of the experimental notes relating to the production of this particular batch of purified adhesin. It was found that the Synsorb H-2 affinity adsorbent used for this batch had been exposed to the purified *U. europaeus* lectin. This had occurred during an experiment to examine the efficiency of elution of H antigen-binding components from Synsorb affinity adsorbent. None of the previous batches of purified adhesin had been contaminated in this way. This accidental contamination in fact provided a useful check on sequence determination. Proteins with known sequences are often included on gels destined for sequence analysis. Also, two or three proteins can be sequenced in the same reaction so long as the amounts of the individual proteins are sufficiently different to allow for sequence discrimination (Phil Jackson, personal communication). Amino

Table 3.4 Peptide data base searches of amino acid sequence 1. and 2.

Amino acid sequence 1.

S	R	D	L	I	I	Q	F	K	N
	|	|	•	|	|	•	|	|	
Y	R	D	I	I	I	R	F	K	F
		270					275		

Human Rotavirus (serotype 4) Outer capsid protein (Haemagglutinin)
75% Identity in an eight amino acid overlap.

Amino acid sequence 2.

S	D	D	L	S	F	K	F	K	N
|	|	|	|	|	|	|	|	|	|
S	D	D	L	S	F	K	F	K	N
1				5					10

Ulex europaeus lectin I. 100% identity in a ten amino acid overlap.

Amino acid sequencing was performed by Sandra Miller in the Dept. of Geology and Applied Geology, University of Glasgow. Purified adhesin was run on a tricine polyacrylamide gel as described in the legend of Fig. 3.7. and then transferred onto ProBlott (Applied Biosystems) according to the method of Towbin and Gordan (1984) in a Trans-Blot Electophoretic Transfer apparatus (Bio-Rad). The ProBlott was cut to include the band of the adhesin seen in a blot of an identical gel of biotinylated adhesin (as described in the legend of Fig. 3.7) from the same purified batch. The material on the ProBlott was then sequenced in an Applied Biosystems 477A Protein Sequencer. The sequences obtained were run through peptide database using the FASTA program developed by Pearson and Lipman (1988) and the closest homologies are shown above. Identical amino acid residues are linked with a line:- (|).Similar amino acid residues are indicated with a full stop:- (.).

Table 3.5 Protein database search results

Amino acid sequence 3* from the purified adhesin was run through the peptide database using the FASTA program developed by Pearson and Lipman (1988). The resulting homologies are shown below. Identical amino acid residues are linked with a line:- (|). Similar amino acid residues are indicated with a full stop:- (.). Legend the same as for Table 3.4.

*FDYEHVDXAV

```
RGBYI3 regulatory protein SIR3 - yeast (Saccharomyces 71.4% identity in 7 aa
overlap
                                        FDYEHVDXAV
                                        ||| |.|.
RGBYI3 TNADDSTKFRLVNDVMDELVTSSARKELPIFDYIHIDALELAGMDALYEKIWPAISKENL
       580       590       600       610       620       630

------------------------------------------------------------------------
S11428 narY protein - Escherichia coli
  66.7% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        |||||...|
S11428 PRLGNKMGVITKIFANPVVPQIDDYYEPFTFDYEHLHSAPEGKHIPTARPRSLIDGKRMD
          80        90       100       110       120       130

------------------------------------------------------------------------
S24599 vitellogenin - Caenorhabditis elegans
 100.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        |||||
S24599 NSMKMLPFERFEEVERMNREHQELLSMPVEFDYEHGLVREIREAENDQPWSENIKRAVIN
          100       110       120       130       140       150

------------------------------------------------------------------------
VIT6_CAEEL VITELLOGENIN-6 PRECURSOR (FRAGMENT).
 100.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        |||||
VIT6_C NSMKMLPFERFEEVERMNREHQELLSMPVEFDYEHGLVREIREAENDQPWSENIKRAVIN
          100       110       120       130       140       150

------------------------------------------------------------------------
RSPB_ECOLI STARVATION SENSING PROTEIN RSPB
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||..||..|.
RSPB_E SSRLNANKPPIVIDWLSKGLIKPEKLITHTFDFQHVADAISLFEQDQKHCCKVLLTFSE
             290       300       310       320       330

------------------------------------------------------------------------
PGH1_MOUSE PROSTAGLANDIN G/H SYNTHASE 1 PRECURSOR (EC
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        |||. ...||
PGH1_M NTSMLVDYGVEALVDAFSRQRAGRIGGGRNFDYHVLHVAVDVIKESREMRLQPFNEYRKR
             420       430       440       450       460       470
```

```
--------------------------------------------------------
WMBEP6 ribonucleoside-diphosphate reductase
  40.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||......||
WMBEP6 FTSTCNLANVCLPKCLNSSNFPYTCSNTAQFDFSKLEYAVQAAVFIINACILSPSPTSSA
           410       420       430       440       450       460

--------------------------------------------------------
JQ1387 NAD(P)H-dependent xylose reductase - yeast
  83.3% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||||.|
JQ1387 LFLIHFPVTFKEVPLEEKYPPGFYCGKGDNFDYEDVPILETWKALEKLVKAGKIRSIGVS
         110       120       130       140       150       160

--------------------------------------------------------
S08450 hypothetical protein 1 - spiroplasma virus 1
  71.4% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||.| ||.
S08450 YSVLQENIRVQQGGSADYENPNKVGTKRIIFDFETVDELDVKNIKKAIYRMILTVDEANL
            290       300       310       320       330       340

--------------------------------------------------------
RIR1_HSVSA RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE LARGE
  40.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||......||
RIR1_H FTSTCNLANVCLPKCLNSSNFPYTCSNTAQFDFSKLEYAVQAAVFIINACILSPSPTSSA
           410       420       430       440       450       460

--------------------------------------------------------
VG1_SPV1R CAPSID PROTEIN.
  71.4% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||.| ||.
VG1_SP YSVLQENIRVQQGGSADYENPNKVGTKRMIFDFETVDELDVKNIKKAIYRMILTVDEANL
            290       300       310       320       330       340

--------------------------------------------------------
A35564 prostaglandin-endoperoxide synthase
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        |||. ...||
A35564 NTSMLVDYGVEALVDAFSRQRAGRIGGGRNFDYHVLHVAVDVIKESREMRLQPFNEYRKR
             420       430       440       450       460       470

--------------------------------------------------------
S30958 gene 13 protein - Mycobacterium phage L5
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||.|....||
S30958 GRTYKKKLKVNASPNNPVAFDMRGQQKRFAFDCERLEDAVLEGEVWHDGNPVLRQHVLNA
      480       490       500       510       520       530

--------------------------------------------------------
FIXB_AZOCA FIXB PROTEIN.
  57.1% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||..||..
FIXB_A AGISGAIQHRVGVEGADMIVAINTDKNAPIFDFAHVGIVTDAIRLLPALTEAFRARLSPH
            310       320       330       340       350       360

--------------------------------------------------------
VG13_BPML5 GENE 13 PROTEIN (GP13).
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||.|....||
VG13_B GRTYKKKLKVNASPNNPVAFDMRGQQKRFAFDCERLEDAVLEGEVWHDGNPVLRQHVLNA
      480       490       500       510       520       530
```

```
--------------------------------------------------------------
S14071 fixB protein - Azorhizobium caulinodans
  57.1% identity in 7 aa overlap
                                          FDYEHVDXAV
                                          ||..||..
S14071 AGISGAIQHRVGVEGADMIVAINTDKNAPIFDEAHVGIVTDAIRLLPALTEAFRARLSPH
            310       320       330       340       350       360


--------------------------------------------------------------
S12955 calcium channel protein type L - rabbit
  50.0% identity in 6 aa overlap
                                          FDYEHVDXAV
                                          ||...|
S12955 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
      70        80        90       100       110       120


--------------------------------------------------------------
A40695 alpha 1(V) procollagen - chicken (fragment)
  75.0% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        ||.||.||
A40695 DAPQSQDPNQDEYYTDGEGEGDTYYYEYPYYEDVDEAVKPEAPTTKPAPPGVAAGERPET
        250       260       270       280       290       300


YMR1_YEAST HYPOTHETICAL PROTEIN IN MRF1 5'REGION (FRAGMENT)
  44.4% identity in 9 aa overlap
                                          FDYEHVDXAV
                                          |||... .X
YMR1_Y SYIEYNRFFNSLAHWDSRRSGDIIIYYKLPFDYSDIPAADI
       240       250       260       270


--------------------------------------------------------------
S05054 calcium channel alpha-1 chain, dihydropyridine
  50.0% identity in 6 aa overlap
                                          FDYEHVDXAV
                                          ||...|
S05054 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
        1100      1110      1120      1130      1140      1150


--------------------------------------------------------------
GWEBT indole-3-glycerol-phosphate synthase
  55.6% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||.|||. .
GWEBT  QAYVNALREALPKQVQIWKALSVSDALPARDYHHVDKYIFDNGQGGSGQRFDWSLLQGQP
      340       350       360       370       380       390


--------------------------------------------------------------
WMBEB1 ribonucleoside-diphosphate reductase
  40.0% identity in 10 aa overlap
                                          FDYEHVDXAV
                                          ||.... .||
WMBEB1 TRIVHPASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMVNIMIDSTLQPTPQ
           800       810       820       830       840       850


--------------------------------------------------------------
YK62_CAESL HYPOTHETICAL 105.4 KD PROTEIN C29E4.2
  75.0% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        ||.||.||
YK62_C LLIDKALPLLKKFAENRTDREQMAYEMAKAYEDVDVAVSTLQEHVDKWHSRMEPILEEGE
         770       780       790       800       810       820


--------------------------------------------------------------
TRPC_SALTY INDOLE-3-GLYCEROL PHOSPHATE SYNTHASE
  55.6% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||.|||. .
TRPC_S QAYVNALREALPKQVQIWKALSVSDALPARDYHHVDKYIFDNGQGGSGQRFDWSLLQGQP
      340       350       360       370       380       390
```

```
--------------------------------------------------------------
A44467 voltage-dependent calcium channel complex alpha
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
A44467 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
        1070      1080      1090      1100      1110      1120


--------------------------------------------------------------
A45290 calcium channel protein type L - human
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
A45290 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
        1090      1100      1110      1120      1130      1140


--------------------------------------------------------------
JH0427 voltage-dependent calcium channel complex alpha
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
JH0427 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
     1070      1080      1090      1100      1110      1120


--------------------------------------------------------------
C25877 hypothetical protein 1 - Crithidia fasciculata
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||| .||...
C25877 FLRLFDMRMSILLCKQCFFVGFFVFGFVCLFDYMYVDVTIETIISLFYSLWCCILPGCSF
     220       230       240       250       260       270


--------------------------------------------------------------
B43921 dihydropyridine receptor, DHP receptor - rat
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
B43921 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
           30        40        50        60        70        80


--------------------------------------------------------------
CICC_RAT DIHYDROPRYRIDINE-SENSITIVE L-TYPE, CARDIAC MU
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
CICC_R EAESKGNYITYKTGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
         1100      1110      1120      1130      1140      1150


--------------------------------------------------------------
S11339 calcium channel protein - rabbit
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
S11339 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
             1100      1110      1120      1130      1140      1150


--------------------------------------------------------------
S05007 calcium channel alpha-1 chain, dihydropyridine
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
S05007 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
             20        30        40        50        60        70


--------------------------------------------------------------
S44769 C29E4.2 protein - Caenorhabditis elegans
  75.0% identity in 8 aa overlap
                                      FDYEHVDXAV
                                      ||.||.||
S44769 LLIDKALPLLKKFAENRTDREQMAYEMAKAYSDVDVAVSTLQEHVDKWHSRMEPILEEGE
          770       780       790       800       810       820
```

```
S29994 Type V collagen, alpha 1 chain, NH2-terminal do
  75.0% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        ||-||.||
S29994 DAPQSQDPNQDEYYTDGEGEGDTYYYEYPYYEDVDEAVKPEAPTTKPAPPGVAAGERPET
         250       260       270       280       290       300


------------------------------------------------------------
RIR1_HSV11 RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE LARGE
  40.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||.... .||
RIR1_H TEIVHPASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMVNIMIDSTLQPTPQ
           800       810       820       830       840       850


------------------------------------------------------------
F22848 hypothetical ORF-4 protein - Sauroleishmania ta
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||| .||...
F22848 FLRLFDMRMSILLCKQCFFVGFFVFGFVCLFDYMYVDVTIETIISLFYSLWCCILPGCSF
     200       210       220       230       240       250


------------------------------------------------------------
CICC_RABIT DIHYDROPRYRIDINE-SENSITIVE L-TYPE, CARDIAC
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
CICC_R EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
         1100      1110      1120      1130      1140      1150


------------------------------------------------------------
WMBEB7 ribonucleoside-diphosphate reductase
  40.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        ||.... .||
WMBEB7 TEIVHPASKRSSGVCNLGSVNLARCVSRQTFDFGRLRDAVQACVLMVNIMIDSTLQPTPQ
           800       810       820       830       840       850


------------------------------------------------------------------------
S28601 hypothetical protein (MRF1 5' region) - yeast
  44.4% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||... .X
S28601 SYIEYNRFFNSLAHWDSRRSGDIIIYYKLPFDYSDIPAADI
       240       250       260       270


------------------------------------------------------------
JH0426 voltage-dependent calcium channel complex alpha
  50.0% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||...|
JH0426 EAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSI
         1070      1080      1090      1100      1110      1120


------------------------------------------------------------
C40049 virC-region protein yscL - Yersinia enterocolit
  44.4% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||..||...
C40049 ETQLQCQQFYRHVEQQMSEVVLLAVRKILNDYDQVDMTLQVVREALALVSNQKQVVVRVN
      90        100       110       120       130       140


------------------------------------------------------------
TRPF_KLULA N-(5'-PHOSPHORIBOSYL)ANTHRANILATE ISOMERASE
  62.5% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        |||||...
TRPF_K IKEYRSLIPSSIPIIKRFQFPQDCELLLDLYEHVDNVLTLFDSGEGGTGEKLNWSAISSW
             100       110       120       130       140       150
```

```
--------------------------------------------------------------
SLAP_BACSH SURFACE-LAYER 125 KD PROTEIN PRECURSOR.
 100.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          |||||
SLAP_B TIKNVKDATKLNNTMLDYTGKFTRSDKEGPDYEHVINADAKAKKVVLKFDKKMDAASLAD
         640       650       660       670       680       690


--------------------------------------------------------------
ISVKNL phosphoribosylanthranilate isomerase
  62.5% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        |||||...
ISVKNL IKEYRSLIPSSIPIIKRFQFPQDCELLLDLYEHVDNVLTLFDSGEGGTGEKLNWSAISSW
             100       110       120       130       140       150


--------------------------------------------------------------
MPP1_RAT MITOCHONDRIAL PROCESSING PEPTIDASE CATALYTIC
  50.0% identity in 8 aa overlap
                                        FDYEHVDXAV
                                        |||...|.
MPP1_R KPEDIKRVASKMLRGKPAVAALGDLTDLPTYEHIQAALSSRDGRLPRTYRLFR
             480       490       500       510       520


--------------------------------------------------------------
S29854 spectrin beta chain - dog (fragment)
 100.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          |||||.
S29854 RLFQLNREVDDLEQWIAEREVVAGSHELGQDYEHVTMLQERFREFARDTGNIGQERVDTV
             50        60        70        80        90       100


--------------------------------------------------------------
HRPN_ERWAM HARPIN (HARPIN-EA).
  44.4% identity in 9 aa overlap
                                          FDYEHVDXAV
                                          ||.....||
HRPN_E LGGGQGGNAGTGLDGSSLGGKGLRGLSGPVDYQQLGNAVGTGIGMKAGIQALNDIGTHRH
           230       240       250       260       270       280


--------------------------------------------------------------
S20477 ribulose-bisphosphate carboxylase
  57.1% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||...||.
S20477 LAQEGNDILRQAGKWSPELAAACEVWKEIRFDFKPVDTLDPNDKKQRDNEDTLADKLFGD
         440       450       460       470       480       490


--------------------------------------------------------------------------
ETS1_MOUSE C-ETS-1 PROTEIN (P54).
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        |||||  .|.
ETS1_M ESYDSCDRLTQSWSSQSSFNSLQRVPSYDSFDYEDYPAALPNHKPKGTFKDYVRDRADLN
         260       270       280       290       300       310


--------------------------------------------------------
YSCL_YEREN YSC OPERON PROTEIN L.
  44.4% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||..||...
YSCL_Y ETQLQCQQFYRHVEQQMSEVVLLAVRKILNDYDQVDMTLQVVREALALVSNQKQVVVRVN
       90        100       110       120       130       140


--------------------------------------------------------
S25021 probable polyketide synthase - Bacillus subtilis
  66.7% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        |||||..|.
S25021 ETMALAEELSAYFSTYRIFDSQELDRVSAADYEHVAGAIDLIGCGTSHEHSMGWINWLQK
             3410      3420      3430      3440      3450      3460
```

```
--------------------------------------------------------------
CC10_SCHPO START CONTROL PROTEIN CDC10.
  83.3% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        |||.||.
CC10_S SYFNISQILRLAGTSSSENAKELDDIIESGDYENVDSKHPQIDGVWVPYDRAISIAKRYG
          100       110       120       130       140       150

--------------------------------------------------------------
COZPCD cdc10 start control protein - fission yeast
  83.3% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        |||.||.
COZPCD SYFNISQILRLAGTSSSENAKELDDIIESGDYENVDSKHPQIDGVWVPYDRAISIAKRYG
          100       110       120       130       140       150

--------------------------------------------------------------
GNNYH4 genome polyprotein - human rhinovirus 14
  40.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        |||.. |...
GNNYH4 PGVLTGSAVGCDPDVFWSVIPCLMDGHLMAFDYSNFDASLSPVWFVCLEKVLTKLGFAGS
            1930      1940      1950      1960      1970      1980

--------------------------------------------------------------
S33821 median body protein - Giardia lamblia
  55.6% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        |||.||...
S33821 LKENSYNFDQLLEQKQQMRSDLNALREKAADYERVDRELRLKDKELEEKNAEIERLLEDR
            490       500       510       520       530       540

--------------------------------------------------------------
A36205 mitochondrial processing peptidase
  50.0% identity in 8 aa overlap
                                      FDYEHVDXAV
                                      |||...|.
A36205 KPEDIKRVASKMLRGKPAVAALGDLTDLPTYEHIQAALSSRDGRLPRTYRLFR
            480       490       500       510       520

--------------------------------------------------------------
RBL_CUSRE RIBULOSE BISPHOSPHATE CARBOXYLASE LARGE CHAIN
  57.1% identity in 7 aa overlap
                                      FDYEHVDXAV
                                      ||...||.
RBL_CU LAQEGNDILRQAGKWSPELAAACEVWKEIRFDFKPVDTLDPNDKKQRDNEDTLADKLFGD
       440       450       460       470       480       490

--------------------------------------------------------------
TRJ6_ECOLI TRAJ PROTEIN (RELAXOSOME PROTEIN).
 100.0% identity in 5 aa overlap
                                      FDYEHVDXAV
                                      |||||
TRJ6_E AIEEKAAAAGMSLSAYLLAVGQGYKITGVVDYEHVRELARINGDLGRLGGLLKLWLTDDP
          30        40        50        60        70        80

--------------------------------------------------------------
JC1378 aryldialkylphosphatase (EC 3.1.8.1) - Nocardia
  62.5% identity in 8 aa overlap
                         FDYEHVDXAV
                           ||..|.||
JC1378 MSAEFVIRNAKVFDGERVYERLDVAVREGRIAQVGTDLATPADGDELDAAGQALLPGLID
            20        30        40        50        60

--------------------------------------------------------------
S23002 traJ protein - Escherichia coli plasmid RP4
 100.0% identity in 5 aa overlap
                                      FDYEHVDXAV
                                      |||||
S23002 AIEEKAAAAGMSLSAYLLAVGQGYKITGVVDYEHVRELARINGDLGRLGGLLKLWLTDDP
          30        40        50        60        70        80
```

```
--------------------------------------------------------------
A35875 transforming protein (ets-1) - mouse
  50.0% identity in 10 aa overlap
                                          FDYEHVDXAV
                                          ||||| .|.
A35875 ESYDSCDRLTQSWSSQSSFNSLQRVPSYDSEDYEDYPAALPNHKPKGTFKDYVRDRADLN
         260       270       280       290       300       310

--------------------------------------------------------------
POLG_HRV14 GENOME POLYPROTEIN (COAT PROTEINS VP1 TO VP2)
  40.0% identity in 10 aa overlap
                                          FDYEHVDXAV
                                          |||.. |...
POLG_H PGVLTGSAVGCDPDVFWSVIPCLMDGHLMAFDYSNFDASLSPVWFVCLEKVLTKLGFAGS
             1930      1940      1950      1960      1970      1980


--------------------------------------------------------------
A33856 surface-layer 125K protein precursor - Bacillus
 100.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          |||||
A33856 TIKNVKDATKLNNTMLDYTGKFTRSDKEGPDYEHVINADAKAKKVVLKFDKKMDAASLAD
         640       650       660       670       680       690


--------------------------------------------------------------
A44159 spectrin beta-G chain - human
 100.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          |||||.
A44159 RLFQLNREVDDLEQWIAEREVVAGSHELGQDYEHVTMLQERFREFARDTGNIGQERVDTV
     1700      1710      1720      1730      1740      1750


--------------------------------------------------------------
A34172 traJ protein - Escherichia coli plasmid RP4
 100.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          |||||
A34172 AIEEKAAAAGMSLSAYLLAVGQGGYKITGVVDYEHVRELARINGDLGRLGGLLKLWLTDDP
          30        40        50        60        70        80


--------------------------------------------------------------
QQSA4E hypothetical protein C-403 - Staphylococcus aur
  80.0% identity in 5 aa overlap
                                          FDYEHVDXAV
                                          ||||.
QQSA4E KQELQEVKDELQKANKQLQSGIEHMRSTKPFDYENERTGLFSGREETGRKILTADEFERL
           210       220       230       240       250       260


--------------------------------------------------------------
Z3BPFD coat protein A precursor - phage fd
  55.6% identity in 9 aa overlap
                                          FDYEHVDXAV
                                          ||||....|
Z3BPFD GSEGGGSEGGGSEGGGSEGGGSGGGSGGSGDFDYEKMANANKGAMTENADENALQSDAKGK
         250       260       270       280       290       300


--------------------------------------------------------------
IJCHCL E-cadherin precursor, hepatic - chicken
 100.0% identity in 4 aa overlap
                                          FDYEHVDXAV
                                          ||||
IJCHCL NPDEIGNFIDENLKAADTDPTAPPYDSLLVFDYEGGGSEATSLSSLNSSASDQDQDYDYL
        810       820       830       840       850       860


--------------------------------------------------------------
ACTP_ACACA ACTOPHORIN.
 100.0% identity in 4 aa overlap
                                          FDYEHVDXAV
                                          ||||
ACTP_A EVVVEHVGGPNATYEDFKSQLPERDCRYALFDYEFQVDGGQRNKITFILWAPDSAPIKSK
          40        50        60        70        80        90
```

```
B41870 DNA-directed DNA polymerase III
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
B41870 AARSLPARPPAPVLAGLLLKAEEGQLSLSSFDYEVSARVSVEAEIEEEGTVLVSGRLLAD
          20        30        40        50        60        70


A38122 ard protein - Escherichia coli plasmid ColIb-P9
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
A38122 GEADSEEAFAVEFASDTGLLADVPETVALYFDYEAYARDLFLDSFTFIDGHVFRR
              120       130       140       150       160


CPG1_RABIT CYTOCHROME P450 IIG1
  80.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        ||||.
CPG1_R RKTKGAPIDPTFFLSRTVSNVISSVVFGSRFDYEDKQFLSLLRMINESFIEMSTPWAQLY
               170       180       190       200       210       220


YBK6_YEAST HYPOTHETICAL 111.7 KD PROTEIN IN PKC1 5'REG
  50.0% identity in 10 aa overlap
                                        FDYEHVDXAV
                                        |||..  .||
YBK6_Y HDVSNGINSKFFDTKKIGTYGINGRITVTTFDYTQSLLAVATTAGEIHVYGQKQIEVVFT
                30        40        50        60        70        80


A32470 cytochrome P450 2C13, hepatic - rat
  71.4% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||||. |.
A32470 KKTNGSPCDPQFIMGCAPGNVICSIILQNRFDYEDKDFLNEIEKVNEAVKIISSPGIQVF
         160       170       180       190       200       210


JT0948 maltodextrin glucosidase - Escherichia coli
  83.3% identity in 6 aa overlap
                                       FDYEHVDXAV
                                       || |||.
JT0948 KLPYLKKLGVTALYLNPVFKAPSVHKYDTEDYRHVDPQFGGDGALLRLRHNTQQLGMRLV
           190       200       210       220       230       240


CPA4_MOUSE CYTOCHROME P450 IIA4
  57.1% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||||. ..
CPA4_M RKTNGAFIDPTFYLSRTVSNVISSIVFGDRFDYEDKEFLSLLRMMLGSLQFTATSMGQVY
               170       180       190       200       210       220


YO81_CAEEL HYPOTHETICAL 65.0 KD PROTEIN ZK1236.1
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||.
YO81_C RLVILWRLPLAEVAVDFFERLKKLTSGYASFDYEPDGWQDTRLVKLTILINSKEVSEFSQ
       420       430       440       450       460       470


S30718 metR protein - Escherichia coli
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
S30718 DPQPALQQGELDLVMTSDILPRSGLHYSEMFDYEVRLVLAPDHPLAAKTRITPEDLASET
       130       140       150       160       170       180
```

```
IJHUCN N-cadherin precursor, neuronal - human
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
IJHUCN HPGDIGDFINEGLKAADNDPTAPPYDSLLVFDYEGSGSTAGSLSSLNSSSSGGEQDYDYL
       830       840       850       860       870       880


CPH1_CHICK CYTOCHROME P450 IIH1 (EC 1.14.14.1) (PB15)
  80.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        ||||.
CPH1_C RKTHEEPFNPGKFLIHAVANIICSIVFGDRFDYEDKKFLDLIEMLEENNKYQNRIQTLLY
     160       170       180       190       200       210


VCNV87 p87 capsid protein - Orgyia pseudotsugata nucle
  80.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        ||||.
VCNV87 LPPYSSDEEDAAPPRSPKRKESLSSSEEDEFDYEREQKRRREEDKNFLRLKALELSKYAG
              380       390       400       410       420       430


ANGR_VIBAN ANGR PROTEIN.
  66.7% identity in 6 aa overlap
                                        FDYEHVDXAV
                                        ||||...
ANGR_V LPLTSNQNWQLSTQRQRTEKKSITNFTYQEFDYENISRDTLERCLTTIIKHHPIFGAKLS
           20        30        40        50        60        70


A36122 cytochrome P450 2C13 - rat
  71.4% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||||. |.
A36122 KKTNGSPCDPQFIMGCAPGNVICCIILQNRFDYEDKDFLNLIEKVNEAVKIISSPGIQVF
        160       170       180       190       200       210


CHLB_EQUAR PROTOCHLOROPHYLLIDE REDUCTASE CHLB SUBUNIT
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
CHLB_E TPMGIIDTADFIRQVQKNVNKLAPFFLNKTFDYESYIDYQTKFV
      60        70        80        90       100


B38170 cytochrome d complex terminal oxidase
  66.7% identity in 6 aa overlap
        FDYEHVDXAV
        |||| ..
B38170 MFDYSTLKLVWWGLIGVLLIGLALTDGFDMGAMALMPFLAKTDNERRVAINTVAPHWDGN
             20        30        40        50        60


DEJJGC glyceraldehyde-3-phosphate dehydrogenase (EC 1.
  50.0% identity in 8 aa overlap
                                      FDYEHVDXAV
                                        ||....||
DEJJGC NGKLTGMSMRVPTSNVSVVDLTVRIEKGATYEQIKTAVKKAADGPLKGVLAYTEDDVVST
          230       240       250       260       270       280


B49349 possible succinoglycan transport protein ExoP -
  66.7% identity in 9 aa overlap
                                        FDYEHVDXAV
                                        ||| ||.|.
B49349 AGGASHQRHQSNELLASPAMANLIRNARNAFDYVVVDLAALAPVVDAKAFAPLADGILFV
       660       670       680       690       700       710
```

```
--------------------------------------------------------------------
IJMSCN N-cadherin precursor, neuronal - mouse
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
IJMSCN HFGDIGDFINEGLKAADNDPTAPPYDSLLVFDYEGSGSTAGSLSSLNSSSSGGDQDYDYL
       830       840       850       860       870       880

IJMSCP P-cadherin precursor - mouse
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
IJMSCP NPDEIGNFIIENLKAANTDPTAPPYDSLMVFDYEGSGSDAASLSSLTTSASDQDQDYNYL
            750       760       770       780       790       800

--------------------------------------------------------------
DYR_PNECA DIHYDROFOLATE REDUCTASE
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
DYR_PN WSSVWKKEKHSDLESWVGTKVPHGKINEDGFDYEFEMWTRDL
         170       180       190       200

--------------------------------------------------------------
PGL2_ASPTU POLYGALACTURONASE II PRECURSOR
  80.0% identity in 5 aa overlap
                                        FDYEHVDXAV
                                        ||||.
PGL2_A LDNIEVEAGTTLDLTGLTSGTKVIFEGTTTFDYEEWAGPLISMSGKDITVTGASGHLINC
      50        60        70        80        90       100

--------------------------------------------------------------
B33531 cytochrome P450 2A3.2 - mouse
  57.1% identity in 7 aa overlap
                                        FDYEHVDXAV
                                        ||||. ..
B33531 RKTNGAFIDPTFYLSRTVSNVISSIVFGDRFDYEDKEFLSLLRMMLGSFQFTATSMGQLY
               170       180       190       200       210       220

--------------------------------------------------------------
A36066 metR protein - Escherichia coli
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
A36066 DPQPALQQGELDLVMTSDILPRSGLHYSPMFDYEVRLVLAPDHPLAAKTRITPEDLASET
      130       140       150       160       170       180

--------------------------------------------------------------
A48340 capsid protein - peanut stripe virus (fragment)
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
A48340 PYIAESALKTLYTNKRTRIEELAKYLAVLDFDYEVGCGESVHLQSGSSTTQSPVLDAGVD
      40        50        60        70        80        90

--------------------------------------------------------------
QQUTC5 NADH dehydrogenase (ubiquinone)
 100.0% identity in 4 aa overlap
                                        FDYEHVDXAV
                                        ||||
QQUTC5 FFTMIYNYFLLFFLMFVFKCFCLVDCLFLLFDYECCLVYCLISLYMCILSIFFIIDFVCI
     420       430       440       450       460       470

                        END OF DATA
```

acid sequence 3 was run through the peptide database and the sequences with similarities are shown in Table 3.5. There was no complete match found in the protein database but the purified adhesin sequence contains an interesting motif, FDYE, that is found in a wide range of proteins from a variety of sources. The proteins that contain this sequence have diverse functions ranging from viral coat proteins to yeast enzymes. The partial amino acid sequence obtained for the adhesin is most probably an internal sequence produced as a result of enzymic cleavage by the action of papain. Yu *et al*, (1994a) attempted to sequence a fimbrial adhesin of *C. albicans* but were unsuccessful due to N-terminal blockage of the protein. Similarly, Klotz *et al.* (1994) partially characterised the fibronectin adhesin of *C. albicans* which binds the fungus to subendothelial extracellular matrix and matrix proteins The N-terminus of the 60-kDa glycoprotein was blocked to Edman degradation. Proteolytic cleavage prior to sequencing would get round the problem of N-terminal blockage.

4. IDENTIFICATION OF THE *CANDIDA ALBICANS* RECEPTOR ON BUCCAL EPITHELIAL CELLS

A number of workers have found that fibronectin, a common mammalian cell surface glycoprotein, adheres to *C. albicans in vitro* (Skerl *et al.*, 1984; Scheld *et al.*, 1985; Klotz, 1990). *Candida* have been seen to bind to fibrin-platelet matrices (Maisch and Calderone, 1980). Mammalian proteins thought to mediate *C. albicans* attachment include fibrinogen (Tronchin *et al.*, 1987), laminin (Bouchara *et al.*, 1990), types I and IV collagen (Klotz, 1990). The fibronectin-cell binding domain, which has the amino acid sequence Arg-Gly-Asp (RGD), has been examined for its ability to block adherence of *C. albicans*. RGD-containing peptides were shown not to inhibit adhesion of *C. albicans* to BEC by Brassart *et al.* (1991). However, Ollert *et al.* (1993) found that *C. albicans* attachment to keratinocytes was inhibited by RGD-containing peptides derived from laminin. Similarly, Bendel *et al.* (1993) reported that

adhesion of *C. albicans* to HeLa S3 (an epithelial cell line) was inhibited by RGD-containing peptides.

A number of studies have examined the role of carbohydrates in the adhesion of *C. albicans* to epithelia. This work has mainly involved the use of lectins and/or monosaccharides (Centeno *et al.*, 1983; Collins-Lech *et al.*, 1984; Critchley and Douglas, 1987b). A more sophisticated study demonstrated that *C. albicans* can be inhibited from binding to BEC by L-fucose-containing complex carbohydrates (Brassart *et al.*, 1991). Other recent studies have found that *C. albicans* binds to the glycolipids lactosylceramide (Jimenez-Lucho *et al.*, 1990), asialo-GM_1 and asialo-GM_2 (Yu *et al.*, 1994b, c).

4.1 RGD peptide as a receptor for *C. albicans* on buccal epithelial cells.

To examine the possible role of RGD peptide in the adhesion of *C. albicans* to oral epithelium, *C. albicans* GDH 2346 cells were pre-treated with RGD peptide prior to adhesion-inhibition assays with BEC. Pre-incubation with RGD peptide in the presence and absence of Mg^{2+} and Ca^{2+} was found not to inhibit adhesion of yeasts to BEC. There was no significant difference in the adhesion values obtained for treated and untreated yeasts. The results are shown in Table 4.1. These results are in agreement with work by Brassart *et al.* (1991) but contradict the work of Bendel *et al.* (1993) who found that adhesion of *C. albicans* to HeLa S3 cells (epithelial cell line) was inhibited by RGD containing peptides.

4.2 The role of glycolipids in the adhesion of *C. albicans* to epithelial cells

A number of studies have examined the role of lipids on the adhesion of *C. albicans* to epithelia. Ghannoum *et al.* (1986) investigated the adhesion-blocking action of lipids extracted from three *Candida* spp. Three more recent studies have employed a TLC chromatogram overlay assay, previously used in the characterisation of bacterial adhesion mechanisms, to examine the ability of pathogenic yeasts to bind to a variety of lipid types (Jimenez-Lucho *et al.*, 1990; Yu *et al.*, 1994b; Prakobphol *et al.*, 1994).

Table 4.1 Effect of RGD peptide on adhesion of *C. albicans* GDH 2346 to buccal epithelial cells.

Incubation conditions	Mean no. ± SEM of adherent yeasts/100 epithelial cells	% inhibition	P[5]
PBS[1]	1147.22 ± 54	-	-
PBS+RGD[2]	1149.89 ± 61	-0.23	NS
Tris[3]	1267.56 ± 86.64	-	-
Tris+RGD[4]	1278.44 ± 86.32	-0.85	NS

[1] Yeast cells were pre-treated with a solution of PBS[1] at 37^{0}C for 30 min and then used in adhesion assays.

[2] Yeast cells were pre-treated with a solution of RGD peptide (0.5 mgml^{-1}, in PBS) at 37^{0}C for 30 min and then used in adhesion assays.

[3] Yeast cells were pre-treated with a solution of 50 mM Tris-HCl (pH 7.3) containing 0.02% CaCl and 0.02% MgCl at 37^{0}C for 30 min. The cells were then used in adhesion assays.

[4] Yeast cells were pre-treated with a solution of 50 mM Tris-HCl (pH 7.3) containing 0.02% CaCl, 0.02% MgCl and RGD peptide (0.5 mgml^{-1}) at 37^{0}C for 30 min and then used in adhesion assays.

[5] Probability values relative to adhesion of PBS and Tris controls; NS, not significant.

4.2.1 Isolation of BEC glycolipids and their separation on TLC plates

With a view to identifying glycolipid receptors for *C. albicans* in the oral cavity, BEC were isolated from four healthy human donors (all blood group A). Total glycolipids were isolated from the BEC by chloroform-methanol extraction for use in a glycolipid chromatogram overlay assay. This system has been used to characterise a number of adhesin-receptor relationships (Hansson *et al.*, 1985). In the following experiments, BEC lipids, along with control glycolipids (Calbiochem) and sheep red blood cell (sRBC) glycolipids (a gift from Chris Brotherston, Dept. of Microbiology, University of Glasgow), were separated on thin layer chromatography (TLC) plates. The control glycolipids comprised galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS). The structures of these lipids are shown in Table 4.2.

Various methods of lipid visualisation were examined. Iodine vapour was found to stain the separated lipids quite well, but not well enough to allow for clear photography of the plates. Anisaldehyde reagent stained the lipids clearly (blue-green on a pink-red background) and provided good contrast for photography (Fig. 4.1). However, the images obtained were quite variable due to difficulty in judging the optimum amount of anisaldehyde to be sprayed onto the chromatogram. If too much was applied, then intense background staining of the plates was encountered. If too little was used then the lipid bands were barely visible. Resorcinol and orcinol reagent staining both gave satisfactory results that were easy to reproduce (Figs. 4.2 and 4.3). Resorcinol reagent is commonly used to stain separated gangliosides and orcinol is used for both neutral glycolipids and gangliosides. Orcinol reagent showed lipid spots as reddish purple on a white background whereas resorcinol reagent gave spots that were brownish yellow in colour. The contrast achieved with orcinol reagent was marginally better than that obtained with resorcinol and so total lipid was subsequently visualised by treatment with orcinol reagent.

Different solvent systems for the separation of lipids were also assessed. All those tested contained chloroform, methanol and water but in varying proportions. The

Table 4.2 Neutral Glycolipid TLC Standards

Structure	Name	Designation
Galβ1-1Cer	Galactosylceramide (ceramide mono-hexose)	CMH
Galβ1-4Glcβ1-1Cer	Lactosylceramide (ceramide di-hexose)	CDH
Galα1-4Galβ1-4Glcβ1-1Cer	Globotriaosylceramide (ceramide tri-hexose)	CTH
GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Globotetraosylceramide (globoside)	GL4
GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer	Globopentaosylceramide (Forssman glycolipid)	FORS

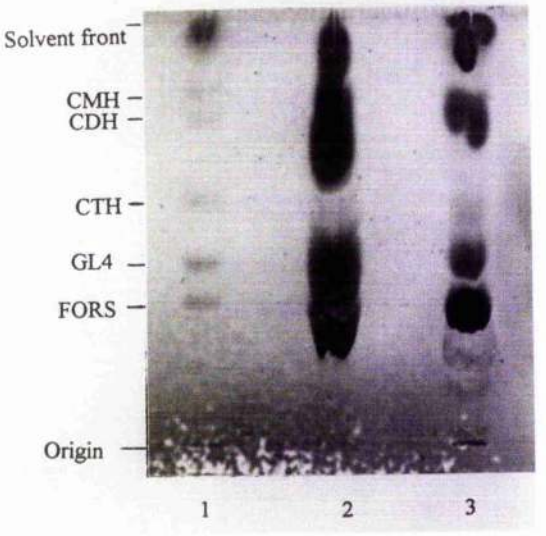


Fig 4.1 Thin-layer chromatogram of neutral glycolipid standards, BEC glycolipids, and sRBC glycolipids

Glycolipids were separated using chloroform-methanol-water (55:45:10) and then visualised using anisaldehyde reagent. Lane 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 200 μg BEC total lipid extract; lane 3, 600 μg sRBC total lipid extract.

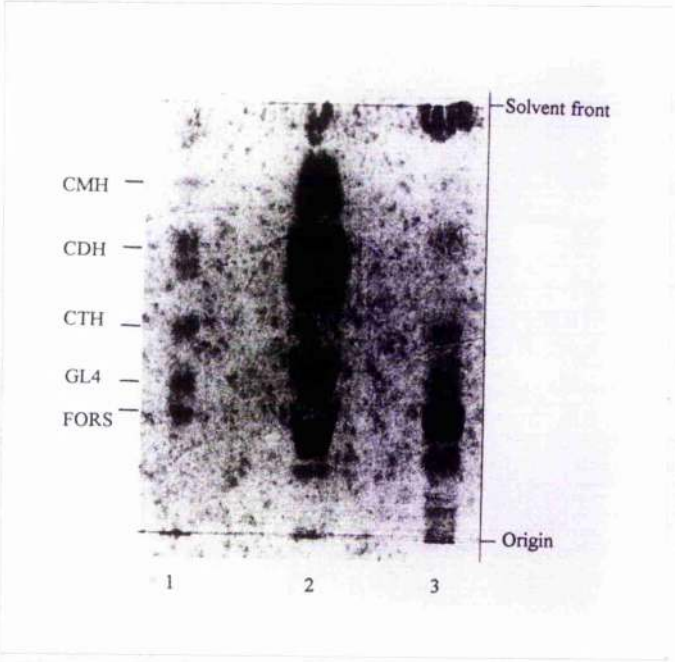


Fig 4.2 Thin-layer chromatogram of neutral glycolipid standards, BEC glycolipids, and sRBC glycolipids

Glycolipids were separated using chloroform-methanol-water (60:35:8) and then visualised using orcinol reagent. Lane 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 150 μg BEC total lipid extract; lane 3, 200 μg sRBC total lipid extract.

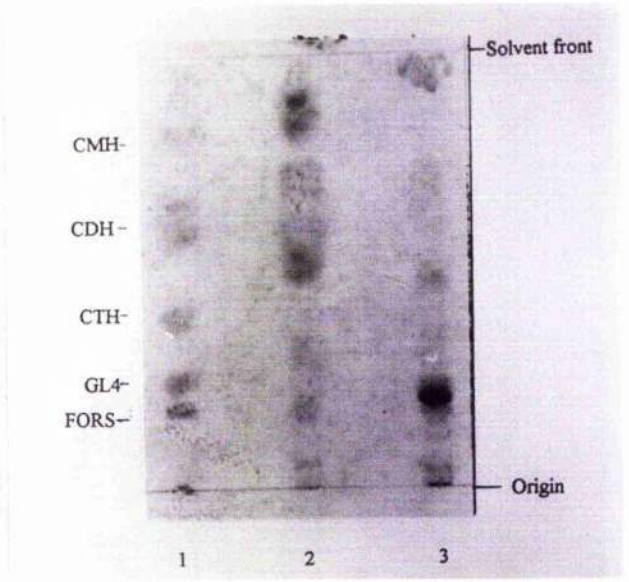


Fig 4.3 Thin-layer chromatogram of neutral glycolipid standards, BEC glycolipids, and sRBC glycolipids

Glycolipids were separated using chloroform-methanol-water (65:25:4) and then visualised using resorcinol reagent. Lane 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 50 μg BEC total lipid extract; lane 3, 200μg sRBC total lipid extract.

solvent systems containing chloroform, methanol and water in the ratios 55:45:10 (Fig. 4.1) and 60:35:8 (Fig. 4.2) allowed for adequate lipid separation but a system with chloroform, methanol and water in a ratio of 65:25:4 was found to give the most reproducible separation (Fig. 4.3). The mobility of a glycolipid on TLC gives a rough indication of the number of sugar residues present in the lipid. This is clearly demonstrated by the separation of the standard glycolipid mixtures on all the chromatograms. Those glycolipids with the few sugar residues (e.g. CMH and CDH) run close to the solvent front. With each additional residue, mobility is reduced. CDH is often seen as a double spot (Fig 4.4) due to heterogeneity in ceramide structure. The amount of lipid applied to the TLC plate was found to affect the subsequent separation of lipid components. If too much lipid was applied then streaking of the bands was encountered. Also, some bands seemed to run a little slower when too much lipid was applied. Fig. 4.1 demonstrates the effect of overloading lipid onto TLC plates. The BEC lipid track shows clear signs of streaking. Fig. 4.4 demonstrates the change in mobility pattern of different amounts of the same lipid mixture. BEC lipids were loaded at 80 μg and 160 μg. There is a clear reduction in migration distance between the two lipid lanes which becomes more pronounced towards the top of the chromatogram. Having optimised all the parameters relating to the separation of glycolipids and their subsequent staining, a chromatogram like that shown in Fig. 4.5 was routinely produced.

4.2.2 Binding of ^{125}I-EP from strain GDH 2346 to glycolipids separated on TLC plates

EP from *C. albicans* GDH 2346 was radioiodinated using the Iodogen procedure (Pierce). Glycolipids (from BEC, sRBC, and control lipids) were separated on TLC plates using chloroform-methanol-water in the ratio 65:25:4. After development, TLC plates to be used in the overlay analysis were air-dried and briefly dipped in polyisobutylmethacrylate (PIBMA) in hexane. The plates were again air-dried and then placed in PBS-BSA blocking solution. The blocking solution was tipped off and ^{125}I-EP

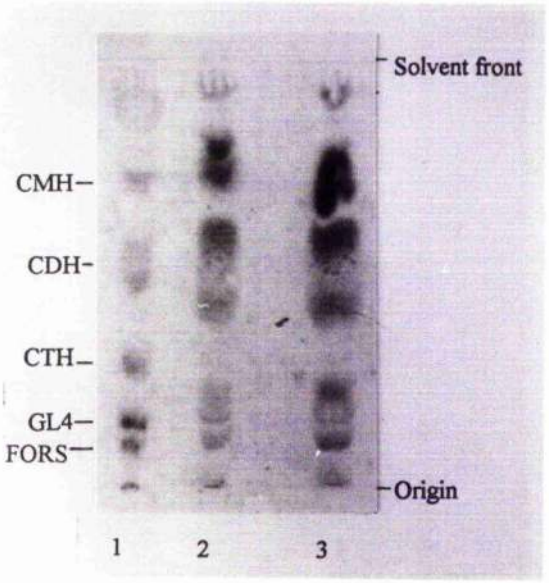


Fig 4.4 Thin-layer chromatogram of neutral glycolipid standards and BEC glycolipids

Glycolipids were separated using chloroform-methanol-water (65:25:4) and then visualised using orcinol reagent. Lane 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 80 μg BEC total lipid extract; lane 3, 160μg BEC total lipid extract.

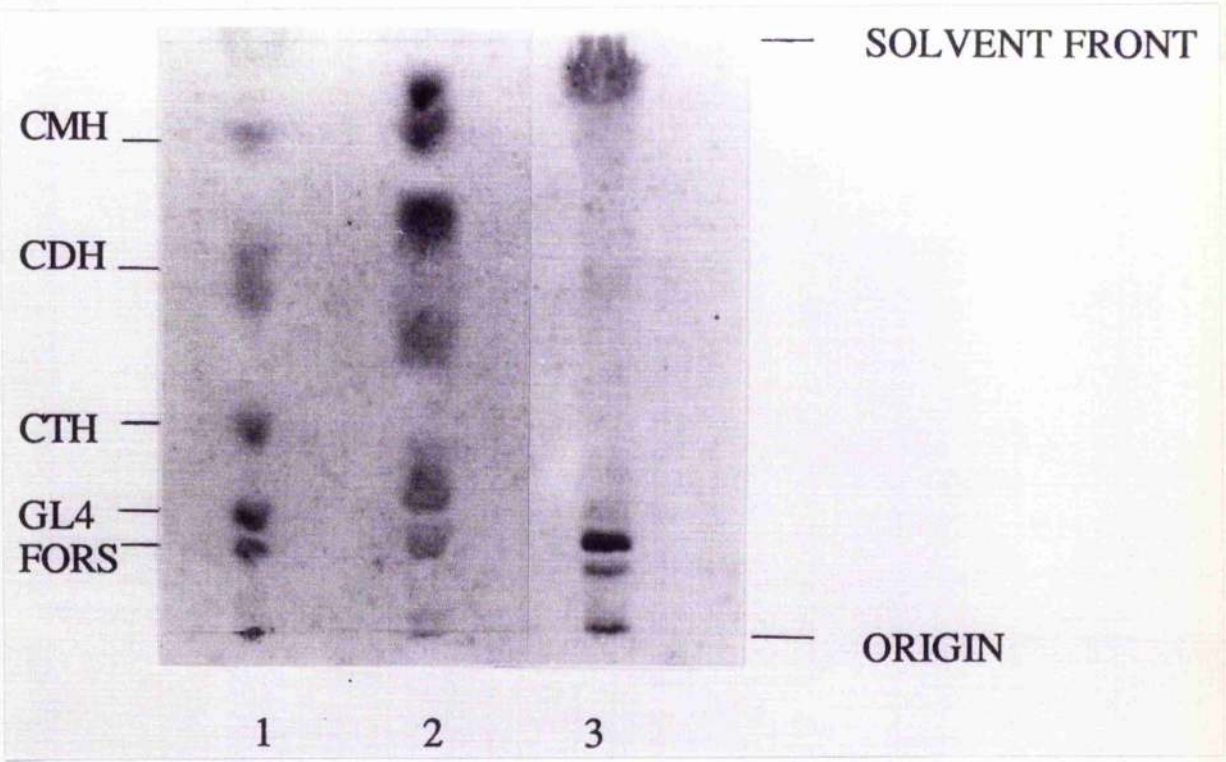


Fig 4.5 Thin-layer chromatogram of neutral glycolipid standards, BEC glycolipids, and sRBC glycolipids

Glycolipids were separated using chloroform-methanol-water (65:25:4) and then visualised using orcinol reagent. Lane 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 80 μg BEC total lipid extract; lane 3, 500 μg sRBC total lipid extract.

in fresh blocking solution was added. After incubation, unbound ^{125}I-EP was removed by washing the plates in PBS. The plates were then air dried, placed inside plastic and sealed, and exposed to X-ray film for 72 h. Despite the high background, the autoradiogram obtained (Fig. 4.6) shows that ^{125}I-EP to bound to two lipid components from sRBC. There did not appear to be any binding to either the control lipids or the lipids from BEC. Some clear zones were seen through the background in the BEC lipid lane. Some pitting of the silica was seen on the TLC plate after the washing steps were complete. The pits were seen to correspond to radioactive hot spots on the autoradiograph. This suggests that the pitting resulted in the exposure of silica that was not blocked to the same extent as the surrounding silica. This may have allowed for non-specific binding of ^{125}I-EP.

4.2.3 Binding of ^{125}I-EP from strain GDH 2023 to glycolipids separated on TLC plates

The protocol for this experiment was identical to that described previously except that ^{125}I-EP from strain GDH 2023 was used. The autoradiogram obtained is shown in Fig. 4.7. ^{125}I-EP bound to four lipid components from sRBC. There did not appear to be any binding to lipids from BEC or to the control lipids. There were again clear zones in the BEC lipid lane. The pitting that had been seen in the previous experiment was not as pronounced in this experiment. There was significant loss of silica on the edge of the TLC plates which was probably a result of the plates being free to move inside the reaction tray. In all subsequent experiments, the plates were cut to the exact dimensions of the reaction tray to minimise this collision damage.

4.2.4 Binding of ^{125}I-labelled whole cells and EP of *C. albicans* GDH 2346 to glycolipids separated on TLC plates

The binding of ^{125}I-labelled whole cells and EP from strain GDH 2346 to glycolipids was examined by the chromatogram overlay assay. The overlay procedure was similar to that used in the experiments described above, but in an attempt to reduce

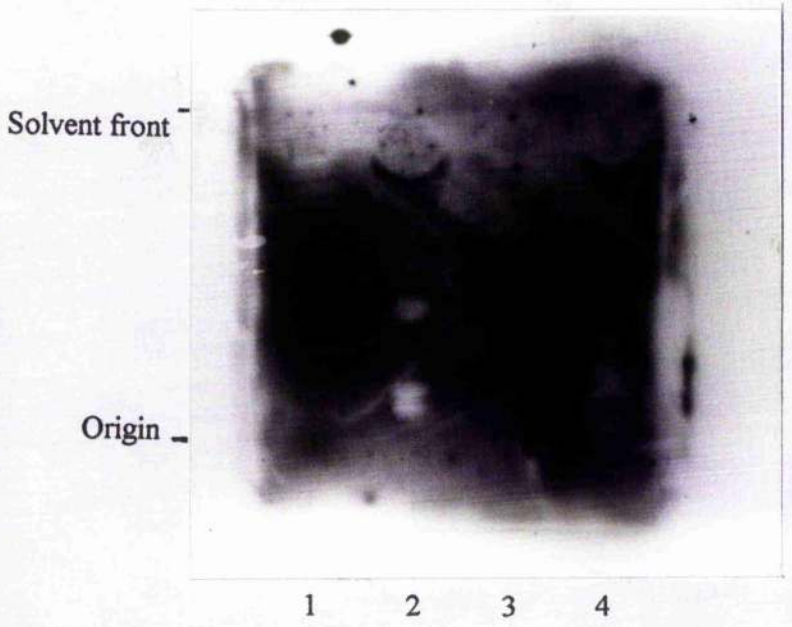


Fig 4.6 TLC overlay analysis of the binding of ^{125}I-EP from *C. albicans* GDH 2346 to separated glycolipids

EP was iodinated as described in Materials and Methods. TLC plate was prepared and blocked as described in Results 4.2.2 and then overlaid with ^{125}I-EP. Binding was detected by autoradiography. Lanes 1 and 3, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 80 μg BEC total lipid extract; lane 4, 500 μg sRBC total lipid extract.

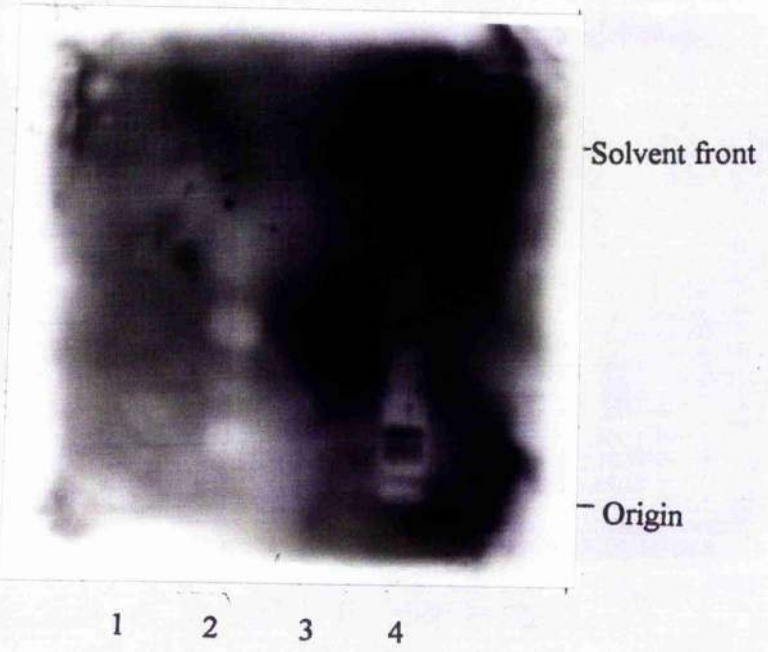


Fig 4.7 TLC overlay analysis of the binding of ^{125}I-EP from *C. albicans* GDH 2023 to separated glycolipids

EP was iodinated as described in Materials and Methods. The TLC plate was prepared and blocked as described in Results 4.2.2 and then overlaid with ^{125}I-EP. Binding was detected by autoradiography. Lanes 1 and 3, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), globotriaosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lane 2, 80 μg BEC total lipid extract; lane 4, 500 μg sRBC total lipid extract.

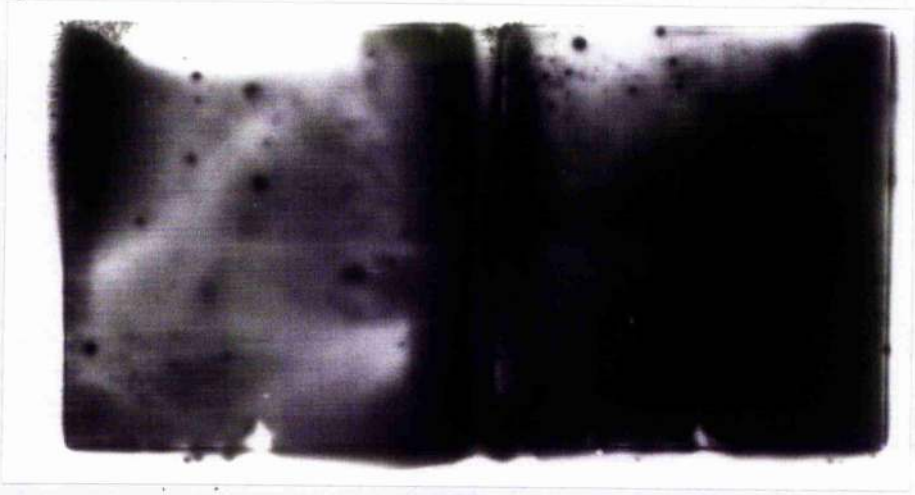
the intense background encountered previously, an alternative blocking solution (Tris-BSA buffer) was employed after PIBMA treatment. This solution was also used for the overlay incubation steps. The autoradiographs obtained (Fig. 4.8 A and B) show that the background was even higher than that previously encountered. Although there did seem to be some binding of ^{125}I-labelled material, the degree of background made useful interpretation impossible. The pitting seen was almost as bad as that observed seen in the first overlay experiment.

4.2.5 Binding of lectins to glycolipids separated on TLC plates

BEC were collected from one donor (blood group A) over a period of two weeks and stored at -20°C until required. The total glycolipids were then extracted from the pooled BEC. This BEC lipid preparation replaced the lipid extract used previously that had been isolated from the BEC of four donors (section 4.2.1). Biotinylated lectins from *Bauhinia purpurea, Triticum vulgaris* and *Vicia villosa* were purchased from Sigma. The use of lectins would allow for partial characterisation of the separated lipids. TLC plates were prepared as previously and PBS-BSA was used as the blocking and binding buffer. The lectins were diluted in blocking buffer prior to overlay analysis. Unbound lectin was removed by washing and biotinylated material was detected by probing with ^{125}I-streptavidin followed by autoradiography. *Bauhinia purpurea* agglutinin has an affinity for Gal(β1-3)GalNAc, N-acetylgalactosamine (GalNAc) and D-galactose (Irimura and Osawa, 1972), the lectin from *Triticum vulgaris* (wheatgerm agglutinin) has an affinity for GlcNAc and *Vicia villosa* lectin has an affinity for GalNAc. The autoradiographs obtained for these experiments are shown in Fig. 4.9 A, B and C.

The autoradiogram of the TLC plate overlaid with *Bauhinia purpurea* agglutinin showed a number of lipid components that were able to bind to this lectin. This lectin will bind to a number of different carbohydrate structures. It has highest affinity for compounds containing the Gal(β1-3)GalNAc structure. It was seen to bind to two of the control glycolipids (CDH and CTH) but to none of the other controls. Two lipid

A ^{125}I-EP B ^{125}I-whole cells



1 2 3 1 2 3

Fig 4.8 TLC overlay analysis of the binding of ^{125}I-EP and whole cells from *C. albicans* GDH 2346 to separated glycolipids

EP and whole cells were iodinated as described in Materials and Methods. TLC plates were prepared and blocked as described in Results section 4.2.4 and then overlaid with ^{125}I-EP and ^{125}I-whole cells. Binding was detected by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

components from the BEC lipid extract with mobility similar to CDH and CMH were also bound by this lectin (Fig. 4.9A). This suggests that the BEC extract contains lipid components that are similar to CDH and CMH both in lectin binding ability and TLC migration. Two additional BEC lipid components were bound by this lectin. One was very close to the origin (R_f 0.08) which suggests that it contained a large number of sugar residues, and the other was close to the solvent front (R_f 0.79). Three components from sRBC lipid extract were also bound by the lectin from *Bauhinia purpurea*. One component was very close to the origin with an R_f value similar to a component from BEC (R_f 0.08). The second component had a similar R_f value to CDH (R_f 0.62)and the third component (close to the solvent front) had a similar R_f value to another BEC lipid component (R_f 0.79).

The lectin from *Triticum vulgaris* (wheatgerm agglutinin) binds to molecules containing GlcNAc with preferential binding to dimers and trimers of GlcNAc. Not surprisingly, none of the lipid standards was bound by this lectin, as they lack GlcNAc. Similarly, not one of the BEC lipids bound the lectin (Fig. 4.9B). This was slightly surprising as one would expect GlcNAc to be relatively common on BEC. Four sRBC lipid components were seen to bind to this lectin, however. These components had R_f values between those of CDH and CMH. One component was intensely labelled with the other three relatively poorly indicated.

The lectin from *Vicia villosa* is specific for GalNAc-containing molecules. As expected, the two lipid standards with terminal GalNAc bound the lectin, but not very intensely. Three components from the BEC lipid extract were bound by this lectin (Fig. 4.9C). Two very poorly labelled components were seen close to the origin (R_f 0.05 and 0.08). Above them was a large and intensely stained component which migrated to a region that covered the area that FORS and GL4 migrated to in the control lipid lane (R_f 0.12-0.26). This could indicate that components similar to both FORS and GL4 are present in BEC. Two lipid components from sRBC bound to this lectin. One was close to the origin (R_f 0.05), the other had an R_f value similar to GL4 (R_f 0.24).

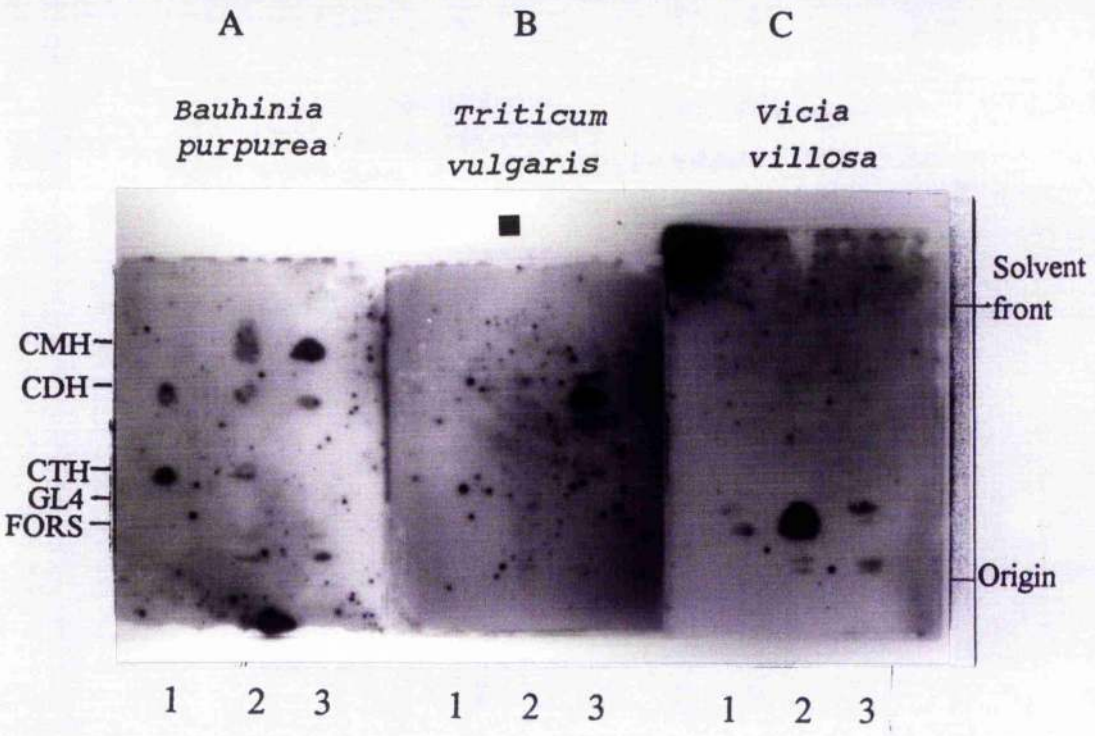


Fig. 4.9 Binding of lectins to glycolipids separated on thin-layer chromatograms.

TLC plates were prepared and blocked as described in Results 4.2.5 and then overlaid with biotinylated lectins from *Bauhinia purpurea, Triticum vulgaris* and *Vicia villosa*. Each lectin was used at a concentration of 10 μgml^{-1} (10 ml reaction volume). Plates were then overlaid with ^{125}I-streptavidin (30 $\mu Ci/\mu g$; 5 μCi in 10ml reaction volume) and binding detected by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycosphingolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

There was less background on these autoradiographs than had been encountered previously. This may have been due to the use of ^{125}I-streptavidin. However, there was clearly some deterioration of the TLC plates. Pitting was again visible on the surface of the plates which corresponded to "hot spots" on the autoradiograph. The pitting is probably due to silica loss during the extensive incubation and washing stages. Non-specific binding of biotinylated lectin and/or ^{125}I-streptavidin to these pits is likely as the blocking activity of the PIBMA coating will have been lost at these places.

4.2.6 Binding of biotinylated EP from *C. albicans* GDH 2346 and GDH 2023 to glycolipids separated on TLC plates

EP preparations from strains GDH 2346 and 2023 were first biotinylated and then subjected to TLC-overlay analysis. The protocol was similar to that used previously (section 4.2.5) but with minor modifications intended to reduce background and TLC plate deterioration. The TLC plates were loaded with lipid and run as before. After air drying, the plates were subjected to an enhanced PIBMA treatment as follows. Each plate was submerged in the PIBMA solution for 1 min, removed and allowed to air-dry. This was repeated a further two times and then the plates were blocked as previously and overlaid with biotinylated EP. The autoradiographs obtained are shown in Fig. 4.10 A and B. The binding of biotinylated EP from strain GDH 2346 to some separated lipids on the TLC plate was clearly visible. Binding was seen to two lipid components from the sRBC and one lipid component from BEC. Less intense binding to one further component from BEC was also observed. There was no binding to any of the neutral glycolipid controls. The background seen in this experiment was the lowest yet encountered and the pitting observed previously was virtually abolished. This may have been due to the use of ^{125}I-streptavidin to detect biotinylated proteins in place of the direct protein iodination system that had been used previously. The enhanced PIBMA treatment may have also contributed to the reduction in background. Biotinylated EP from strain GDH 2023 did appear to bind some lipid components from sRBC but only very poorly (Fig. 4.10B). In a previous experiment (section 4.2.3), there

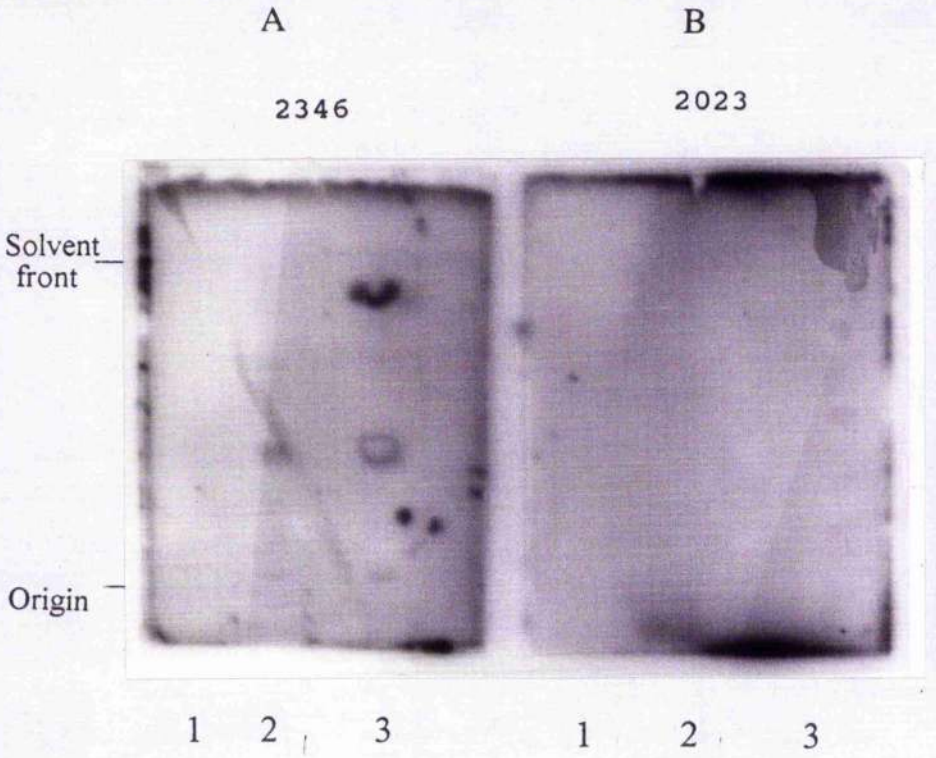


Fig. 4.10 Binding of biotinylated EP from *C. albicans* GDH 2346 and 2023 to glycolipids separated on thin-layer chromatograms.

EP was biotinylated as described in Materials and Methods. TLC plates were prepared and blocked as described in Results section 4.2.6, and overlaid with biotinylated EP. Plates were then overlaid with ^{125}I-streptavidin (30 μCi/μg; 5 μCi in 10ml reaction volume) and binding detected by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

was binding of ^{125}I-EP from GDH 2023 to four lipid components from sRBC. The weak binding seen in this experiment may have been due to poor biotinylation or damage to EP as a result of biotinylation, or it may have been a consequence of the enhanced PIBMA treatment.

4.2.7 Binding of biotinylated whole cells of different *C. albicans strains* to glycolipids on TLC plates.

The binding of biotinylated whole cells from five *C. albicans* strains (GDH 2346, GRI 681, GRI 682, MRL 3153 and "outbreak") to glycolipids from BEC, sRBC and a number of neutral glycolipid standards (CMH, CDH, CTH, GL4, FORS), was examined by the chromatogram overlay assay. Bound cells were visualised by the addition of ^{125}I-streptavidin followed by autoradiography. The autoradiographs from these experiments are shown in Fig. 4.11. All five strains were found to have similar binding patterns. By comparison with an identical chromatogram overlaid with biotinylated *Ulex europaeus* lectin (UE) it appeared that the cells were able to bind to two glycolipids with mobilities similar to those of lipids expressing H-blood group antigen. The cells also bound to an additional lipid from BEC, which was not bound by UE, and which had a mobility (R_f 0.04) similar to that of a lipid bound by the GalNAc-specific lectin from *Helix pomatia* (Fig. 4.11). However, GalNAc alone is unlikely to be the receptor as the yeast cells did not bind to the GalNAc containing neutral glycolipid standards, GL4 and FORS, both of which were detected by *Helix pomatia* lectin. None of the strains tested bound to lactosylceramide, recently proposed as a receptor for *C. albicans* (Jimenez-Lucho *et al.*, 1990). These results are consistent with previous experiments involving sugars and lectins (Critchley and Douglas, 1987b) which suggested that strains GDH 2346, GRI 681, GRI 682, and MRL 3153 all share a common adhesion mechanism involving fucose-containing receptors.

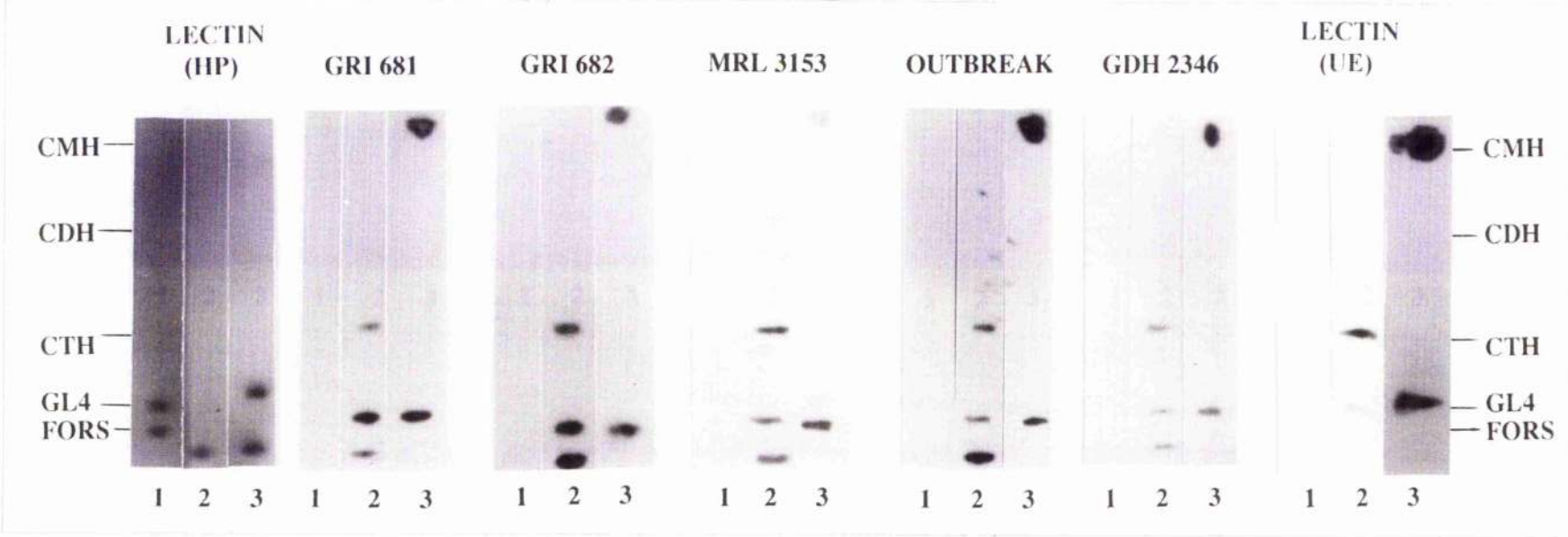


Fig 4.11 Binding of *C. albicans* and lectins to glycolipids separated on thin-layer chromatograms.

Glycolipids were obtained and separated on TLC plates as described in Materials and Methods. The chromatograms were then ovelaid with biotinylated *C. albicans* strains GRI 681, GRI 682, MRL 3153, outbreak, GDH 2346, and the biotinylated lectins from *Ulex europaeus* (UE) and *Helix pomatia* (HP). After washing off unbound material, biotin containing components were detected by the addition of ^{125}I-streptavidin followed by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

4.2.8 Binding of biotinylated EP and purified adhesin from *C. albicans* GDH 2346 to glycolipids on TLC plates.

The binding of EP and PA from strain GDH 2346 to glycolipids was examined by the chromatogram overlay assay. Bound material was visualised by the addition of ^{125}I-streptavidin followed by autoradiography. The autoradiographs from these experiments are shown in Fig. 4.12. The binding patterns observed for EP and PA were remarkably similar. Both bound to glycolipids from BEC and sRBC but not to any of the neutral glycolipid standards (including lactosylceramide). By comparison with a chromatogram overlaid with biotinylated *Ulex europaeus* lectin (UE) it appeared that the EP and PA were able to bind to two glycolipids with a similar R_f value to lipids expressing H-blood group antigen. Interestingly, in the previous experiment (section 4.2.7), whole cells from strain GDH 2346 (and four other strains) were found to bind three lipid components from BEC - two with mobilities similar to those of lipids expressing H-blood group antigen, and a third with a mobility similar to that of a GalNAc-containing lipid. The component on the *C. albicans* cell surface that binds the GalNAc- containing lipid may not be released as part of EP, or the ability of EP to bind to this component may be lost during the purification procedure.

4.2.9 Binding of biotinylated whole cells and EP from strain GDH 2023 to glycolipids on TLC plates.

The binding of whole cells and EP (both biotinylated) from strain GDH 2023 to glycolipids was examined by the chromatogram overlay assay. Biotin-labelled material was visualised by the addition of ^{125}I-streptavidin followed by autoradiography. The autoradiographs from these experiments are shown in Fig. 4.13. Whole cells and EP from strain GDH 2023 were found to bind to four glycolipids from both BEC and sRBC (not bound to by whole cells and EP from strain GDH 2346). By comparison with an identical chromatogram overlaid with biotinylated *Griffonia simplicifolia* lectin II (GlcNAc specific) it appeared that the cells were able to bind to glycolipids with similar R_f values to lipids expressing GlcNAc from BEC and sRBC. This strain did not

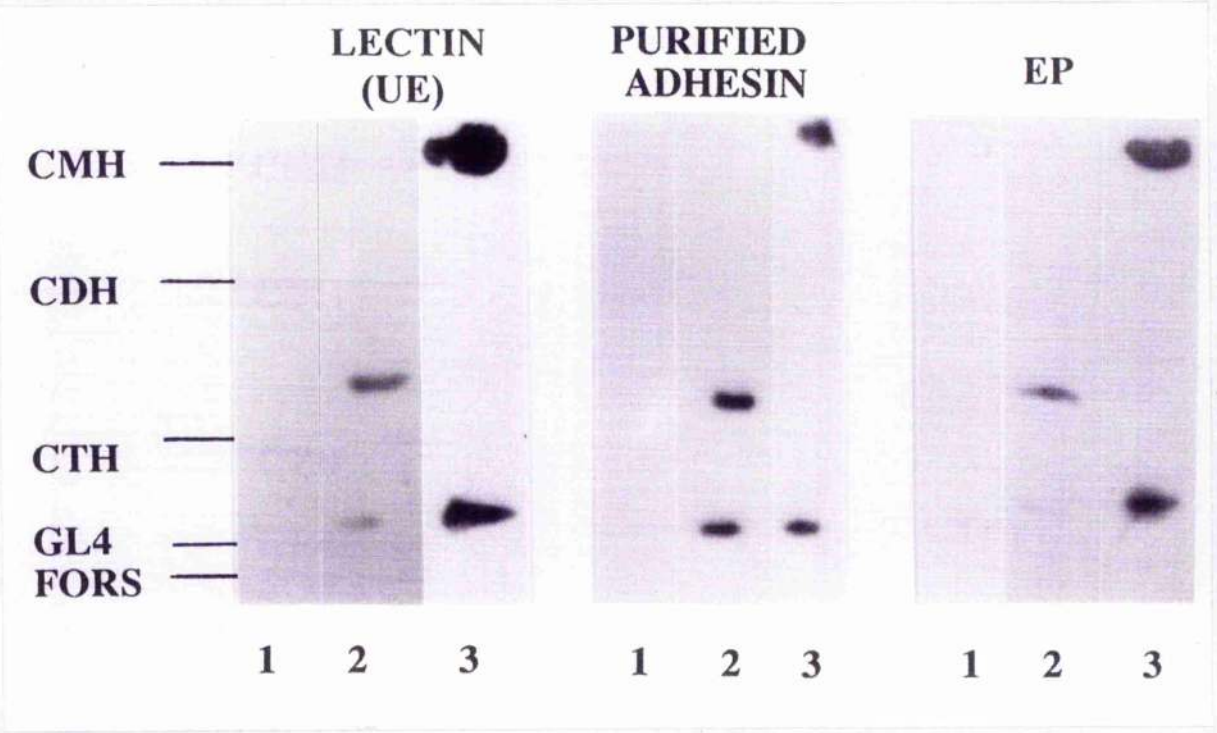


Fig 4.12 Binding of *C. albicans* GDH 2346 EP, and PA to glycolipids separated on thin-layer chromatograms.

Glycolipids were obtained and separated as described in Materials and Methods. Glycolipids were overlaid with biotinylated EP and PA, and the biotinylated lectin from *Ulex europaeus* (UE). After washing, the plates were overlaid with ^{125}I-streptavidin and binding was detected by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

bind to any of the neutral glycolipid standards (including lactosylceramide). These results are consistent with previous experiments involving sugars and lectins (Critchley and Douglas, 1987b) which suggested that strain GDH 2023 has an adhesion mechanism involving GlcNAc containing receptors.

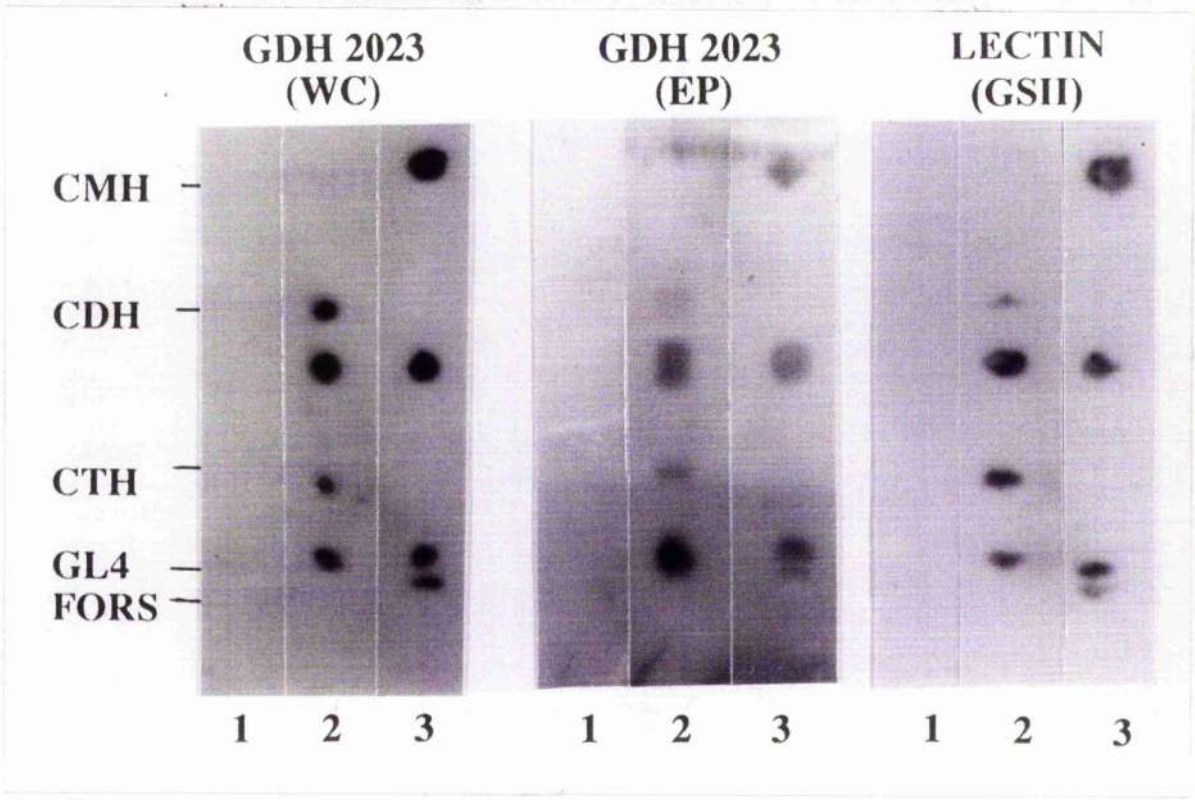


Fig 4.13 Binding of *C. albicans* GDH 2023 (whole cells and EP) and *Griffonia simplicifolia* lectin II (GS II) to glycolipids separated on thin-layer chromatograms.

Glycolipids were obtained and separated as described in Materials and Methods. Glycolipids were overlaid with biotinylated *C. albicans* GDH 2023 whole cells, EP and biotinylated GS II. Plates were then overlaid with ^{125}I-streptavidin and binding detected by autoradiography. Lanes 1, 2μg each of galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman glycolipid (FORS); lanes 2, 80 μg BEC total lipid extract; lanes 3, 500 μg sRBC total lipid extract.

DISCUSSION

1. ADHESION OF *C. ALBICANS IN VITRO*

Adhesion plays an important part in the pathogenesis of many microbial infections. *Candida* species have been shown to bind to a variety of surfaces, including neutrophils (Diamond *et al.*, 1978), vascular endothelium (Klotz *et al.*, 1983), and inert materials such as denture acrylic (Samaranayake, and MacFarlane, 1980; McCourtie and Douglas, 1981). A number of investigators have studied the adherence of *C. albicans* to buccal epithelial cells (BEC) (King *et al.*, 1980; Macura *et al.*, 1983; Critchley and Douglas, 1985; Brassart *et al.*, 1991). *C. albicans* has been found to be the most adherent *Candida* species with *C. tropicalis* moderately adherent and *C. parapsilosis* only slightly adherent to BEC.

There seems to be a clear relationship between ability to adhere to epithelial cells and the capacity for colonisation and infection of mucosal surfaces. Liljemark and Gibbons (1973) measured the attachment of *C. albicans* to rat buccal cells employing a method that had been previously developed for quantitating bacterial adherence. A modification of this procedure was used by Kimura and Pearsall (1980) which has been adopted by a number of other laboratories (Douglas, 1987). Radiolabelling (King *et al.*, 1980) and visual determination (Douglas *et al.*, 1981) of yeast adherence to epithelial cells have been used. The visual method employed in this study involves the incubation of standardised suspensions of yeasts and BEC. After incubation, the cell suspensions are passed through transparent polycarbonate filters that have a pore size of 12μm. This pore size is sufficient to let unbound yeasts pass through but prevents the passage of BEC and attached yeasts. The cells on the filters are fixed, stained, and the number of adherent yeasts can be determined by microscopy. This method has been used extensively in previous studies (Douglas *et al.*, 1981; McCourtie and Douglas, 1984; Critchley and Douglas,1987a, b; Tosh, 1991) and it permits the examination of a number of factors affecting the adhesion process.

The adhesion of *Candida* can also be measured by the use of radiometric assays (King *et al.*, 1980). These assays involve growing yeasts in medium containing ^{14}C-glucose prior to incubation with epithelial cells. This approach is less laborious and time

consuming than the visual technique described above, but does not permit monitoring of adhesion to individual epithelial cells or yeast to yeast coadherence (Douglas, 1987; Ghannoum and Abu-Elteen, 1991).

Odds (1988) criticised the filtration method of separating non-adherent yeasts from epithelial cell-*Candida* mixtures. He suggested that shear forces from currents in the washing fluids may remove adherent yeasts and impact non-adherent yeasts on to epithelia. However, it is possible that the shear forces described by Odds would only remove loosely associated yeasts, leaving those yeasts that are firmly attached. Also, the impacted non-adherent yeasts on the epithelial cells are likely to be removed by the washing steps that normally follow the filtration procedure. Another possible criticism of the in-vitro adhesion assay is the variation encountered, both from donor to donor, and even from day to day with the same donor. In this study, a number of steps were taken to minimise this variation. The same BEC donor was used throughout the study, and cells were collected at the same time of day for each experiment. The assays were performed three times in triplicate with at least a 48-hour gap between assays to allow for buccal epithelial cell regeneration in the donor. Counting bias was minimised by coding the assay filters and reading them 'blind'. For every sample, 100 epithelial cells were examined and the number of adherent yeasts counted. Control filters were always included to check for the presence of endogenous yeast flora.

1.1 Effect of EP on yeast adhesion in vitro

Previous work in this laboratory has shown that the adhesion of some strains of *C. albicans* to BEC is substantially increased after growth in defined media containing certain sugars (notably galactose) as the carbon source (Douglas *et al.*, 1981; McCourtie and Douglas, 1984; Critchley and Douglas, 1987a). This increase in adhesive ability seems to be a result of the production of an additional fibrillar-floccular layer on the yeast cell surface (Douglas, 1987). Prolonged incubation of *C. albicans* in medium containing galactose causes release of surface fibrils, and EP can be isolated from culture supernatants. Pretreatment of BEC with EP has been shown to inhibit adhesion of *C.*

albicans. The interaction seems to be strain specific as EP from one strain, GDH 2023, failed to inhibit the adhesion of a second strain, GDH 2346 (Critchley and Douglas,1987a). Pretreatment of EP with heat, dithiothreitol, and proteolytic enzymes (except papain) significantly reduced the blocking ability of EP, whereas pretreatment with sodium periodate and α-mannosidase had no effect on this ability (Critchley and Douglas, 1987a). These findings suggest that EP (composed primarily of mannoprotein) contains an adhesin, and that the protein portion of the mannoprotein is more important in the adhesion process than the carbohydrate portion.

A variety of methods have been employed in the preparation of EP from culture supernatants. McCourtie and Douglas (1985) isolated EP by acetone precipitation, Critchley and Douglas (1987a) used prolonged dialysis, and Tosh and Douglas (1992) employed dialysis followed by ultrafiltration. Throughout this study, EP was produced by a method very similar to that of Tosh and Douglas (1992). The exclusion limit of the ultrafiltration membrane employed in this study was 3.5 kDa in place of the 5 kDa filter used previously. EP (strain GDH 2346) prepared in this study had a protein content of 8.04% and carbohydrate content of 82% (Table 1.1). EP from strain GDH 2023 prepared in this study had a protein content of 5.5% and a carbohydrate content of 84.4% The values for strain 2346 are similar to those obtained by Tosh (7.2% and 79.2% for protein and carbohydrate respectively). However, the values for strain GDH 2023 differ slightly from those obtained by Tosh (10.5% and 72.4% for protein and carbohydrate, respectively). The only difference in the method of EP preparation between the two studies was the ultrafiltration membrane employed. The 3.5 kDa filter used in this study may have retained slightly more carbohydrate-containing material than a 5 kDa filter which would result in a higher percentage of carbohydrate and a lower percentage of protein. The values obtained for EP from strain GDH 2346 are very similar to those obtained by Tosh. This may mean that the supernatant of this strain did not contain additional carbohydrate components, or that both additional protein and carbohydrate components were retained resulting in a compositional analysis similar to that previously obtained by Tosh.

1.1.1 Effect of EP concentration on yeast adhesion

For adhesion-inhibition experiments with EP, yeasts were grown to stationary phase at 37°C with shaking in yeast nitrogen base (Difco) containing 500mM galactose as a carbon source. Under these conditions, *C. albicans* grows exclusively in the yeast phase. EP from two strains (GDH 2346 and GDH 2023) was prepared and used to pre-treat BEC prior to adhesion assays with yeast cells from the homologous strains. The concentrations of yeast cells used were $5x10^7$ and $1x10^8$ cells ml^{-1} for strains GDH 2346 and GDH 2023 respectively. Pre-incubation of BEC with increasing concentrations (2-20 mg ml^{-1}) of EP from GDH 2346 inhibited yeasts from the homologous strain by almost 60% (Fig.1.1). This result is consistent with the findings of Critchley and Douglas (1987a). In a similar experiment involving strain GDH 2023, yeast adhesion was inhibited by just over 40% (Fig.1.1). Tosh (1991) performed two adhesion-inhibition assays using EP derived from GDH 2023 to block the adherence of the homologous strain. Adhesion was inhibited by 52.8 and 47.8%. These values are higher than the maximum value obtained for this strain in the present study (40.9%). As mentioned previously, EP from strain GDH 2023 prepared in this study had a lower protein content (5.5%) and higher carbohydrate content (84.4%) than that prepared by Tosh (10.5% and 72.4% for protein and carbohydrate respectively). The lower adhesion-inhibition activity may have been due to the lower protein content of the EP prepared in this study. However, the graph of the effect of EP concentration on yeast adhesion suggests that maximum inhibition had been reached at around 40%. The most likely explanation for the differences between the two studies is the difference in BEC donor.

Two other groups have demonstrated the ability of EP from *C. albicans* to inhibit adhesion of yeasts to BEC. Brassart *et al.* (1991) found that EP could inhibit the adhesion of *C. albicans* cells of the homologous strain by up to 52%. They prepared EP using the method of Critchley and Douglas (1987a). More recently, Jenq *et al.* (1994) isolated EP with adhesion-inhibition activity of 42%. This level of inhibition is similar to that for the EP from GDH 2023 prepared in this study.

1.1.2 Effect of yeast cell concentration on adhesion-inhibition values

It has previously been reported that the attachment of yeasts to epithelial cells gradually increases as the ratio of yeasts to epithelial cells increases in assay mixtures (Kimura and Pearsal, 1980; King *et al*, 1980). BEC were pretreated with EP from strain GDH 2346 prior to incubation with yeast cells from the homologous strain at two different concentrations; $1x10^7$ and $5x10^7$ yeasts ml^{-1}. The results are shown in Table 1.2. Inhibition of adhesion for the two concentrations of yeasts was not significantly different. Yeasts at a concentration of $1x10^7$ ml^{-1} were inhibited by 49.1% while yeasts at $5x10^7$ yeasts ml^{-1} were inhibited by 48.5%, when compared to PBS-treated controls. The counting of attached yeasts was more difficult and time consuming when the higher yeast concentration was used, as some BEC had as many as 130 bound yeasts. A yeast concentration of $1x10^7$ was adopted for all subsequent adhesion-inhibition assays.

Data from King *et al.* (1980) indicates that the relationship between ratio of yeast cell to epithelial cell in adhesion assays is not linear. They found that yeast-yeast co-adherence was more common when higher yeast : epithelial cell ratios were employed. This was apparently due to the attachment of unbound yeasts to those already bound to the epithelial cells, and not a result of yeast aggregation in solution. Yeast-yeast co-adherence could inflate the adhesion values obtained for the assay. High numbers of attached yeasts are difficult to count and could result in an underestimate of yeast numbers.

1.1.3 Distribution of bound yeasts among BEC

The number of yeasts attached to individual BEC can vary greatly in *in-vitro* adhesion-inhibition assays. A large number of BEC appear to have no yeasts attached while some have their entire surface covered in yeasts. Sandin *et al.* (1987) performed adhesion assays with *Candida* and BEC in the ratio of 10:1 and found that 41% of BEC had no yeasts attached. They also observed that only 12% of BEC had 9 or more attached yeasts and that 52% of the total yeasts were attached to this 12% of cells. In the present study, a similar experiment was performed which examined the distribution of bound yeast on BEC at two different yeast-BEC ratios. At a yeast-BEC ratio of 50:1, 33.7% of BEC

had no yeasts attached and only 13.9% of BEC had 9 or more attached yeasts. However, 46% of the total yeasts were attached to this 13.9%. These results are comparable to the results of Sandin *et al.* (1987) and are shown in Table 1.3. When the yeast-BEC ratio was increased to 250:1, only 21.1% of BEC had no yeasts attached and 32.9% of BEC had 9 or more attached yeasts. Sandin *et al.* took their study a stage further by examining the size of BEC cells and assessing the number of yeasts bound to four size subgroups. The subgroups had size ranges 0-35, 36-70, 71-105, and > 106μm. The most abundant size of BEC was 36-70 μm, making up 73% of the total population. The 71-105 μm group represented 24% and the 0-35 and > 106μm groups made up < 3 and < 1 % respectively. The 36-70 μm group had the highest average number of attached yeasts per BEC at 3. Both the 0-35 and 71-105 μm groups had an average of 2.7 attached yeasts. The > 106 μm averaged only 2.1 yeasts per BEC. They suggested that the differences between sub-populations could be attributed to different levels of receptor expression and/or the presence of endogenous bacteria.

Oral thrush and acute atrophic candidiasis are associated with oral antibiotic therapy, suggesting that endogenous bacteria normally inhibit the attachment of pathogenic yeasts to the oral mucosa. Cox (1983) studied the adherence of *C. albicans* to BEC in children undergoing antibiotic therapy. Adherence was tested daily in 15 initially noncolonised, previously healthy children receiving antibiotics. The number of adherent background bacteria decreased significantly on the first day of narrow- or broad-spectrum antibiotic therapy. *C. albicans* adherence increased significantly on the second day and oral colonisation with *C. albicans* occurred on the third day of therapy.

The buccal mucosa is composed of stratified squamous epithelium. BEC are initially plump cuboidal cells with rounded upper ends. Above this basal layer are additional layers of irregularly polyhedral cells. BEC migrate towards the free surface of the mucosa as new cells are formed beneath them (Fawcett, 1986). As they migrate, the cells become more differentiated and cell surface receptors, such as blood group antigens, undergo chemical changes brought about by glycosylation enzymes (Vedtofte *et al.*, 1984). They also become more flattened and appear larger due to this shape change. They

are eventually colonised by endogenous bacteria when access is made possible by the sloughing off of the cells immediately above them. They undergo further flattening before they themselved are sloughed off. This neatly explains the results obtained by Sandin *et al.* (1987). The smallest cells, that probably originated from the deepest epithelial layer (present only in small numbers and not fully differentiated), had quite a few yeasts attached. These cells are unlikely to have been colonised by bacteria. However, because they were less mature, they will have had few receptors. The larger cells (most abundant and fully developed) had the most attached yeasts. Only very few bacteria will have been able to colonise these cells, and so a large number of receptors would be available. The closer the cells get to the surface of the mucosa, the larger (flatter) they become, and the greater the chance of bacterial colonisation. Bacteria are difficult to remove from BEC prior to adhesion assays and so the larger, more superficial cells exhibited relatively low yeast adhesion.

1.1.4 Effect of papain degradation on the adhesion-inhibition activity of EP

McCourtie and Douglas (1985) and Critchley and Douglas (1987a) were able to inhibit the adhesion of *C. albicans* to BEC by pretreatment of the BEC with EP derived from culture supernatants of the yeast grown in medium rich in galactose. This finding suggested that the EP (composed primarily of mannoprotein) contained an adhesin. EP subjected to heat, chemicals, and enzymes was examined for blocking activity. Critchley and Douglas, 1987a). Heat (60^{o}C, 15min), dithiothreitol (DTT), mild acid, and the proteases chymotrypsin, bromelain, and pronase significantly destroyed the blocking ability of EP. However, periodate and α-mannosidase had no effect on this ability. This suggests that the protein part of the mannoprotein is important in mediating adherence.

Earlier work had demonstrated that treatment of whole cells with proteolytic enzymes (Sobel *et al.*, 1981) and reducing agents (Lee and King, 1983) such as β–mercaptoethanol and DTT caused a reduction in yeast adhesion. Interestingly, Critchley and Douglas (1987a) demonstrated that papain treatment of EP appeared to enhance its ability to inhibit adhesion. This effect was attributed to the possible production of peptide

fragments that could more easily bind to BEC receptors and hence block adhesion. Papain is a cysteine protease i.e. it has an essential cysteine residue at its active site that must be maintained in the reduced form for activity (Butler, 1989). However, papain was previously used to digest EP under non-reducing conditions (Critchley and Douglas, 1987a; Tosh and Douglas, 1992).

In this study, EP was treated with papain under both reducing and non-reducing conditions (Table 1.4). EP treated with the enzyme under non-reducing conditions retained its ability to inhibit adhesion whereas EP treated with papain under reducing conditions was found to have lost this activity. It appears that the use of papain to degrade EP under non-optimal conditions in previous studies resulted in the production of EP fragments (possibly peptides) that were capable of binding to epithelial cell receptors more efficiently than untreated EP. When papain is used under reducing conditions, the adhesion-inhibition activity of EP is abolished, presumably, because of extensive damage to the protein portion of the molecule. This result confirms the importance of the protein portion of the candidal mannoprotein in the adhesion process.

2. HPLC ANALYSIS OF EP

Previous work in this laboratory has examined the biochemical properties of EP. The search for the component(s) in EP that is responsible for adhesion-inhibition has involved the use of a number of chemical and enzymic treatments. In this study, EP was subjected to some of the treatments previously found to have an effect on its adhesion-inhibition activity, prior to analysis by reverse-phase high performance liquid chromatography (RP-HPLC). This HPLC system is commonly used to resolve and prepare products of proteolysis for further analysis.

The column used in this study contained a hydrophobic stationary phase octadecyl (C18) silica. The solvent system employed was a gradient of acetonitrile, in a weak aqueous solution of trifluoroacetic acid (TFA), an ion-pairing agent which also acts to maintain protein solubility. The majority of proteins and peptides adsorb to the stationary phase under these conditions and are subsequently eluted by decreasing the polarity of the

mobile phase. This is achieved by the slow introduction of a water miscible organic solvent (HPLC grade acetonitrile). Eluted proteins and peptides are detected by U.V. absorbance. At a wavelength of 280nm, only proteins and peptides that contain aromatic residues will be detected. However, at wavelengths of between 200-230nm, the peptide bond absorbs light and this range is usually very sensitive. Detection sensitivity at 280 nm and 208 nm was examined. The absorbance values obtained for 280nm were very poor so all subsequent HPLC analyses were carried out at 208 nm.

On each occasion the HPLC machine was to be used, a number of steps were taken to prepare the column and to ensure that the solvent was of a suitable quality. The HPLC pumps were flushed with HPLC-grade water then HPLC-grade acetonitrile (both degassed). The column was attached and flushed with HPLC-grade water then HPLC-grade acetonitrile. The absorbance was monitored while the column was primed with acetonitrile-TFA and, once a stable baseline was achieved, a blank run was started. This consisted of loading a 200µl sample of acetonitrile-TFA, to check for injection effects, and also to monitor the effect of the acetonitrile gradient on absorbance. The blank run was followed by the sample runs. Periodically, insulin was loaded as a sample to check the efficiency of the column and detection system. A fine sharp peak was always observed (Fig.2.1 C).

EP from *C. albicans* strain GDH 2346 was subjected to HPLC analysis. This run indicated that there are a large number of distinct proteins present in EP from this strain, with varying degrees of hydrophobicity. Four major peaks and a number of smaller peaks were seen. Similar traces were obtained each time a sample of untreated EP was run.

In a recent study, Hazen and Hazen (1992) used hydrophobic interaction (HIC)-HPLC to examine cell wall proteins extracted from both hydrophobic and hydrophilic *C. albicans* cells by β1-3 glucanase digestion. Both HIC-HPLC and RP-HPLC separate proteins on the basis of their surface hydrophobicity. Proteins are eluted from RP-HPLC columns using organic solvents whereas proteins are eluted with descending salt gradients from HIC-HPLC columns. The most striking difference observed observed by Hazen and Hazen between the surfaces of hydrophobic and hydrophilic cells was in the large amount

of relatively hydrophilic protein derived from the surface of hydrophilic cells in comparison with hydrophobic cells. The amounts of hydrophobic proteins seen in the two cell types were similar. It was concluded that hydrophobic proteins are present on both cell types. However, in hydrophilic cells, hydrophobic proteins are masked by the hydrophilic proteins resulting in cells that are hydrophilic. *C. albicans* cell-surface hydrophobicity influences both adhesion and virulence of this organism and it was suggested that a simple mechanism, such as decreased glycosylation, may result in changes in cell-surface-hydrophobicity. Hazen and Glee (1994) took these results a stage further. They examined the glycosylation level of three hydrophobic cell wall proteins and one hydrophilic protein derived from the surface of yeast cells. They found that the hydrophobic proteins were glycosylated to a far lesser extent than the hydrophilic proteins and suggested that changes in glycosylation may determine exposure of hydrophobic protein regions at the cell surface. The effect of deglycosylation on the hydrophobicity of EP components was examined in the present study.

The enzyme N-glycanase cleaves the N-glycosidic bond between N-acetyl-D-glucosamine and asparagine on the protein backbone of glycoproteins, without damaging peptide bonds (Tarentino *et al.*, 1985). Previous work by Tosh and Douglas (1992) had shown that N-glycanase treatment of EP enhanced the adhesion inhibition activity of EP. This enzyme has also been used in the preparation of deglycosylated glucomannoproteins from *Saccharomyces cerevisiae* (Van Rinsum *et al*, 1991). Two samples of EP were prepared: one was treated with the enzyme, the other was treated under the same conditions but in the absence of the enzyme. A third sample of enzyme alone was included as a further control. The HPLC trace of N-glycanase-treated EP (Fig. 2.2 A) was not greatly different from the trace of EP incubated under the same conditions but in the absence of N-glycanase. The four major peaks were still visible but a number of new peaks could also be seen. The first two peaks appeared split in two and there followed a new small sharp peak. The largest peak appeared unchanged as did the final sharp peak. A control HPLC run with N-glycanase alone produced a trace that was not visibly different from that of the blank run. These results suggest that the N-glycanase does not have a

great effect on the RP-HPLC retention of most EP components but that certain visible changes are apparent. Some of these changes may be associated with the production of more adhesive fragments that have greater adhesion-inhibition activity than the parent EP molecules.

The second experiment on EP degradation involved the treatment of EP with NaOH. Base-labile oligosaccharides linked to the protein through serine or threonine residues are removed by the action of NaOH (Ballou, 1976). Mild alkali was previously found to enhance the ability of EP to inhibit adhesion of *C. albicans* to BEC (Critchley and Douglas, 1987a). The optimum conditions for this effect were determined by Tosh (1991). He found that 0.1M NaOH produced maximal inhibitory effect. Van Rinsum *et al.* (1991) performed some work on the breakdown of glycoproteins from the cell wall of *Saccharomyces cerevisiae* by enzymic and chemical means They employed both N-glycanase and NaOH treatments to remove all traces of carbohydrate from the protein backbone. In the present study, NaOH treatment was observed to have a profound effect on the HPLC trace of EP when compared with a trace of EP incubated under the same conditions but with PBS in place of NaOH. In general, there was a clear shift from a relative abundance of hydrophilic proteins to more hydrophobic proteins. This change in the proportion of hydrophilic components could be consistent with the removal of oligosaccharide chains from Ser and Thr residues on the protein backbones of the glycoproteins. This is likely to have resulted in the exposure of hydrophobic regions of the protein that were previously unavailable for interaction with the hydrophobic column packing. NaOH treatment may also produce some protein degradation. Van Rinsum *et al.* (1991) employed both N-glycanase and NaOH treatment on *S. cerevisiae* proteins and were able to remove all traces of carbohydrate from the protein backbone when both the treatments were used in conjunction. In their study, the NaOH was used at a concentration of 0.1 M and the reaction mixture was incubated at 20^{o}C for 24H. They found that longer incubation times or higher temperatures resulted in protein damage. In the present study, the conditions used were similar to those employed by Van Rinsum *et al.* (1991); however, the temperature of incubation employed was 30^{o}C. This higher temperature may

have resulted in some protein degradation. Previous work by Tosh and Douglas (1992) found that this higher temperature was of use in the preparation of adhesion-blocking material from EP. It is probable that if protein was being damaged by the action of NaOH, the treatment was not damaging to those protein fragments that are able to block adhesion, and may even aid their production. Van Rinsum *et al.* (1991) found that approximately 70% of the mannose residues on *S. cerevisiae* mannoproteins were O-glycosidically linked to the protein. They also found that the removal of O-linked carbohydrate chains had no effect of the ability of N-glycanase to act on N-linked chains. This suggests that N-glycanase is not sterically hindered by O-linked carbohydrate chains and can be used to treat proteins prior to β-elimination by NaOH.

The third experiment on EP degradation involved the treatment of EP with papain. Previous work by Critchley and Douglas (1987a) showed that a number of proteases, chymotrypsin, bromelain, and pronase, significantly decreased the ability of EP to inhibit adhesion. This suggested that the protein part of the mannoprotein is important in adhesion-inhibition. Interestingly, the protease papain or dilute alkali treatment of EP appeared to enhance its ability to inhibit adhesion. When these two treatments were combined, substantially greater inhibition of adhesion was observed. This increased inhibition was attributed to the production of peptide fragments which could bind epithelial cell receptors more easily than the parent molecule. An experiment was performed in order to examine the effect of papain treatment (under non-reducing conditions) on EP. The HPLC trace of papain-treated EP was considerably different from the trace of EP incubated under the same conditions but in the absence of papain. The EP mannoproteins appeared greatly fragmented with a large number (19) of sharp peaks, supporting the theory of peptide production. Most of the fragments produced were of relatively high hydrophobicity compared to the trace of EP not exposed to papain. A control HPLC run with papain and α_2-macroglobulin alone produced a trace that was not greatly different from that of a blank run. A small peak was observed which was most likely to be of papain-α_2-macroglobulin complexed. The corresponding region on the EP, papain and α_2-macroglobulin trace shows no sign of this complex due to the presence of

large amounts of EP digestion products. As was mentioned previously, papain is a cysteine protease i.e. it has an essential cysteine residue at its active site that must be maintained in the reduced form for activity (Butler, 1989). However, papain was previously used to digest EP under non-reducing conditions (Critchley and Douglas, 1987a; Tosh and Douglas, 1992). It appears that the use of papain to degrade EP under non-optimal conditions, as in previous studies, results in the production of EP fragments (possibly peptides) that are capable of binding to epithelial cell receptors more efficiently than untreated EP. When papain was used to degrade EP under reducing conditions, the adhesion-inhibition activity of EP was abolished, presumably because of extensive damage to the protein portion of the molecule.

The above mentioned treatments have been used as the basis of a purification protocol for the yeast adhesin. The first stage of the protocol involves the sequential treatment of EP with N-glycanase, papain and dilute alkali to cleave the protein and carbohydrate portions of the mannoprotein (Tosh and Douglas, 1992). The combined effect of the above treatments on EP were examined. A number of differences were seen between this HPLC trace (Fig. 2.5A) and that of EP incubated under the same conditions but in the absence of N-glycanase, papain, and NaOH (Fig. 2.5B). Tosh and Douglas (1992) showed that the combined treatment of EP with N-glycanase, papain and mild alkali produced an EP digest that was able to inhibit adherence by a far greater extent than untreated EP. A control HPLC run with N-glycanase, papain, α_2-macroglobulin and NaOH produced a trace that was slightly different from that of a blank run (Fig. 2.5 C). A small peak was seen and some smaller, less defined peaks were eluted soon after. These peaks indicate that NaOH had an effect on the papain and α_2-macroglobulin complex that had been seen in Fig. 2.4C. The corresponding region on the EP, N-glycanase, papain, α_2-macroglobulin, NaOH trace shows no sign of this complex due to the presence of large amounts of EP digestion products. Clearly the combined treatment of EP with the above reagents results in the production of a number of fragments.

The full purification protocol for the yeast adhesin described by Tosh and Douglas (1992) has, as its final step, an affinity adsorption with H-2 blood group substance

immobilised on a silica matrix. Tosh and Douglas (1992) obtained a single peak for the adhesin on fast high pressure liquid chromatography (FPLC). This indicated that the adhesin was a single component. Attempts in the present study to repeat this result on HPLC proved unsuccessful.

3. THE *CANDIDA ALBICANS* ADHESIN

3.1 The search for adhesive components in *C. albicans* mannoprotein

Most of the major cell wall components of *C. albicans*, including chitin (Lehrer *et al.*, 1983) and lipids (Ghannoum *et al.*, 1986) have been implicated as adhesins. However, the majority of reports indicate that surface mannoprotein acts as the adhesion molecule (Calderone and Braun, 1991). As mentioned previously, the adhesion of *C. albicans* to buccal epithelial cells (BEC) has been shown to be inhibited by pretreatment of the BEC with extracellular polymeric material (EP) derived from culture supernatants of the yeast grown in medium rich in galactose (McCourtie and Douglas, 1984; Critchley and Douglas, 1987a). These finding suggest that EP (composed primarily of mannoprotein) contains an adhesin for epithelia.

A considerable amount of evidence suggests that the protein part of the mannoprotein is important in mediating adherence. As mentioned earlier, pretreatment of EP with heat, DTT, and proteolytic enzymes (except papain) significantly reduced the blocking ability of EP, whereas pretreatment with sodium periodate and α–mannosidase had no effect on this ability (Critchley and Douglas, 1987a). Interestingly, the protease papain or dilute alkali treatment appeared to enhance the ability of EP to inhibit adhesion and when these two treatments were combined, substantially greater inhibition of adhesion was observed. In a related study, possible receptor molecules were identified (Critchley and Douglas, 1987b). By the incorporation of lectins and sugars in adhesion-inhibition assays, it was found that most strains tested were primarily inhibited by L-fucose and *Lotus tetragonolobus* lectin (L-fucose specific). One other strain tested was found to be inhibited by N-acetylglucosamine and wheat germ agglutinin. These results suggested that the protein portion of yeast cell-surface mannoproteins may bind in a lectin-like manner to

glycosides containing L-fucose or N-acetylglucosamine expressed on epithelial cell-surface glycoconjugates.

L-Fucose is found on epithelial cell surfaces as a component of blood group antigens on glycoproteins or glycolipids. The interaction between *C. albicans* and host cell L-fucose residues may be of great benefit to the organism in the colonisation of epithelial surfaces, but at other body sites, this interaction may be to the organism's detriment. Sawyer *et al.* (1992) examined the effect of lectins on hepatic clearance and killing of *C. albicans* by the isolated perfused mouse liver. When *Ulex europeaus* lectin (binding specificity for L-fucose) was included in preperfusion buffers, hepatic trapping of *C. albicans* was decreased by 37%. The lectin also eluted trapped *C. albicans* from the liver when added to postperfusion buffer. By comparison, treatment of *C. albicans* with *U. europeaus* lectin before infusion had no effect on the trapping or killing of yeast cells. These results suggest that a fucose-containing receptor on the surface of either sinusoidal endothelial cells or Kupffer cells is involved in the trapping of *C. albicans* by the perfused mouse liver.

3.2 Purification of the yeast adhesin

A purification scheme for the isolation of the fucoside-binding adhesin of *C. albicans* was described by Tosh and Douglas (1992). The method made use of the findings of the above mentioned experiments on adhesion-inhibition activity of EP treated with various enzymes and chemicals (Critchley and Douglas, 1987a), combined with a knowledge of receptor specificity derived from work on lectin and sugar blocking studies (Critchley and Douglas, 1987b). The purification protocol involves the stepwise treatment of EP with N-glycanase, papain, and dilute alkali followed by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen on Synsorb silica beads (Fig. 3.1). Adhesin purified in this way is devoid of carbohydrate, can inhibit adhesion of yeasts to BEC by up to 80%, and consists of one major component as determined by reverse-phase fast protein liquid chromatography (FPLC).

Synsorb affinity matrices have also been used to purify L-fucose-binding adhesins from other microbes. Quinn *et al.* (1993) described the isolation of possible L-fucose-binding adhesins from *Aeromonas hydrophila* using affinity matrices similar to those used in this, and previous, studies on *C. albicans* adhesins. They purified carbohydrate-reactive outer membrane proteins (CROMPs) by affinity chromatography on two different affinity matrices composed of trisaccharides resembling H type 1 and 2 antigens. Reactive proteins were successfully eluted under alkaline conditions (pH 11.0) and in the presence of soluble H substance prepared from group O secretor saliva, but not by 60 mM α-L-fucose or under acid conditions (pH 3.0). The eluate contained at least three components (of molecular mass 43, 40, and <14 kDa) as detected by immunoblot analysis. In this study, the bound proteins were eluted from the H-2 affinity adsorbent under alkaline conditions.

Synsorbs have also been used to purify L-fucose-binding adhesins from *Staphylococcus aureus* (Saadi *et al.*, 1994). Tosh (1991) examined the ability of EP from three *C. albicans* strains to bind to four different oligosaccharide affinity matrices. The adsorbents used were H-1, H-2, Lewis^{a} and Lewis^{b}. It was found that EP from GDH 2346 was bound to a similar degree by H-2, Lewis^{a} and Lewis^{b} adsorbents but relatively poorly by H-1. However, the material eluted from H-12 adsorbent was found to have the highest adhesion-inhibition activity and so the H-2 adsorbent was incorporated into the adhesin purification protocol.

The purification protocol for the yeast adhesin developed by Tosh and Douglas (1992) was used throughout this study. One modification to the procedure was the use of a 3.5 kDa Amicon ultrafiltration filter in place of the 5 kDa filter employed previously. When protein and carbohydrate analyses were performed on the purified adhesin, it was found to be devoid of carbohydrate. This is consitent with the findings of Tosh and Douglas (1992). To ensure that the purified adhesin prepared in this study was of the same activity as that obtained previously, BEC were treated with the new material prior to adhesion assays with *C. albicans* GDH 2346. This material inhibited adhesion by up to 74.81% and the adhesion inhibition index value (AII), which is a measure of the ability of EP or its derivatives to block adhesion, was calculated to be 178 (Table 3.1). These results

are comparable with those obtained by Tosh and Douglas (1992). The lower adhesion-inhibition values of this study is most likely due to different BEC donor. In the previous study, the purified adhesin produced one major peak when subjected to fast high pressure liquid chromatography (FPLC). This indicated that the adhesin was a single component. Attempts to repeat this result on HPLC proved unsuccessful.

The protease inhibitor, human plasma α_2-macroglobulin (Sigma), is normally used to inactivate papain after incubation of EP with papain. It is a 772 kDa protein that contains four near identical disulphide linked polypeptide chains and inhibits proteases by entrapment in a cleft called the bait region. The protease is then covalently anchored by transacylation (Neurath, 1989). There are two possible problems associated with the use of α_2-macroglobulin. One problem is that α_2-macroglobulin may not completely inhibit the activity of papain (Salvesen and Nagase, 1989). The binding of proteases to α_2-macroglobulin is irreversible but the complexed enzyme retains full activity against small substrates such as peptides. It is inactive against substrates greater than 30 kDa but the purified adhesin has an estimated molecular weight of 15.7 kDa which is theoretically susceptible to further proteolytic degradation. Another problem relating to the production of purified adhesin has recently come to light. Matsui *et al.* (1993) identified ABO(H) blood group structures on human plasma α_2-macroglobulin. They employed blood group-specific monoclonal antibodies and *Ulex europaeus* lectin I in Western blotting and sandwich enzyme-linked immunosorbent assays. Direct sequence analysis of one of the blood group antigen-containing proteins identified it as α_2-macroglobulin. This was confirmed by the use of anti-α_2-macroglobulin. The presence of a protein that contains blood group antigens may cause problems when trying to isolate H-2 blood group antigen-binding components from the degradation mixture of EP.

3.3 Amino acid composition of the yeast adhesin

Amino acid analysis was performed on the purified adhesin by Dr. M. Cusack in the Dept. of Geology and Applied Geology, University of Glasgow. A sample of purified adhesin was prepared by the normal procedure. A total of 18 μg of material was used for

amino acid analysis. A buffer blank sample that had undergone the entire purification protocol was used to adjust the background on the sample run. The results obtained are shown in Fig. 3.3. The purified adhesin was found to be mainly composed of the amino acids Thr, Pro, Ala, Ser, Val, Gly, Asx and Glx, which made up around 80% of the total amino acids. No Cys was detected and very little Met, Phe or Arg was present. This composition is remarkably similar to a large number of microbial adhesins (see below) and lectins, including the fucose binding lectins (reviewed in Goldstein and Poretz, 1986). This would appear to indicate a relationship between the amino acid composition of lectins and lectin-like proteins and their function as carbohydrate binding molecules. The composition is also consistent with the adhesin being a glycoprotein that would have carbohydrate chains linked to the protein backbone via serine, threonine and asparagine residues.

In a recent study by Yu *et al.* (1994a), a fimbrial adhesin of *C. albicans* was partially characterised. Its composition differed from the adhesin described in this study in that it contained approximately 15% protein (the remainder being D-mannose) and had a molecular mass of around 66 kDa. The molecular mass of the protein portion was assumed to be about 8.6 kDa. Amino acid analysis of the fimbrial adhesin indicated that the most abundant amino acids were Val, Asx, Glx, Ser, Thr, Gly, and Ala, all of which are in abundance in PA. Another similarity was the very small amount of Met in both analyses. The most striking difference between the two compositions was in the amount of Pro. This was only a minor constituent in the fimbrial adhesin but was the second most abundant amino acid in PA. Amino acid sequence comparison is required to determine if the two adhesins are in any way related.

In a further study, Yu *et al.* (1994b) investigated the mechanism of adherence of *Pseudomonas aeruginosa* and *C. albicans* to asialo-GM_1. Their results suggest that common cell surface receptors are recognised by the *P. aeruginosa* and *C. albicans* adhesins because of a conserved receptor-binding domain on their adhesins. By using monoclonal antibodies (MAb) against purified pili of *P. aeruginosa* and monospecific anti-peptide antibodies against the PAK pilin peptides (anti-PAK 128-144 and anti-PAK 134-140), they demonstrated that the antibodies agglutinated *C. albicans* whole cells and

cross-reacted with *C. albicans* fimbriae in immunoblots. A control antibody, PKL1, and anti-PAK 75-84 peptide antibodies failed to agglutinate *C. albicans* whole cells or cross-react with the fimbrial proteins. Conversely, the anti-*C. albicans* fimbrial MAb Em16 agglutinated *P. aeruginosa* whole cells. *P. aeruginosa* and *C. albicans* are evolutionarily distant organisms but they appear to share a portion of adhesin molecule. Again, sequencing of the *C. albicans* adhesin is required to confirm the similarity at the molecular level.

Some bacterial adhesins have amino acid compositions that have similarities with the purified adhesin described in this study. Kist *et al.* (1990) described the purification and characterisation of some fibrillar hemagglutinins expressed by two uropathogenic *Escherichia coli* strains. The haemagglutinins were mechanically sheared from whole cells and then purified by anion-exchange high-pressure liquid chromatography. The amino acid compositions of the two haemagglutinins were highly similar to each other and had a number of similarities with the *C. albicans* adhesin described in this study (Table 3.2). Sajjan and Forstner (1992) identified a *Pseudomonas cepacia* fimbrial mucin-binding adhesin. The strains tested were isolated from patients with cystic fibrosis. The amino acid composition of the 22-kDa protein was similar to the *C. albicans* adhesin. Compositional similarities with the *C. albicans* adhesin are not limited to microbial proteins. Allen *et al.* (1991) isolated and characterised galaptin, a β-galactoside-binding lectin from human peripheral leukocytes, by affinity chromatography. The galaptin showed the presence of a single polypeptide when subjected to RP-HPLC. The subunit molecular mass was 14.5 kDa and its amino acid composition was again similar to that of the *C. albicans* adhesin.

Not all carbohydrate binding proteins share the compositional similarities outlined above. There are a number of lectins and lectin-like proteins, of both plant and microbial origin, that are rich in cysteine. Wright *et al.* (1991) studied a family of GlcNAc binding proteins containing the disulfide-rich domain of wheat germ agglutinin. This 43-residue domain includes eight disulphide-linked cysteines and has been implicated in the binding of GlcNAc and its polymers.

Tannich *et al.* (1991) examined the interaction of a galactose-binding surface lectin from the pathogenic amoeba *Entamoeba histolytica* with colonic mucins and host cells. The extracellular portion of the lectin was found to contain a cysteine-rich domain which shows a degree of sequence homology to wheat germ agglutinin. Gillin and Diamond (1981) suggested that the cysteine-rich surface proteins may be important for survival in the harsh environment of the gut. Saukkonen *et al.* (1992) examined pertussis toxin (PT) carbohydrate recognition domains. *Bordetella pertussis* binds to glycoconjugates on human cilia and macrophages by multiple adhesins, including PT. Comparison of the peptide sequences of the carbohydrate binding regions of PT to those of plant and animal lectins revealed that regions essential for function of the prokaryotic lectins are strongly related to a subset of eukaryotic carbohydrate recognition domains. Of particular interest was the effect of amino acid substitutions on receptor specificity of the PT. Substitution of certain residues resulted in a shift of carbohydrate recognition from lactosylceramide to gangliosides. Mutational exchange of amino acid residues between two domains interchanged their carbohydrate and target cell specificity. This gives an indication of a possible mechanism by which organisms can alter their tissue specificity by means of a mutation at an adhesin's carbohydrate binding site.

3.4 Analysis of EP and purified adhesin by SDS-PAGE

Previous attempts in this laboratory to visualise EP and purified adhesin by SDS-PAGE have met with mixed success. Critchley (1986), was unable to visualise EP after SDS-PAGE using either Coomassie blue R250 or silver staining. However, Tosh (1991) visualised EP components using both staining procedures. Indeed, when EP prepared in earlier studies was subjected to SDS-PAGE along side EP that Tosh had prepared, similar protein components were seen. This suggests that the SDS-PAGE procedure used by Critchley was in some way flawed.

Tosh (1991) made a number of unsuccessful attempts to visualise purified adhesin by SDS-PAGE, using both Coomassie blue R250 and silver staining. A number of factors

may have contributed to this failure. The adhesin may have been lost from the gel during the staining procedures. The acid-alcohol solutions used in Coomassie blue staining do not completely fix proteins in the gel which can result in the loss of some low molecular weight proteins (Garfin, 1990). This, however, does not explain the failure of silver staining. A more likely explanation is that the Laemmli gel system employed by Tosh (1991) is not ideally suited to the separation of small proteins and peptides. The stacking limit for large proteins in the Laemmli system lies far beyond 100 kDa, but proteins below 20 kDa are only partly or not at all separated from the bulk of SDS (Schagger and Von Jagow, 1987). This property causes streaking of small proteins and so proteins present in small amounts are less likely to stain well. Moreover, the amino acid composition of the adhesin may be a contributing factor in the failure of conventional protein stains. Certain types of synthetic peptides are undetectable with both Coomassie blue R250 and silver staining (Wallace and Saluz, 1992). The peptides that exhibit this property are rich in Asp, Ala, and Pro and are relatively poor in Cys, Met, and aromatic amino acids. (Andrew Wallace, personal communication). The amino acid composition of the purified adhesin fits the above description of a peptide likely to stain poorly by conventional means.

Two SDS-PAGE systems were employed in this study: the procedures of Laemmli (1970) and of Schagger and Von Jagow (1987). Initial attempts to visualise the purified adhesin used the Laemmli procedure. Coomassie blue staining (Weber and Osborne, 1969) and silver staining (Oakley *et al.*, 1980) were then used to visualise separated proteins after electrophoresis. As was found previously by Tosh (1991), purified adhesin could not be visualised in this way.

The second SDS-PAGE system, described by Schagger and Von Jagow (1987), was then employed in an attempt to visualise purified adhesin. This system is able to resolve proteins and peptides in the range 2.5 kDa-17.0 kDa, and because of the incorporation of tricine in place of glycine in the running buffer, there is no possibility of glycine contamination in subsequent sequencing analysis. A number of attempts were made to visualise the purified adhesin using both Coomassie blue staining and silver staining but without success. An alternative approach to protein visualisation was required.

In a previous study, Sweet and Douglas (1991) examined the cell-surface composition of radio-iodinated whole cells from strain GDH 2346 grown in different concentrations of iron. The isolated cell wall mannoproteins were subjected to SDS-PAGE and then visualised by autoradiography. A number of protein components were resolved by this procedure.

In this study, an experiment was performed in which EP was first labelled with ^{125}I using the Iodogen procedure (Fraker and Speck, 1978) and then subjected to the purification protocol for the yeast adhesin. Samples were removed after each step of the procedure to monitor the effects of the various chemicals and enzymes on EP. Five samples were taken in total and were run together on SDS-PAGE using the procedure of Schagger and Von Jagow (1987). The lanes with molecular weight standards were cut off and stained with Coomassie blue. With this gel system, small proteins and peptides within the range 2.5 kDa-17.0 kDa should appear as distinct bands but proteins of higher molecular weight are likely to be bunched towards the top of the separating gel. The autoradiograph is shown in Fig. 3.4. Untreated EP appeared as three distinct bands. Two strong bands were seen at the very top of the gel, one a short way into the separating gel, the other at the junction of the separating gel and the stacking gel. The third band which was less intense than the other two, was seen in the region of the 3.5 kDa marker. The lane containing N-glycanase treated EP was not remarkably different from the untreated EP lane except for a noticeable reduction in the intensity of the band at the junction of the separating gel, and the stacking gel. The lane containing N-glycanase- and papain-treated EP showed only one clear band a short way into the separating gel. There was also a diffuse streak of ^{125}I-labelled material visible in the region of the 2.5 kDa marker. The lane containing N-glycanase-, papain-, and NaOH-treated EP had only one band a short way into the separating gel. There was also a diffuse streak of ^{125}I-labelled material running the entire length of the gel. No ^{125}I-labelled material was visible in the lane that should contain purified adhesin (the N-glycanase-, papain-, NaOH-treated EP and bound to Synsorb H-2). This could have been a result of insufficient ^{125}I-labelled material at this point in the degradation. Another possibility is that iodination may have damaged the adhesin

molecules and prevented binding to the Synsorb matrix. Between pH 7 and 8, the ortho position in the aromatic ring of tyrosine in peptides and proteins is activated for electrophilic attack, due to the electron-donating effect of the neighbouring hydroxyl group. Similarly, at pH 9, the the two nitrogen atoms in the imidazole ring of histidine have an electron donating effect on their neighbouring carbon atom (Bolton, 1977). So, iodine ($^{125}I^{+}$) acts as the electrophilic agent to produce the radiolabelled protein. Direct radiolabelling with iodine is a very sensitive method, but it can change the physical and biological properties of proteins (Wallace and Saluz, 1992).

Although iodination of protein is a relatively simple procudure, the use of ^{125}I-labelled proteins was problematic. The handling of labelled material, and the subsequent steps involving SDS-PAGE, produced large amounts of contaminated waste and equipment. Also, the ionination procedure may adversely affect the integrity of the proteins and is relatively inefficient. An alternative labelling procedure, biotinylation, was used in subsequent experiments. Biotinylation has been used by Casanova *et al.* (1992) to label cell wall proteins from yeast and mycelial cells of *C. albicans*. They demonstrated that the labelling of intact, living *C. albicans* cells with biotin allows characterisation of cell surface proteins and glycoproteins following SDS-PAGE and Western blot analyses. Casanova *et al.* (1992) detected proteins on nitrocellulose by incubation with Extravidin-peroxidase conjugate (Sigma). In this study, ^{125}I-streptavidin was used in an attempt to maximise the sensitivity of the procedure. Biotinylation allows for the safe handling of proteins right up to the point of detection with ^{125}I-streptavidin. As this is the final step prior to autoradiography, the amount of radioactive waste produced is far less. Also, the total activity of the material required is greatly reduced.

In the first experiment of this type, EP was biotinylated using a commercial kit from Amersham. Biotinylated EP and biotinylated SDS molecular weight standards (SDS-6B, Sigma) were separated using the Laemmli procedure on a mini gel apparatus. After electrophoresis, the separated proteins were transferred on to nitrocellulose and biotinylated material was detected by probing with ^{125}I-streptavidin followed by autoradiography (Fig. 3.5). Four bands were visible in the biotin-EP lanes. An intense

band was visible in the region of the 97.4 kDa marker and a broad diffuse band was seen in the region of the 39.8 kDa marker. Two additional bands were seen in the region between the 20.1 and 14.3 kDa markers but these were relatively faint. Any other bands present were obscured by the streak of biotinylated material that ran the length of the gel. Sweet and Douglas (1991) examined ^{125}I-proteins released from the surface of GDH 2346 cells grown in medium with varying iron concentrations, by boiling the cells in the presence of SDS and DTT, prior to SDS-PAGE. It is not possible to directly compare the result shown in Fig. 3.5 with the result of Sweet and Douglas (1991) due to the different growth conditions and method of purification of the cell surface proteins. However, some proteins visualised in both studies appear similar. Sweet and Douglas (1991) identified five main protein components in cells grown under reduced iron conditions (16, 26, 39, 47, and 85 kDa). By contrast, six components appeared in increased amounts in organisms grown in high iron medium (18, 19, 35, 49, 51, and 64 kDa). In this study, four components were most dominant with approximate molecular weights of 100, 40, 18, and 16 kDa. More accurate estimation of the weights was not possible due to the intense background in the sample tracks.

EP was labelled with biotin and then subjected to the purification protocol for the yeast adhesin. Samples were removed after each step of the procedure. Three samples were taken in total, to monitor the effects of the various chemicals and enzymes on EP and were run together on SDS-PAGE using a mini gel and the Laemmli procedure (Fig. 3.6). The results obtained in this experiment were poor possibly due to insufficient biotinylation of the EP although the degree of biotinylation seemed adequate in the previous experiment (Fig. 3.5). A more likely explanation is that the degradation treatments had an adverse effect on the protein-bound biotin. The biotin may have been cleaved from the proteins or the ability of the bound biotin to bind to ^{125}I-streptavidin may have been affected.

Overall, the results of these SDS-PAGE experiments were rather disappointing, with poor band resolution a major problem. However, the experiments were valuable in demonstrating that biotin-labelling could be used as an alternative to direct radiolabelling of EP components, prior to SDS-PAGE and Western blot analyses.

Purified adhesin was biotinylated prior to SDS-PAGE on a mini-gel, using the procedure of Schagger and Von Jagow (1987). After electrophoresis, the gel was cut and the markers were stained with Brilliant Blue G (Sigma).The biotinylated purified adhesin lanes were transferred onto nitrocellulose for Western blot analysis. The protein was then visualised by probing with ^{125}I-streptavidin followed by autoradiography. The purified adhesin was seen as a strong band with a molecular mass of approximately 15.7 kDa with two possible breakdown products also visible (Fig. 3.7). This was the first time that purified adhesin had been seen by SDS-PAGE. The successful visualisation of the purified adhesin meant that partial amino acid sequencing could be attempted. In a recent study by Yu *et al.* (1994a), a fimbrial adhesin was partially characterised. Its composition differed from the adhesin described in this study in that it contained approximately 15% protein (the remainder being D-mannose) and had a molecular mass of around 66 kDa. The molecular mass of the protein portion was assumed to be around 8.6 kDa.

3.5 Amino acid sequencing of the purified adhesin

Purified adhesin was subjected to sequencing on an Applied Biosystems 477A Protein Sequencer. The sequence determined initially was :-

Ser-Arg-Asp-Leu-Ile-Ile-Gln-Phe-Lys-Asn (Amino acid sequence 1).

When this sequence was run through the FASTA program developed by Pearson and Lipman (1988), the closest homology was found to be an outer capsid protein (VP4 haemagglutinin) from human Rotavirus serotype 4 with 75% identity in an eight amino acid overlap. However, when the sequence data from the run was re-examined by Dr. Phil Jackson of Applied Biosystems, he determined that there were two distinct sequences present. The computer system attached to the sequencer had miss-read the data due to a high amount of background in the sample. The most prominent sequence seen after re-examination of the data was :-

Ser-Asp-Asp-Leu-Ser-Phe-Lys-Phe-Lys-Asn (Amino acid sequence 2),

and the second sequence was :-

Phe-Asp-Tyr-Glu-His-Val-Asp-X-Ala-Val (Amino acid sequence 3).

The amino acid at position 8 in Amino acid sequence 3 could not be determined with any certainty.

When Amino acid sequence 2 was run through the data base it had 100% homology with the first 10 amino acids at the N-terminus of the anti H (O) binding lectin from *Ulex europaeus*. As mentioned in the Results section, the experimental notes relating to the production of this particular batch of purified adhesin were carefully examined. It was found that the Synsorb H-2 affinity adsorbent used had been exposed to the purified *U. europaeus* I lectin. This had occurred during an experiment to examine the efficiency of removal of H antigen binding components from Synsorb affinity adsorbent. This suggests that the removal of Synsorb bound material in by no means complete even after prolonged NH_4OH treatment.

Amino acid sequence 3 was run through the peptide database. Although no complete matches were found for the peptide sequence there was a predominance of microbial protein homologies including quite a few yeast proteins (Fig.3.5). Among the closest matches for the peptide was the *Saccharomyces cerevisiae* regulatory protein SIR 3 (Silent information regulator) with 71.4 % identity in a 7 amino acid overlap. The partial amino acid sequence obtained is most probably an internal sequence produced as a result of enzymic cleavage by the action of papain.

Recent studies have sought to characterise and sequence adhesins isolated from *C. albicans*. Yu *et al.* (1994a) attempted to sequence a fimbrial adhesin of *C. albicans* but were unsuccessful due to N-terminal blockage of the protein. However, in a later study (Yu *et al.* 1994b), the same workers were able to demonstrate that the fimbrial adhesin was antigenically similar to a *P. aeruginosa* adhesin. Klotz *et al.* (1994) partially characterised the fibronectin adhesin of *C. albicans* which binds the fungus to subendothelial extracellular matrix and matrix proteins. The adhesin was found to be composed of 75 to 80% carbohydrate and approximately 20 to 25% protein by weight. HPLC of material eluted from a fibronectin-agarose affinity column demonstrated the presence of three components, all of which migrated as one protein of approximately 60 kDa on SDS-PAGE. Molecular weight sizing column chromatography demonstrated that

the adhesin eluted with an apparent molecular mass of 42 kDa. The N-terminus of the 60-kDa glycoprotein was blocked to Edman degradation.

A considerable amount of work remains to be done on all the reported *C. albicans* adhesins. Extensive sequence analysis should provide valuable information on the relationships between carbohydrate-binding adhesins and fibronectin-binding adhesins. Proteolytic cleavage prior to sequencing would circumvent the problem of N-terminal blockage encountered by the groups mentioned above. The cleavage can be performed prior to SDS-PAGE or HPLC. Alternatively, the in situ enzymic cleavage of proteins on nitrocellulose followed by RP-HPLC, would produce fragments suitable for gas-phase microsequencing (Aebersold *et al.*, 1987).

The next stage in the characterisation of the *C. albicans* adhesin described in this study should involve the identification of the receptor-binding domain. The molecular details of how glycoconjugate-binding proteins interact with their ligands have been revealed by a variety of techniques, as reviewed by Holt (1991). For example, proteases, chemical-modifying reagents and antibodies have been useful probes for locating lectin binding domains. Protein crystallography has shown how lectins are structured, and identified which amino acids in these proteins are positioned appropriately for bond formation with glycoconjugates. Studies of lectin mutants produced by site-directed mutagenesis, and characterisation and sequencing of naturally occurring, non-functional lectin variants have demonstrated the importance of particular amino acids for glycoconjugate binding.

The first step in this work would require the determination of the primary structure of the *C. albicans* adhesin. This could be achieved by using methods previously employed in the study of lectin binding domains. Yamamoto *et al.* (1992b) identified carbohydrate binding peptides from several blood group H-binding lectins. The peptides obtained from the endoproteinase digestion were subjected to affinity chromatography, to isolate those components with an affinity for the H-antigen, then the peptides were sequenced. A problem with this approach is that a binding portion of the adhesin may be destroyed during endoproteinase digestion. Konami *et al.* (1991) determined the primary structures

of two *Ulex europaeus* lectins by endoproteinase digestion, RP-HPLC purification and peptide sequencing. The resultant sequences were aligned to give the complete protein sequence.

This approach could be adopted in the search for the *C. albicans* adhesin binding domain. Once the complete protein sequence of the *C. albicans* adhesin is identified, synthetic peptides could be used to determine the minimum binding domain of the *C. albicans* adhesin. The multipin peptide synthesis technology developed by Geysen *et al.* (1984) could be used to produce a series of overlapping peptides that correspond to the primary structure of the adhesin. The peptides can be made a variety of lengths and could either be N-terminally biotinylated, for use in peptide binding studies, or left unbiotinylated for use in adhesion-inhibition studies. The peptide binding assay would involve mixing the biotinylated peptides with a suspension of the target cells e.g. exfoliated BEC or a cell line of interest. Unbound peptide could be removed by washing, and bound peptide probed for with a streptavidin-conjugate. This rational approach should pave the way to a better understanding of the complexity of *C. albicans* adhesion. It may also help to explain the variable results that have been obtained in sugar and lectin blocking studies.

It could be the case that the basic architecture of the *C. albicans* adhesin is similar for all strains with only a few differences at the carbohydrate-binding domains, resulting in different glycoside specificities. Saukkonen *et al.* (1992) demonstrated that amino acid substitutions at the carbohydrate binding site of *B. pertussis* toxin resulted in altered glycoside specificity. Substitution of certain residues resulted in a shift of carbohydrate recognition from lactosylceramide to gangliosides. Mutational exchange of amino acid residues between two domains interchanged their carbohydrate and target cell specificity. This suggests a possible mechanism by which pathogenic organisms can alter their tissue specificity. Similar alterations in the carbohydrate binding domain of *Bauhinia purpurea* lectin, resulted in altered glycoside-binding specificity (Yamamoto *et al.*, 1992a).

The discovery of amino acid homologies dispersed over long segments of the primary sequences of several lectins has suggested that many of these proteins have a related three-dimensional organisation. In addition, the identification of more highly

focused regions of sequence homology has indicated that many structures within the lectin glycoconjugate-binding domains themselves may be conserved (Holt, 1991). There is no reason to suppose that the same is not also true of microbial lectin-like proteins.

4. IDENTIFICATION OF THE *C. ALBICANS* RECEPTOR ON BUCCAL EPITHELIAL CELLS

C. albicans is a commensal yeast which can cause infections at cutaneous, mucocutaneous and systemic sites. The organism appears to utilise cell surface mannoproteins to colonise host epithelial and endothelial cells (Calderone and Wadsworth, 1993). Such cell-cell interactions are frequently mediated by lectin-carbohydrate recognitions, as already established for numerous viruses, bacteria and protozoa (Karlsson and Stromberg, 1987).

The mechanism of adherence of *C. albicans* to endothelium appears to differ from that for adherence to epithelium (Odds, 1988). Lesions such as endophthalmitis, nephritis, and endocarditis arise as a result of adherence of *C. albicans* to endothelium or exposed subendothelial surfaces (Klotz, 1987). Fibrils on the yeast cell surface have been shown to be responsible for adhesion of *C. albicans* to renal endothelium (Barnes, 1983). Proteolytic treatment of fungi fails to reduce their attachment to endothelium whereas similar treatment is known to inhibit adhesion of *C. albicans* to VEC and BEC (Lee and King, 1983).

The search for host cell receptors for *C. albicans* has, until recently, been limited to blocking studies using monosaccharides and/or lectins in adhesion-inhibition assays. Studies involving sugar inhibition tests have produced a variety of contradictory results. L-Fucose (Tosh and Douglas, 1992), amino sugars (Segal *et al.*, 1982a) and D-mannose (Sobel *et al.*, 1981) have all been reported to be major inhibitors of *C. albicans* adhesion and therefore suggested as likely receptor determinants. More recent work, using both monosaccharides and lectins in adhesion-inhibition assays, demonstrated that a receptor containing L-fucose is required for most strains of the yeast (Critchley and Douglas,

1987a). Although the addition of L-fucose to assay mixtures caused only partial inhibition of adhesion with sensitive strains, it is possible that the natural mucosal receptor is larger than L-fucose or that a particular stereochemical configuration is necessary (Tosh and Douglas, 1992). Brassart *et al.* (1991), demonstrated that *C. albicans* adhesion to BEC *in vitro* was significantly inhibited by the addition of Fucα1-2Galβ-bearing complex carbohydrates. This structure is found on all blood group substances in the ABO system. Blood group antigens are expressed on the carbohydrate side chains of lipids and proteins on erythrocytes and certain epithelial cells (Kabat, 1982).

A number of groups have found that *C. albicans* adheres to fibronectin (FN), a common mammalian cell surface glycoprotein, *in vitro* (Skerl *et al.*, 1984; Scheld *et al.*, 1985; Klotz, 1990). As mentioned earlier, Klotz *et al.* (1994) partially characterised the FN adhesin of *C. albicans* which binds the fungus to subendothelial extracellular matrix and matrix proteins. Other mammalian proteins thought to mediate *C. albicans* attachment include fibrinogen (Tronchin *et al.*, 1987), laminin (Bouchara *et al.*, 1990), and types I and IV collagen (Klotz, 1990).

The FN-cell-binding domain, which has the amino acid sequence Arg-Gly-Asp (RGD), has been examined by two groups for its ability to block adherence of *C. albicans*. RGD-containing peptides were shown not to inhibit adhesion of *C. albicans* to BEC by Brassart *et al.* (1991). However, Ollert *et al.* (1993) found that *C. albicans* attachment to keratinocytes was inhibited by RGD containing peptides derived from laminin.

More recently Santoni *et al.* (1994) investigated the expression of the FN receptor prototype, $\alpha_5\beta_1$ integrin, on *C. albicans* and its involvement in adhesion to FN. Several monoclonal antibodies (mAbs) directed against human α_5 or β_1 integrin subunits, or two different antisera to FN receptor, positively stained *C. albicans* yeasts and germ tubes. Both *C. albicans* yeast and germ tube forms bound to an RGD-containing 120 kDa tryptic fragment of FN and adhesion to FN was markedly inhibited by GRGDSP, but not GRGESP peptides.

Studies that have examined BEC for the presence of FN have produced contradictory results. Woods *et al.*, (1981 a, b) found measurable amounts of FN on scraped BEC from

healthy humans. The level of FN on scraped BEC from ill individuals was found to be reduced. When the level of bacterial adhesion was assessed between the two groups, in an in vitro assay, the BEC of ill patients were found to bind more bacteria than BEC from healthy individuals. There appeared to be an association between reduced FN levels and the susceptibility of BEC to bacterial attachment. Mason *et al.* (1990) however, could not detect FN on BEC in epithelial biopsy specimens from rodents or humans. The presence of FN in the studies by Woods and coworkers may be due to the method of BEC collection. FN from lower levels of the epithelium may have been collected along with the more superficial BEC layers. The increased bacterial adhesion seen in the BEC from the ill subjects may be related to different receptor expression. This possibility will be expanded upon below.

4.1 The effect of RGD peptide on *C. albicans* adhesion to BEC

In this study, the possible role of RGD peptide in the adhesion of *C. albicans* to oral epithelium was examined. Pre-incubation of yeasts (strain GDH 2346) with RGD peptide in the presence and absence of Mg^{2+} and Ca^{2+} did not inhibit adhesion to BEC (Table 4.1 and 4.2). There was no significant difference in the adhesion values obtained for treated and untreated yeasts. These results are in agreement with work by Brassart *et al.* (1991) but contradict the work of Bendel *et al.* (1993) who found that adhesion of *C. albicans* to HeLa S3 cells (an epithelial cell line) was inhibited by RGD containing peptides. However, transformed cells may not express the same receptors as exfoliated epithelial cells. The results obtained in the present study suggest that the oral adhesion of *C. albicans* is not affected by the presence of the FN binding domain peptide, RGD.

4.2 Epithelial glycosides as receptors for *C. albicans*

A number of studies have examined the role of carbohydrates in the adhesion of *C. albicans* to epithelia. This work has mainly involved the use of lectins and/or monosaccharides (Centeno *et al.*, 1983; Collins-Lech *et al.*, 1984; Critchley and Douglas, 1987b). More recent studies on the role of glycosides in candidal adhesion have employed

a chromatogram overlay assay previously used in the characterisation of bacterial adhesion mechanisms.

In studies of the role of glycosides as receptors for bacterial adhesion, most workers have adopted glycosphingolipids over glycoproteins for binding analysis (Karlsson and Stromberg, 1987). Glycosphingolipids carry only one oligosaccharide chain and are easily extracted from tissues. Glycoproteins, on the other hand, may express two or more different glycoside moieties and as a result, make the identification of receptors more difficult.

Glycosphingolipid structure and nomenclature was recently reviewed by Hakamori (1993) and is summarised in the Introduction. Briefly, glycosphingolipids can be classified as neutral glycolipids, sulfatides (sulphate-containing) and gangliosides (sialic acid-containing). They can also be classified according to the number of carbohydrate residues e.g. ceramide monohexose, ceramide dihexose etc. Alternative classification depends on the backbone structure of the carbohydrate. Under this system, the globo series glycosphingolipids contain globotriaosylceramide (Galβ1-4 or 1-3Galβ1-4Glc-Cer). The lactoseries contain lactotriaosylceramide (GlcNAcβ1-3Galβ1-4Glc-Cer). The ganglio series contain gangliotriaosyl ceramide (GlcNAcβ1-4Galβ1-4Glcβ1-1Cer) and the gala series contain galactobiosyl ceramide (Galβ1-4Galβ1-1Cer) as the common structure. The lactoseries show the most extensive substitutions with sialosyl, fucosyl, and α– and β-galactosyl moieties. They can also have extensions or branching by repeating N-acetyllactosamine units which can result in a great number of distinct molecular structures. The fucosylated species include the major blood group antigens.

Glycosphingolipids have been reported to be cell-surface receptors for a number of bacteria. Uropathogenic *E. coli* specifically bind to Galα1-4Gal sequence on urinary tract epithelial cells (Leffler *et al.*, 1982). Also, *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Pseudomonas cepacia,* isolated from cystic fibrosis patients, bind to glycosphingolipids containing GalNAcβ1-4Gal sequences (Krivan *et al.*, 1988).

The TLC overlay assay has the advantage of combining the high-resolution separation of potential receptors with direct solid-phase binding (Karlsson and Stromberg, 1987). Glycolipids are normally separated on silica gel-coated aluminium sheets that tend to be more resistant to layer detachment during washing than glass plates. The aluminium plates are also easy to cut.

The dried chromatogram with separated glycolipids is dipped in a solvent solution of polyisobutylmethacrylate (PIBMA) and then allowed to air dry before being blocked with a buffered protein solution. The hydrophobic film produced by the PIBMA treatment may both optimise the protein blocking of the silica gel and induce presentation of the amphipathic glycolipids to resemble the situation in the natural cell membrane (Karlsson and Stromberg, 1987). The glycoside chains are then free to interact with the ligand while the hydrophobic lipid components are embedded in the plastic layer.

During the assay, the plate is rocked or shaken to reduce the volume of the sample solution required. After incubation, the plate is washed a number of times to remove the unbound ligand, and then treated depending on the ligand used. Detection can be based on direct autoradiography, using a labelled ligand, or a non-labelled ligand can be detected using antibodies (Hansson *et al.* 1985).

An early report on the role of lipids in yeast adhesion was published by Ghannoum *et al.* (1986). They found that various classes of lipids derived from *Candida* cell walls (including ceramide mono- and di-hexosides) were able to inhibit adherence of the yeast to BEC. They subsequently showed that total lipid extracts from BEC also blocked adherence (Ghannoum *et al.*, 1987). Two more recent studies have suggested that *C. albicans* can bind specifically to certain glycosphingolipids. Using a chromatogram overlay assay, Jimenez-Lucho *et al.* (1990) claimed that *C. albicans* and other fungi bound specifically to lactosylceramide (a glycosphingolipid commonly found on epithelial cells. More recently, Yu *et al.* (1994c), employed a similar overlay system to show that *C. albicans* fimbriae bound to asialo-GM_1 and asialo-GM_2, but failed to bind to lactosylceramide. Lipids expressing L-fucosyl residues were not used in either study.

The most recent investigation of this type examined the binding of *C. tropicalis* to a range of lipids (Prakobphol *et al.*, 1994). A lipid library of potential host receptors was screened using the TLC-overlay system and ^{35}S-labelled *C. tropicalis* cells. No interaction was observed with glycolipids or phospholipids. However, lysophospholipids were found to support adherence of *C. tropicalis* but not *Saccharomyces cerevisiae*. Binding resulted in rapid metabolism of the substrate, suggesting that the lipid-metabolising enzymes of the organism are linked to the adhesion process.

L-Fucose-containing glycoconjugates have been proposed as receptors for *C. albicans* on the basis of adhesion-inhibition assays using lectins and sugars as blocking agents (Critchley and Douglas, 1987b). More recently, Brassart *et al.* (1991), using complex carbohydrates derived from human milk, determined that the minimum structure required to inhibit *C. albicans* attachment to BEC was Fuc(α1-2)Galβ1 which is the terminal disaccharide of the H blood-group antigen. Tosh and Douglas (1992) used a Synsorb affinity chromatography step to purify an adhesin from *C. albicans*. The adsorbent had covalently linked H-2 blood group antigen (containing a terminal fucose residue) and the adhesin was able to inhibit the attachment of yeasts to BEC by up to 80%. A similar degree of adhesion-inhibition was achieved in this study (74.81%). These results suggest a role for H blood-group antigen in the adhesion of *C. albicans* to BEC.

With a view to identifying glycolipid receptors for *C. albicans* in the oral cavity, BEC glycolipids were prepared in this investigation and a glycolipid chromatogram overlay assay was employed. As mentioned above, this system has been used to characterise a number of adhesin-receptor relationships (Hansson *et al.*, 1985). Glycolipids were isolated from BEC by chloroform-methanol extraction. In addition to BEC lipids, control glycolipids (Calbiochem) and sheep red blood cell (sRBC) glycolipids (a gift from Chris Brotherston, Dept. of Microbiology, University of Glasgow), were included in most assays. The control glycolipids comprised galactosylceramide (CMH), lactosylceramide (CDH), trihexosylceramide (CTH), globoside (GL4), and Forssman antigen (FORS) (Table 4.3). Various methods of lipid visualisation were investigated including iodine vapour, anisaldehyde (Fig. 4.1), orcinol (Fig. 4.2) and resorcinol (Fig. 4.3). Total lipid was

subsequently visualised by treatment with orcinol reagent (Figs. 4.4 and 4.5). Different solvent systems for the separation of lipids were also assessed. All those tested contained chloroform, methanol and water but in varying proportions. The systems containing chloroform, methanol and water in the ratios 55:45:10 (Fig. 4.1) and 60:35:8 (Fig. 4.2) were found to bunch the majority of lipids. A system with chloroform, methanol and water in a ratio of 65:25:4 (Figs. 4.3, 4.4, and 4.5), gave good separation of lipids and was used in all subsequent experiments.

4.2.1 Binding of ^{125}I-EP from strains GDH 2346 and GDH 2023 to glycolipids separated on TLC plates

The EP from *C. albicans* GDH 2346 was radio-iodinated using the Iodogen procedure as described in Materials and Methods. The TLC plate of separated glycolipids (BEC, sRBC, and control lipids) was air-dried and subjected to a two stage blocking step. Step 1 was PIBMA in hexane; step 2 was PBS-BSA blocking solution. Then the plate was incubated with ^{125}I-EP in fresh blocking solution. After incubation, the unbound ^{125}I-EP was removed by washing the TLC plate in PBS. The TLC plate was then air dried, placed inside a plastic bag and autoradiographed. Despite the high background, the autoradiograph obtained (Fig. 4.6) shows that ^{125}I-EP bound to two lipid components from sRBC. There did not appear to be any binding to either the control lipids or the lipids from BEC. However, some clear zones were seen through the background in the BEC lipid lane. The TLC overlay system showed promise but required considerable optimisation.

Binding of ^{125}I-EP from strain GDH 2023 to glycolipids was also examined using the same procedure. The autoradiograph obtained is shown in Fig. 4.7. ^{125}I-EP bound to four lipid components from sRBC but there did not appear to be any binding to lipids from BEC or to the control lipids. However, clear zones were visible in the BEC lipid lane. Background radioactivity on this autoradiograph was to that seen previously. One possible reason for the intense background could be the ability of *C. albicans* to bind to inert plastic surfaces such as denture base materials (Minagi *et al.*, 1985), and polyvinyl chloride

(Rotrosen *et al.*, 1983). The treatment of TLC plates with PIBMA results in a plastic coating which may promote the adhesion of *C. albicans* whole cells and proteins. To prevent the possibility of this non-specific adhesion to plastic, the TLC plates must be effectively blocked with an appropriate protein-buffer solution. The buffer solution that had been used in the above experiments may not have been appropriate and so other solutions were tested in subsequent experiments.

4.2.2 Binding of ^{125}I-labelled whole cells and ^{125}I-EP from strain GDH 2346 to glycolipids separated on TLC plates

Whole cells and EP from *C. albicans* GDH 2346 were radio-iodinated using the Iodogen procedure as described in Materials and Methods. The overlay procedure was similar to the previous experiments but in an attempt to reduce the intense background, an alternative blocking solution (Tris-BSA buffer) was employed after PIBMA treatment. This solution was also used for the overlay incubation steps. The autoradiographs obtained (Fig. 4.8) show that the background was even higher than that previously encountered. Although there did seem to be some binding of ^{125}I-labelled material, the degree of background made useful interpretation impossible. A number of technical difficulties were encountered, ranging from silica damage due to the TLC plates colliding with the side of the reaction trays, to intense background. The procedure also produced a considerable amount of contaminated equipment and large volumes of radioactive waste.

4.2.3 Binding of lectins to glycolipids separated on TLC plates

The following biotinylated lectins were purchased from Sigma: *Bauhinia purpurea, Triticum vulgaris* and *Vicia villosa. Bauhinia purpurea* agglutinin has an affinity for Gal(β1-3)GalNAc, N-acetyl-galactosamine (GalNAc) and D-galactose (Irimura and Osawa, 1972), the lectin from *Triticum vulgaris* (wheat germ agglutinin) has an affinity for GlcNAc (Nagata and Burger, 1974) and *Vicia villosa* lectin has an affinity for GalNAc (Goldstein and Poretz, 1986). TLC plates were prepared as previously and PBS-BSA was used as the blocking and binding buffer. The lectins were diluted in blocking buffer prior to

overlay analysis and the bound lectins were detected by probing with ^{125}I-streptavidin. The autoradiographs obtained for these experiments are shown in Fig. 4.9 A, B and C. Less background was seen on these autoradiographs than had been encountered previously but there was clearly some deterioration of the TLC plates. Small pits were visible on the surface of the plates and they corresponded to "hot spots" on the autoradiograph. The pitting may have been due to silica loss during the extensive incubation and washing stages. Non-specific binding of biotinylated lectin and/or ^{125}I-streptavidin to these pits is likely as the blocking activity of the PIBMA coating will have been lost. The reduced backgound encountered in the lectin-binding experiment may have been due to the use of biotinylation and ^{125}I-streptavidin instead of directly radiolabelled lectins.

4.2.4 Binding of biotinylated EP from *C. albicans* GDH 2346 and GDH 2023 to glycolipids separated on TLC plates

EP preparations from strains GDH 2346 and 2023 were first biotinylated and then subjected to TLC-overlay analysis. A minor modification was incorporated in the protocol in an attempt to reduce background and TLC plate deterioration. Each plate was submerged in PIBMA solution for 1 min, removed and allowed to air-dry. This was repeated a further two times and then the plates were blocked as previously and overlaid with biotinylated EP. The autoradiographs obtained are shown in Fig. 4.10. The background seen in this experiment was the lowest yet encountered and the pitting observed previously was virtually abolished. The binding of biotinylated EP from strain GDH 2346 to some separated lipids on the TLC plate was clearly visible. Binding was seen to two lipid components from the sRBC and one lipid component from BEC. Less intense binding to one further component from BEC was also observed. There was no binding to any of the neutral glycolipid controls. Biotinylated EP from strain GDH 2023 did not appear to bind any lipid components. In a previous experiment, there was found to be binding of ^{125}I-EP from GDH 2023 to four lipid components from sRBC. The absence of any binding may have been due to poor biotinylation, damage to the biological activity of

EP as a result of biotinylation, or increased blocking as a result of the enhanced PIBMA treatment.

4.2.5 Binding of biotinylated whole cells of different *C. albicans strains* to glycolipids on TLC plates.

The binding of biotinylated whole cells from five *C. albicans* strains (GDH 2346, GRI 681, GRI 682, MRL 3153 and "outbreak") to glycolipids from BEC, sRBC and neutral glycolipid standards (CMH, CDH, CTH, GL4, FORS), was examined by the chromatogram overlay assay. Bound cells were visualised by the addition of ^{125}I-streptavidin followed by autoradiography (Fig. 4.11). All five strains had similar binding patterns. By comparison with an identical chromatogram overlaid with biotinylated *Ulex europaeus* lectin it appeared that the cells were able to bind to four glycolipids expressing H-blood group antigen. The cells also bound to an additional lipid from BEC, not bound by *Ulex europaeus* lectin, with an R_f value similar to a lipid bound by the GalNAc specific lectin from *Helix pomatia* (Hammarstrom and Kabat, 1971). However, GalNAc alone is unlikely to be the receptor as the yeast cells were found not to bind to the GalNAc-expressing neutral glycolipid standards, GL4 and FORS, both of which were detected by *Helix pomatia* lectin. None of the strains tested were found to bind to lactosylceramide, recently proposed as a receptor for *C. albicans* (Jimenez-Lucho *et al.*, 1990). These results are consistent with experiments involving sugars and lectins which suggested that strains GDH 2346, GRI 681, GRI 682, and MRL 3153 all share a common adhesion mechanism involving fucose-containing receptors (Critchley and Douglas, 1987b).

4.2.6 Binding of biotinylated EP and purified adhesin from *C. albicans* GDH 2346 to glycolipids on TLC plates.

To take the study of *C. albicans* GDH 2346 adhesion a stage further, the binding of biotinylated EP and purified adhesin from strain GDH 2346 to glycolipids was examined by the chromatogram overlay assay. Bound material was visualised by the addition of ^{125}I-streptavidin followed by autoradiography. The resulting autoradiograph is shown in Fig.

4.12. The binding patterns observed for EP and purified adhesin were remarkably similar. Both bound to glycolipids from BEC and sRBC but not to any of the neutral glycolipid standards (including lactosylceramide). By comparison with a chromatogram overlaid with biotinylated *Ulex europaeus* lectin (UE) it appeared that the EP and purified adhesin were able to bind to glycolipids with R_f values similar to lipids expressing H-blood group antigen. Interestingly, in the previous experiment, whole cells from strain GDH 2346 (and four other strains) bound to three lipid components from BEC, two with R_f values similar to lipids expressing H-blood group antigen, and a third with an R_f value similar to a lipid expressing GalNAc. The problems of plate deterioration and intense background had been greatly reduced by adopting the modifications outlined in the above experiments.

4.2.7 Binding of biotinylated whole cells and EP from strain GDH 2023 to glycolipids on TLC plates.

The binding of biotinylated whole cells and EP from strain GDH 2023 to glycolipids was then examined by the chromatogram overlay assay. Biotinylated *Griffonia simplicifolia* lectin II (GSII) was purchased from Vector Laboratories. This lectin has an affinity for GlcNAc (Goldstein and Poretz, 1986). Biotin-labelled material was visualised by the addition of ^{125}I-streptavidin followed by autoradiography (Fig. 4.13). Whole cells and EP from strain GDH 2023 were found to bind to four glycolipids from both BEC and sRBC. By comparison with an identical chromatogram overlaid with biotinylated GSII it appeared that the cells were able to bind to glycolipids with R_f values similar to lipids expressing GlcNAc from BEC and sRBC. This strain did not bind to any of the neutral glycolipid standards (including lactosylceramide). These results are in agreement with previous experiments involving sugars and lectins (Critchley and Douglas, 1987b) which suggested that strain GDH 2023 has an adhesion mechanism involving GlcNAc containing receptors.

4.2.8 The role of glycolipids in the adhesion of *C. albicans* to epithelial cells

Six strains in total were examined in this study for their ability to bind to separated glycolipids. Five strains were found to bind to glycolipids expressing H-blood group antigen by comparison with an identical chromatogram overlaid with biotinylated *Ulex europaeus* lectin. These strains bound to an additional lipid from BEC which appeared to have a terminal GalNAc, as determined by comparison with an identical chromatogram overlaid with GalNAc specific lectin from *Helix pomatia*. However, the yeast cells did not bind to control glycolipids with GalNAc as a terminal sugar residue (GL4 and FORS) and so GalNAc is unlikely to be the receptor. By comparing the relative migration of this *Candida* binding lipid with the control lipids a possible explanation is that the yeast cells were binding to blood-group A antigen, to which *Ulex europaeus* lectin cannot bind. This would suggest that more than one fucose-containing glycoside can act as receptor for *C. albicans*. EP and purified adhesin from GDH 2346 were biotinylated and overlaid onto TLC plates of separated glycolipids probed with ^{125}I-streptavidin prior to autoradiography. EP and purified adhesin bound to H-blood group antigen by comparison with an identical chromatogram overlaid with biotinylated *Ulex europaeus* lectin. However, there was no binding to the additional lipid to which whole cells of the same strain bound. The additional lipid (possibly blood-group A antigen) appeared to have a terminal GalNAc, as determined by comparison with an identical chromatogram overlaid with GalNAc specific lectin from *Helix pomatia*. This lectin will bind to blood-group A antigen as it has a terminal GalNAc residue (Fig. 1). There are two possible explanations for this finding. The component on the *Candida* cell surface that binds to the additional component may not be released as part of EP or the ability of EP to bind to this receptor may be lost during the purification procedure.

Biotinylated whole cells and EP from a sixth strain, GDH 2023, were overlaid onto TLC plates of separated lipids as previously described. By comparison of the resultant autoradiographs with an identical chromatogram overlaid with biotinylated GSII lectin (specific for GlcNAc), both EP and whole cells from this strain were seen to bind to glycolipids containing GlcNAc.

The findings of recent reports suggest alternative glycolipid receptors for *C. albicans.* Firstly, Jimenez-Lucho *et al.* (1990) provided evidence that *C. albicans, Cryptococcus neoformans* and *Saccharomyces cerevisiae* could bind to the glycosphingolipid, lactosylceramide. The strains of *C. albicans* tested in the present study did not share this ability. More recently, Yu *et al.* (1994c), employing a similar overlay system, showed that *C. albicans* fimbriae bound to asialo-GM_1 and asialo-GM_2, but failed to bind to lactosylceramide. Neither of the above studies examined the binding of yeasts to lipids expressing L-fucosyl residues. The role of lactosylceramide in the adhesion of *C. albicans* to epithelium remains to be resolved. If lactosylceramide is present in epithelial cells, it could be the case that it is not exposed at the cell surface. Lactosylceramide has been shown to be localised mainly intracellularly in polymorphonuclear neutrophils (Symington *et al.*, 1987) and on the human erythrocyte membrane this glycolipid is not accessible to galactose oxidase (Gahmberg and Hakomori, 1973). In contrast, the presence of blood group-expressing glycolipids is clearly demonstrated on the surface of BEC by the work of Vedtofte *et al* (1984).

Another recent study, Prakobphol *et al.* (1994), examined the binding of *C. tropicalis* to a range of lipids. They screened a lipid library for potential host receptors using the TLC-overlay system and ^{35}S-labelled *C. tropicalis* cells. They found no interaction with glycolipids or phospholipids. However, lysophospholipids were found to support adherence of *C. tropicalis* but not *Saccharomyces cerivisiae*. They additionally found that binding resulted in rapid metabolism of the substrate, suggesting that the lipid-metabolising enzymes of the organism are linked to the adhesion process. *C. albicans* remains to be tested for this ability.

One possible reason for the variability obtained in the above studies is the growth medium empoyed. For example, Jimenez-Lucho *et al.* (1990) used the same medium as this study (yeast nitrogen base, Difco) but the carbon source and culture conditions were quite different (Jimenez-Lucho *et al.*, 1% glucose, cultures unshaken; this study, 9% galactose, with shaking). In a previous investigation of the effect of different growth conditions on the adhesion and virulence of *C. albicans* (McCourtie and Douglas, 1984)

medium containing 0.9% glucose produced cells that were less adherent to BEC and less virulent in mice than cells from the same strain grown in medium containing 9% galactose. Growth of *C. albicans* in high galactose medium has also been shown to result in the production of an outer fibrillar layer (McCourtie and Douglas, 1985). This could result in the masking of other adhesins present on the cell surface and lead to the enhanced expression of adhesins associated with the fibrillar layer. High concentrations of galactose may exist in the oral cavity due to the degradation of lactose by oral bacteria (Chauncey *et al.* 1954), and it is known that a carbohydrate-rich diet often contributes to the development and persistence of oral candidosis (Ritchie *et al.*, 1969; Shuttleworth and Gibbs, 1960).

Yu *et al.* (1994c) found that *C. albicans* fimbriae (from one clinical isolate) did not bind to lactosylceramide (which is in agreement with this study) but bound to asialo-GM_1 and asialo-GM_2. They also reported that the synthetic disaccharide βGalNAc(1-4)βGal was able to inhibit the binding of fimbria to BEC. In the present study, a BEC lipid component that contained GalNac was bound by five strains of *C. albicans*. This component could possibly be asialo-GM_1 but this would require confirmation. It remains for future studies to examine the possibility that *C. albicans* can produce a number of different adhesins, expressed under different environmental constraints. The different adhesins may enable the yeast to bind a variety of glycoside structures on glycolipids and glycoproteins. This may explain how *C. albicans* can colonise so many body sites (epithelial and endothelial), and under certain conditions, become invasive.

The findings of this study are in agreement with the sugar inhibition assay results obtained by Critchley and Douglas (1987b). The findings are also supported by the work of Brassart *et al.* (1991) and Sawyer *et al.* (1992). Brassart *et al.* (1991) used complex carbohydrates derived from human milk and determined the minimum structure required to inhibit *C. albicans* attachment to BEC was Fuc(α1-2)Galβ1, which is the terminal disaccharide of the H blood-group antigen. In addition, the data presented by Sawyer *et al.* (1992) suggested that a fucose-containing receptor on the surface of either sinusoidal endothelial cells or Kupffer cells is involved in the trapping of *C. albicans* by the perfused

mouse liver. The results of sugar inhibition studies can be criticised due to the possibility that the sugar residue involved in binding may be part of either the host cell receptor or the microbial cell adhesin. This study however, made use of the TLC overlay analysis system which can confirm the identity of receptors as glycoconjugates. It appears that the adhesin molecules on the yeast cell surface are binding in a lectin-like manner to the epithelial cell glycoconjugates containing L-fucose or GlcNAc.

Adhesion-inhibition values of 100% have not been attained in previous studies on yeast adhesion. Addition of L-fucose to assay mixtures caused only partial inhibition of adhesion with sensitive strains (Critchley and Douglas, 1987b), the addition of Fucα1-2Galβ-bearing complex carbohydrates resulted in between 30-55% adhesion-inhibition (Brassart *et al.* 1991) and purified adhesin inhibited yeast adhesion by 80% (Tosh and Douglas, 1992). These results suggest that *C. albicans* cells may express at least two types of adhesin simultaneously. The occurrence of a multi-adhesin system has already been proposed for *C. albicans* (Kennedy *et al.*, 1988). Adhesion of *C. albicans* to BEC could resemble that of *Mycoplasma pneumoniae* to human colon adenocarcinoma cells; both sialyllactose and dextran sulphate have been seen to partially inhibit adhesion to these cells, whereas in combination, the compounds inhibited binding by 90% (Krivan *et al.*, 1989; Roberts *et al.*, 1989).

The H and A blood group antigens are both present on the BEC glycolipids of blood group A individuals; the A antigen is synthesised by the addition of a terminal GalNAc to a precursor substance with blood group H activity. Blood group A antigen tends to be on more superficial cells than blood group H and this phenomenon is believed to be related to cell differentiation (Vedtofte *et al.*, 1984). Other fucose-containing molecules, the Lewis antigens, have been studied as possible receptors of *C. albicans.* Unlike ABO antigens, the Lewis antigens are adsorbed onto the surface of oral epithelial cells from the saliva of non-secretors of blood group antigens. Brassart *et al.* (1991) reported that Lewis antigens failed to act as adhesion inhibitors, but two other studies indicate a role for the Lewis^a antigen (May *et al.*, 1989; Tosh and Douglas, 1991). Taken together, it appears that the most likely candidates for the receptors of *C. albicans* on

BEC are fucose-containing glycosides such as the H antigen and blood group A antigen (Tosh and Douglas, 1992). Support for this theory comes from a clinical survey, carried out by Burford-Mason *et al.* (1988), which found that blood group O and non-secretion of blood group antigens were separate and cumulative risk factors for oral candidal carriage in healthy subjects.

C. albicans infections are common among critically ill patients (Widmer, 1994). One reason for this may be that BEC carbohydrates are altered during critical illness. Weinmeister and Dal Nogare (1994) compared respiratory cell and BEC surface carbohydrate levels of normal subjects with those of critically ill patients. Lectins were used to quantitate the amount of mannose, galactose, fucose, and sialic acid on BEC and respiratory cells. The BEC of severely ill patients had decreased amounts of sialic acid and galactose. However, there was no decrease in the level of fucose on the BEC of these patients. No differences were found between the respiratory-cell carbohydrates of normal and critically ill patients. They concluded that severely ill patients have decreased amounts of galactose and sialic acid on their upper-airway epithelial cells, and that loss of these two monosaccharides may explain the high prevalence of gram-negative bacterial colonisation and pneumonia in the critically ill. The same may also apply to *C. albicans* colonisation and infection. Varki (1993) suggested that under normal circumstances, sialic acid and galactose may function to mask internal carbohydrate residues, preventing microbial attachment. Weinmeister and Dal Nogare (1994) proposed two possible mechanisms by which BEC carbohydrates are altered during illness. Firstly, oligosaccharide synthesis may be affected by the cytokines and hormones released during illness. Support for this comes from Wang *et al.* (1989) who demonstrated that glucocorticosteroids affect the activity of a sialyltransferase that normally adds sialic acid to oligosaccharide chains. The second proposed mechanism is that glycocompounds may be degraded externally by enzymes present in respiratory tract secretions. High levels of exoglycosidase activity has been seen in the saliva and tracheal secretions obtained from critically ill patients (Quinn *et al.*, 1994b). Both mechanisms would result in alterations to the carbohydrate chains expressed on BEC and could result in an increase in potential *C. albicans* receptors. The source of

these exoglycosidases remains to be found. Interestingly, there is evidence to suggest that *Pseudomonas aeruginosa* exoproducts modify epithelial cell surface carbohydrates resulting in an increase in the potential receptors for this organism (Saiman *et al.*, 1992). This suggests that some pathogens may modify their epithelial habitat to enhance adhesion.

APPENDIX

BIOCHEMICAL ASSAYS

A. Carbohydrate determination using the phenol-sulphuric acid method (Dubois *et al.*, 1956)

Reagents

1 Concentrated sulphuric acid (Reagent Grade)

2 5% phenol (v/v)

Procedure

Phenol reagent (0.05ml) was added to samples (2ml) containing carbohydrate (0-100μgml^{-1}). Reagent grade concentrated sulphuric acid (5ml) was rapidly added and samples were left for 30 min at room temperature to allow colour development. Samples were read at 485nm in a Shimatzu PR-1 spectrophotometer. A standard curve was produced using mannose.

B. Protein determination using the method of Lowry *et al.*, 1951.

Reagents

A. 2% Na_2CO_3 in 0.1M NaOH

B. 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate (separate double strength solutions mixed 1:1 before use).

C. Folin-Ciocalteau reagent diluted 1:1 with distilled water (to give total acidity of exactly 1.0 N

D. Mix 50 vol of reagent A with 1 vol of reagent B (renewed daily)

Procedure

Reagent D (3ml) was added to each sample (0.6ml), containing 0-200μgml^{-1} of protein, mixed well and left to stand at room temperature for 10 min. Reagent C (0.3ml) was added rapidly with immediate mixing. Samples were left to stand for 30 min at room temperature then read against a reagent blank in a spectrophotometer at 750 nm. A standard curve was produced using bovine serum albumin.

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PUBLICATION

Some of the results in this thesis were presented as a poster at the ASM Conference on *Candida* and Candidiasis: Biology, Pathogenesis and Management, Baltimore, Md, USA, in March, 1993.

ABSTRACTS OF POSTER PRESENTATIONS

A/31 Attempts to Identify a Integrin ß1-Like Molecule from Candida albicans

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The adherence of Candida albicans to host tissue may be mediated by interactions of fungal cell surface molecules with ligands on host cells or molecules of the extracellular matrix. Although integrins have only been described from multicellular eukaryotes e.g. vertebrates and invertebrates like Drosophila, immunological studies with antisera raised to chicken integrin ß1 showed crossreactivity to a 95 kd protein of Candida albicans (Marcantonio, E.E. & Hynes, R.O., 1988, J. Cell. Biol. 106, 1765-1772), suggesting that integrin-like molecules may be involved in Candida adherence.

We tested Candida albicans protein extracts with a commercial antiserum to the human fibronectin receptor (integrin α5ß1) by immunoblotting. We detected a band in the same molecular weight range in both blastospore and hyphal extracts. In hyphae, however, the corresponding protein may be more abundant. A second, direct approach to the identification of an integrin-like molecule was the *in vitro* amplification of a DNA fragment of Candida albicans with degenerate primers directed to evolutionarily conserved regions of integrin ß subunits. Southern hybridization analysis of genomic restriction digests with the amplified fragment as probe, revealed a single band in C. albicans DNA. We have isolated and are now sequencing the DNA fragment of Candida albicans with nucleic acid sequence similarities to integrin ß genes.

A/32 Analyses of Glycoproteins from Blastoconidia and Mycelia of Candida albicans Using Lectins and Treatment with Glycosidases. ELSA WADSWORTH and RICHARD CALDERONE. Georgetown University School of Medicine, Washington, D.C.

Quantitative differences in labelling with a biotin-hydrazide reagent were observed with two major glycoproteins of approximately 50kD from blastoconidia and 60kD from mycelial cells of Candida albicans. Both proteins reacted similarly in Western blot with either a monospecific antiserum (CA-7) or a monoclonal antibody (CA-A) to the C. albicans C3d-binding protein (complement receptor 2 or CR2). Reactivity of these two proteins with lectin-peroxidase reagents differed; also, treatment with N-glycanase and Endoglycosidase F increased the electromobility of the 60kD but not the 50kD species. While both proteins retained their antibody reactivity following glycosidase digestion, neuraminidase treatment abolished reactivity of only the 60kD glycoprotein to the CA-7 antibody. Our data indicate that an epitope common to both a mycelial and blastoconidial glycoprotein is associated with a structurally different oligosaccharide for each growth form.

A/33 Isolation of Avirulent Clones of *Candida albicans* with Reduced Ability to Recognize the CR2 Ligand C3D. S. FRANZKE AND R. CALDERONE, Georgetown University, Washington, D.C.

Four clones of the yeast *Candida albicans* were isolated on the basis of their tolerance to clotrimazole. These clones produced germ tubes or pseudomycelia but no true hyphae in a pattern which was specific for each strain. Growth of each clone at 37°C, under conditions which favor the filamentous growth form of the organism, was equal to the parental strain (H12). In an intravenous mouse model, none of the mice infected with the tolerant clones but all of the mice infected with H12 developed severe renal candidiasis. As a correlate, we measured the complement receptor activity (CR2 and CR3) of each clone. We found that all tolerant clones showed reduced recognition of C3d bearing sheep erythrocytes (EAC3d) in rosetting assays. In comparison, recognition of iC3b (EAiC3b) by each of the clones was similar to wt cells. DTT-extracts of clone P and H12 (wt) were compared by immunoblot. Both quantitative and qualitative differences in immunoblot analyses of clone P were observed with antibodies specific for the *Candida* C3d-receptor.

A/34 A 67-kDa High Affinity Laminin Receptor-like Molecule Is Differentially Expressed in *Candida albicans* Yeast and Mycelial Cells. J.L. LOPEZ-RIBOT[1,2], P. SEPULVEDA[2], C. MONTEAGUDO[2], M. CASANOVA[2], and J.P. MARTINEZ[2]. Texas Tech University Health Sciences Center, Lubbock, Texas 79430, U.S.A.[1], and Univ. de Valencia, Valencia, Spain[2].

Cell interactions with extracellular matrix, as mediated by cell surface receptors, are thought to play an essential role in the dissemination of *C. albicans* cells in vivo. Cell wall extracts and protoplast homogenates from both fungal growth forms (yeasts and germinated yeasts) were analyzed by SDS-PAGE and Western immunoblotting using four affinity-purified rabbit antibodies (Ab) raised to synthetic peptides deduced from the cDNA sequence of the human 67-kDa high-affinity laminin receptor (67LR), that recognize different domains of this molecule. Ab4160 recognized the 37-kDa precursor of the 67LR only in wall extracts from yeasts. This was confirmed by indirect immunofluorescence (the Ab labelled exclusively the yeast cell surface). Ab4056 recognized the 37-kDa precursor in all extracts analyzed. With Ab4099, only a 67-70 kDa species was found in the protoplast lysate from mycelial but not from yeast cells. No specific bands were recognized with Ab4162. The differential expression of 67LR observed in *C. albicans* yeast and mycelial cells may be responsible, at least in part, for the different behaviour of both forms in infected human tissues.

A/35 Effect of Cytotoxic Drugs on *Candida albicans* Adherence to Murine Tissues. J.L. LOPEZ-RIBOT*, and W.L. CHAFFIN. Texas Tech University Health Sciences Center, Lubbock, Texas 79430, U.S.A.

The attachment of *C. albicans* to host cells and tissues is considered to be the initial step leading to establishment of infection. In this era of cytotoxic chemotherapies (among other factors), *C. albicans* is gaining prevalence as an opportunistic pathogen. Mice were treated with lipopolysaccharide and clinically used anticancer drugs doxorubicin, cisplatin and vincristine (2 different concentrations per drug, 5 mice per group). Effects on white blood cell population were monitored. Adherence of *C. albicans* 3153A blastospores to the different tissues was studied using the "ex vivo" assay. No differences were observed in binding of the fungal cells to liver and kidney tissues from treated or untreated animals. All drug-treated spleens displayed altered patterns of adhesion for *C. albicans* compared to the control group, with yeast cells bound not only to the marginal zone but also to the white and red pulp. Immunostaining for macrophages showed no apparent differences between the control and the experimental spleens that could account for the change in adhesion patterns. Preliminary scanning electron microscopy images suggested that yeast binding to the white pulp in treated tissues is mediated through fibers, perhaps exposed extracellular matrix components.

A/36 Fucose-Containing Glycosphingolipids as Receptors for *Candida albicans*.

B.J. CAMERON* and I.J. DOUGLAS. University of Glasgow, Glasgow, Scotland.

L-Fucose inhibits the adhesion of *Candida albicans* GDH 2346 to buccal epithelial cells *in vitro*. This organism produces extracellular polymeric material (EP) which contains a mannoprotein adhesin with a lectin-like affinity for fucose-containing glycosides. To examine the possible role of fucose-containing glycosphingolipids as receptors for *C. albicans*, a chromatogram overlay assay was used.

EP was layered on thin-layer chromatograms of separated glycosphingolipids which had been extracted from human buccal epithelial cells and sheep red blood cells. Two methods for detecting bound EP were used. In the first, EP was labelled with ^{125}I prior to overlay and binding was detected on the chromatograms by autoradiography. The second method involved biotin labelling of EP prior to overlay; binding was detected by the addition of ^{125}I-streptavidin followed by autoradiography. Fucose-containing glycosphingolipids were detected similarly by the use of biotin-labelled lectin from *Ulex europaeus* which has an affinity for L-fucose. The results obtained showed that EP bound to the same glycosphingolipid bands as the fucose-specific lectin suggesting that these lipids can act as receptors for *C. albicans*.