# Investigations on cellular nanoparticles required for synthesis of chitin the precursor for chitosan

## Dissertation

zur Erlangung des Grades eines Doktors
der Naturwissenschaften (Dr. rer. nat.)
an der Universität Osnabrück

**vorgelegt**
**von Mayur Kumar Kajla**
**aus Himachal Pradesh, Indien**

**September 2005**

1. **Berichterstatter : Prof. Dr. H. Schrempf**

2. **Berichterstatter : Prof. Dr. Jürgen Hofemeister**

## Index Page

**1.0 Introduction** **1**

**2.0 Material and methods** **7**

**2.1 Materials** **7**

**2.1 Media** **7**

2.1.1 Yeast peptone adenine dextrose (YPAD) 7

2 Yeast malt extract (YM) 7

3 Luria Burtani (LB) 8

4 M9 Minimal Medium 8

**2.2 Buffers and stains** **9**

2.2.1 Standard buffers 9

2 Western transfer buffer 9

3 Acetonitrile buffer 9

4 Buffers for blue native PAGE 10

5 Protein loading buffer for SDS-PAGE 10

6 Coomassie staining solution 10

7 Silver and zinc staining of polyacrylamide gels 11

7.1 Silver staining 11

2 Zinc staining 12

**2.3 *E. coli* and *S. cerevisiae* strains** **12**

**2.4 Antibodies** **13**

**2.5 Biochemical methods** **14**

2.5.1 Cultivation of yeast 14

2 Chitin synthase assay 14

2.1 WGA based assay 14

2 CHB1 based assay 16

**2.6 Isolation and purification of chitosomes 17**

**2.7 Separation of the proteins 20**

2.7.1 SDS-PAGE 20

2 2-dimensional blue native PAGE 20

**2.8 Western blotting 22**

**2.9 Isolation and purification of chitin binding protein 23**

**3.0 Differential extraction of the proteins 25**

**3.1 Determination of protein concentration 26**

**3.2 Purification of proteins via HPLC and gel filtration 26**

**3.3 Electron microscopy analysis of chitosomes 26**

**3.4 Additional enzymatic assays 28**

3.4.1 UDP-*N*-acetyl-glucosamine pyrophosphorylase assay **28**

3.4.2 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay 28

3.4.3 Enolase assay 28

3.4.4 Pyruvate kinase assay 28

3.4.5 Pyruvate decarboxylase assay 29

**3.5 Sample preparation for MALDI-MS analysis 29**

**4.0 Results 31**

**4.1 Identification and purification of chitosomal complexes 31**

**4.2 CHB1 as a tool for chitosomal quality control 37**

**4.3 Structural analysis of complexes via electron microscopy 39**

**4.4 Evaluation of different protein enrichment conditions 41**

**4.5 Identification of major proteins in chitosomal fractions 44**

**4.6 Characterization of identified proteins 46**

**4.7 Studies on CSI-HA strain 56**

**5.0 Discussion 60**

**6.0 Summary** **72**

**7.0 Bibliography** **73**

**Affidavit**

**Acknowledgements**

## List of figures and tables Page

Fig. 4.1 Purification of chitosomes from wild type strain 32

Fig. 4.2 Purification of chitosomes from *chs3* mutant 34

Fig. 4.3 Comparison of the CSI activity assays 36

Fig. 4.4 Purification of chitin binding protein CHB1 38

Fig. 4.5 EM examination of chitosomal complexes 41

Fig. 4.6 Enrichment of chitosomal proteins using salts 43

Fig. 4.7 Separation of proteins via 2-D PAGE 45

Fig. 4.8 Clustal W alignment of N-termini of two proteins 47

Table 4.9 (i) List of peptide masses 48

Table 4.9 (ii) List of peptide masses 49

Table 4.10 Summary of the identified proteins 50

Fig. 4.11 and 4.12 Evaluation of enzymatic activities 52, 53

Fig. 4.13 Immunodetection of proteins in chitosomal fractions 54

Fig. 4.14 Immunodetection of GAPDH and Pdc 55

Fig. 4.15 Analysis of CSI-HA tagged protein 57

Fig. 4.16 Purification of chitosomes from CSI-HA strain 58

Fig. 4.17 Detection of CSI-HA in chitosomal fractions 59

## Abbreviations used in the text

| | |
|---|---|
| ACN | **Ac**eto**ni**trile |
| ADH | **A**lcohol **de**h**y**drogenase |
| AGM | **A**ngewandte **g**enetic der **m**ikroorganismen |
| APS | **A**mmonium **p**er**s**ulphate |
| $A_{600}$ | **A**bsorbance at **600** nm |
| BSA | **B**ovine **s**erum **a**lbumin |
| $^{0}C$ | **Degree c**elcius |
| Ca | **C**irc**a** |
| CAPS | 3-[**c**yclohexyl**a**mino]-1-**p**ropane**s**ulfonic acid |
| CHB1 | **Ch**itin **b**inding protein **1** |
| cm | **C**enti**m**eter |
| CSI | **C**hitin **s**ynthase **I** |
| CSII | **C**hitin **s**ynthase **II** |
| CSIII | **C**hitin **s**ynthase **III** |
| 1D | **One d**imensional |
| DMSO | **Di**m**e**thyl **s**ulph**o**xide |
| *E. coli* | ***E**scherichia **c**oli* |
| EDTA | **E**thylene **d**iamine **t**etra-chloro **a**cetic acid |
| Eno | **Eno**lase |
| FAS | **F**atty **a**cid **s**ynthase |
| FC | **F**olin-**C**iocalteu reagent |
| FKS | Glucan synthase |
| g | **G**ram/s |
| GAPDH | **G**lycer**a**ldehyde 3-**p**hosphate **de**h**y**drogenase |

| | |
|---|---|
| GdHCl | **G**uanidine **h**ydro**ch**l**o**ride |
| GlcNAc | *N*-**ac**etyl-D-**gl**u**c**osamine |
| HA | **H**aem**a**gglutinin |
| HPLC | **H**igh **p**ressure **l**iquid **c**hromatography |
| HRP | **H**orse**r**adish **p**eroxidase |
| Hrs | **H**ou**rs** |
| IPTG | **I**so**p**ropyl-**t**hio-**g**alactoside |
| kDa | **K**ilo **Da**lton |
| LB | **L**uria-**B**urtani |
| M | **M**olar |
| mg | **M**illi**g**ram |
| µl | **M**icro**l**iter |
| ml | **M**illi**l**iter |
| mM | **M**illi**m**olar |
| β-ME | **β**-**m**ercapto**e**thanol |
| MALDI-MS | **M**atrix **a**ssisted **l**aser **d**esorption **i**onization **m**ass **s**pectrometry |
| Min | **Min**utes |
| MW | **M**olecular **w**eight |
| N | **N**ormal |
| NADH | **N**icotinamide **a**denine **d**inucleotide **h**ydroxide |
| NCBI | **N**ational **c**enter for **b**iotechnology **i**nformation |
| Ni-NTA | **Ni-n**itrilo**tri**a**c**etic acid |
| nm | **N**ano**m**eter/s |
| ORF | **O**pen **r**eading **f**rame |
| PAGE | **P**oly**a**crylamide **g**el **e**lectrophoresis |
| PBS | **P**hosphate **b**uffered **s**aline |
| Pdc | **P**yruvate **d**e**c**arboxylase |

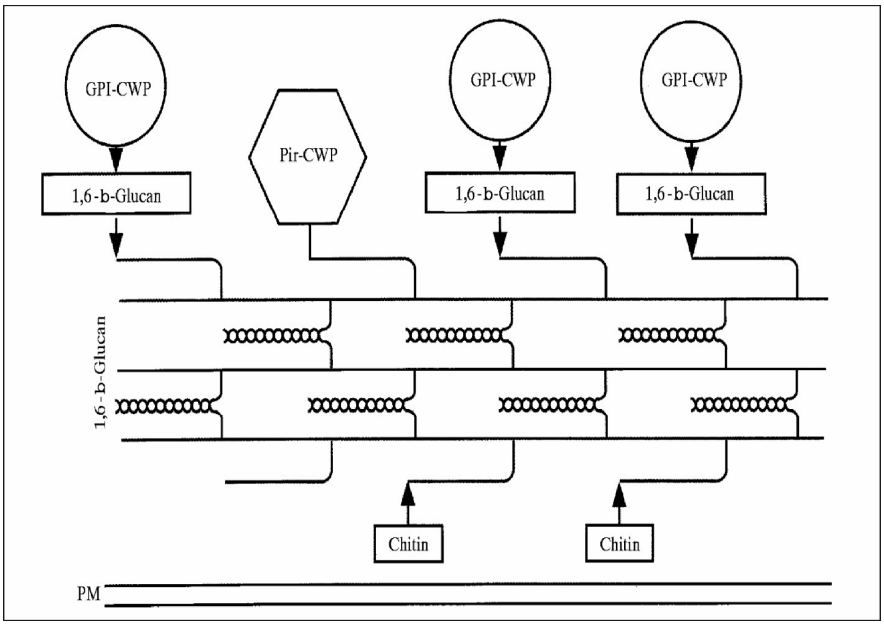
| | |
|---|---|
| PEP | **P**hospho**e**nol **p**yruvate |
| Ppm | **P**arts **p**er **m**illion |
| PVDF | **P**oly**v**inyli**d**ene **f**luoride |
| Pyk | **Py**ruvate **k**inase |
| rpm | **R**otations **p**er **m**inute |
| *S. cerevisiae* | ***S**accharomyces **c**erevisiae* |
| SDS | **S**odium **d**odecyl **s**ulphate |
| Sec | **Sec**onds |
| SGD | **S**accharomyces **g**enome **d**atabase |
| TEMED | N, N, N', N'-**te**tra**me**thylethylene**d**iamine |
| TFA | **T**ri**f**luoroacetic **a**cid |
| TMB | **T**etra **m**ethyl **b**enzidine |
| UDP | **U**ridine-**d**i-**p**hosphate |
| UDP-GlcNAc | **U**ridine-**d**iphos**p**hate-***N***-**a**cetyl-D-**glu**cosamine |
| U/ml | **U**nits per **m**illi**l**iter |
| UTP | **U**ridine **t**ri**p**hosphate |
| V | **V**olts |
| WGA | **W**heat **g**erm **a**gglutinin |
| YM | **Y**east **m**alt extract |
| YPAD | **Y**east **p**eptone **a**denine **d**extrose |

## 1.0 Introduction

Chitin is the second most abundant biopolymer present in nature. It is an unbranched polysaccharide of β(1-4) linked *N*-acetyl, D-glucosamine units. As much as 10 giga tones of chitin is produced and degraded by microorganisms each year. Chitin is present in the cell walls of fungi, exoskeletons of insects and crustaceans. It plays an important role in morphogenesis and survival of the organisms. In addition to the protective covering that maintains structure, it also serves as a dynamic interphase between cytosol and the outer environment which allows changes during growth and differentiation.

In *S. cerevisiae* chitin is an important component of the cell wall and the septum, a structure that separates the mother cell from the daughter. Within the cell wall, chitin cross-links with glucan (Kapteyn *et al*., 1999; Cabib and Duran, 2005). Glucan comprises a major portion (about 60%) of the yeast cell wall. Most of it is β(1-3) glucan and gives rigidity to the cell wall. A small amount of β(1-6) glucan is present interspersed among glucan-protein network. The outer side of the cell wall consists of a highly glycosylated layer of mannoproteins (see pictorial diagram, page 2). Chitin is highly concentrated at the site of the primary septum where it is deposited as ring at the site of incipient bud. This ring remains in the neck during bud emergence and growth. Once the bud has acquired sufficient growth, the plasma membrane invaginates and the disk of chitin is deposited in the neck. The secondary septum is then formed around both sides of the disk forming a trilayered septum. Later after the septation is complete, chitin is deposited in the lateral walls of the bud. Though present in small amount (1-3%), its presence in the cell wall is vital for growth and survival in yeast (Shaw *et al*., 1991). In coordination with the other components, chitin provides rigidity to the cell wall and maintains

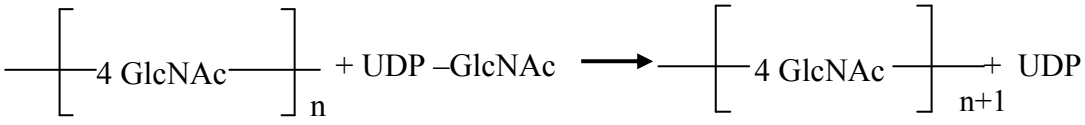
integrity during cell growth and developmental programs like mating and sporulation.




**A model for molecular architecture of the yeast cell wall**.
The internal skeletal layer consists of β(1-3) glucan molecules. At the outer side, cell wall proteins are linked to the non-reducing ends of β(1-3) glucan molecules either directly or indirectly through an interconnecting β(1-6) glucan moiety. A small amount of chitin is present linked to glucan network (Adapted from Smits *et al*., 2001). GPI-CWP-GPI anchored cell wall protein; 1, 6-b-Glucan - β(1-6) glucan.

At the molecular level chitin is synthesized by chitin synthases (CS, EC-2.4.1.16). CS enzymes catalyze the transfer of *N*-acetyl glucosamine (GlcNAc) to the growing chain of β(1-4) linked *N*-acetyl glucosamine residues.

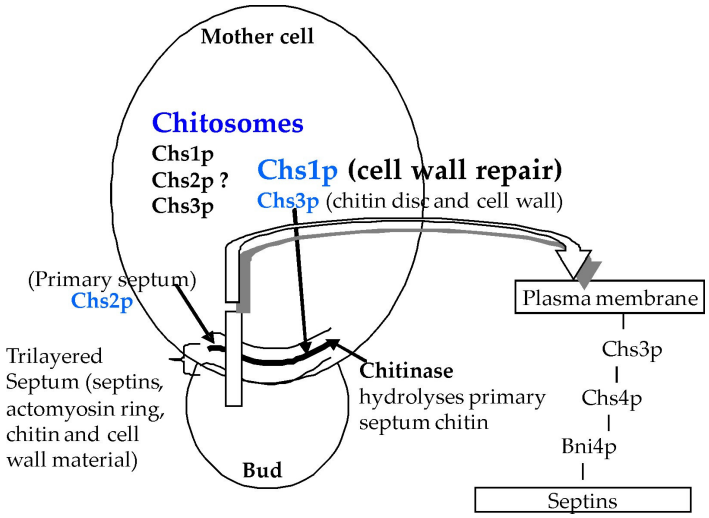
Chemically the reaction proceeds as follows:




Association of the linear chains through extensive hydrogen bonding leads to the formation of chitin. In its deacetylated form, known as chitosan, it is also present in

the spores of *S. cerevisiae* and various fungi (Britza *et al*., 1988). Cell walls of *Mucor rouxii* contain chitin and chitosan in the ratio of 1:3 (Bartnicki-Garcia and Nickerson, 1962). *In vivo* chitosan is formed by concomitant deacetylation of nascent chitin chains by chitin deacetylase (EC 3.5.1.41; Davis and Bartnicki-Garcia, 1984). Owing to unique properties such as fat absorption and cell binding chitosan is considered potentially useful for numerous applications ranging from biomedical to industrial.

Biosynthesis and incorporation of chitin during the life cycle of *S. cerevisiae* is a complex process. As can be presumed, the cell employs more than one CS enzymes to carry out these tasks. In *S. cerevisiae*, chitin synthesis is carried out by three main chitin synthase proteins Chs1p, Chs2p and Chs3p which are required for CSI, CSII and CSIII activity respectively (Bulawa 1993; Cabib *et al*., 1982; Orlean, 1987; Sburlati & Cabib, 1986) (see following Cartoon).




**Diagrammatic model describing the interplay of various proteins involved in chitin formation during septation and cell wall synthesis**
Chs1p - Chitin synthase 1 protein; Chs2p - Chitin synthase 2 protein; Chs3p - Chitin synthase 3 protein; Chs4p, Bni4p and Septins - proteins involved in anchoring Chs3p to the plasma membrane.

CSI activity is found located in the plasma membrane and has been concluded as zymogenic protein that requires proteolytic activation (Duran & Cabib, 1978; Kang *et al*., 1984). In recent reports it is implicated to be required in cell wall repair. In the absence of CSI, holes are formed in the birth scar of the daughter cells after separation (Cabib *et al*., 1989). Chs1p (CSI protein) is quite stable in the cells (Choi *et al*., 1994a) and its levels do not fluctuate significantly during the cell division cycle (Ziman *et al*., 1996).

CSII functions in the synthesis of chitin during primary septum formation. Its activity is tightly regulated and has an optimum just before the cell separation (Choi *et al*., 1994a). CSII activity requires processing of Chs2p.

CSIII is involved in chitin synthesis of the ring at the future bud site and lateral cell walls. Thus it contributes to synthesize the major part (90%) of the chitin in yeast (Valdivieso *et al*., 1991; Orlean, 1997). It is also implicated in chitin formation at the base of Schmoo projection during mating and sporulation. In contrast to CSI and CSII, CSIII activity can be measured without the proteolytic treatment (Choi *et al*., 1994b). The gene *CHS3* encodes Chs3p for CSIII activity (Valdivieso *et al*., 1991) and requires several other proteins including products of genes *CHS4*, *CHS5*, *CHS6*, *CHS7*, *MYO2*, *TIG1* and *BNI4* (Bulawa, 1992; DeMarini *et al*., 1997; Santos and Snyder, 1997; Trilla *et al*., 1997 and Ziman *et al*., 1998). Chs5p, Myo2p and Tig1p help in polarized localization of Chs3p, whereas Chs6p mediates its translocation from chitosomes to the cell surface. Chs7p is required for the exit of Chs3p from endoplasmic reticulum (Trilla *et al*., 1999). Chs4p is required to activate Chs3p and to anchor it to the septins via Bni4p (DeMarini *et al*., 1997). Two other proteins namely Ptdlns(4)p and Pik1p help in the forward transport of Chs3p to the cell periphery (Schörr *et al*., 2001). Interestingly, none of the CS enzymes has a signal sequence but all of the three enzymes use a secretory pathway to reach its site of action (Cabib *et al*., 2001).

On the basis of fractionation studies, it has been shown that Chs1p and Chs3p are present both in the plasma membrane and vesicular compartments called as chitosomes (Leal–Morales *et al*., 1988, 1994; Chuang and Schekman, 1996; Ziman *et al*., 1996; Flores and Schwenke, 1988). These vesicles are assumed to be involved in CS transport to the sites of action. Chitosomes can be gained best from the fungi *Mucor rouxii* and *Neurosopra crassa*. Sedimentation analysis of the chitosomes has revealed that they have a low buoyant density (1.14-1.15 g/cm$^2$; Ruiz Herrera *et al*., 1984; Kamada *et al*., 1991) and can be fractionated distinctively from the other cellular structures. Chitosomal particles from *M. rouxii* (Ruiz Herrera *et al*., 1975, 1976) as well as from *S. cerevisiae* (Leal-Morales *et al*., 1988, 1994) measure between 40-70 nm in size. However due to rapid protein transport *in vivo* a steady state detection of these vesicles have been difficult (Novick *et al*., 1980). Treatment of the chitosomes with digitonin dissociates them into smaller (16S) particles of molecular weight of approximately 5 X $10^5$ still retaining CS activity (Ruiz-Herrera, 1980).

Although in recent years a vast knowledge about the presence and function of different CS proteins has been generated, data on their biogenesis has not increased further. Recent research had focused more on investigating new genes and the associated ORFs involved in chitin synthesis. With the possibility of generating mutants for each gene it has been possible to distinguish their individual function. Using new methods of molecular cloning, numerous chitin synthase genes have been identified in several organisms such as insects, bacteria, protozoa and vertebrates (Bulawa and Wasco, 1991; Gagou *et al*., 2002; Semino *et al*., 1996). On the basis of the conserved regions and the presence of accessory domains, chitin synthases are classified in six categories from class I to VI (Bowen *et al*., 1992; Ruitz-Herrera *et al*., 2002; Roncero, 2002). Their number ranges from one in *Schizosaccharomyces pombe* to seven in *Aspergillus fumigatus* (Roncero, 2002).

Several CS isoenzymes have been reported from some species in varying numbers (for e. g. from filamentous fungus *Aspergillus nidulans*, human pathogen *Wangiella dermatitidis*, *Cenorhabditis elegans*, *Aedes aegypti* and *Fusarium oxysporum*). The transport and localization of some CS proteins have been investigated by protein tagging and immunofluorescence microscopy.

Using advanced techniques of protein separation (2-dimensional gel electrophoresis, HPLC) and identification (Mass spectrometry); it is now possible to work with very small quantities of protein samples. A few micrograms of a protein are sufficient for its identification using mass spectrometry.

In the present study it is attempted to identify and characterize interacting protein partners of the chitosomal complex using a combination of these advanced techniques that has not been attempted and/or possible in earlier times.

# 2.0 Material and methods

## 2.1 Materials

All the standard chemicals used in this study were purchased from Difco Labs, Sigma, Serva, Fluka, Roth, Roche, Fermenta Pharma, Amersham Biosciences, Riedel de Häen, Qiagen and Merck. Matrices and columns (used for protein purification) were obtained from Pharmacia Biotech and Amersham Biosciences. Sources of the specific materials used are indicated as and where they appear.

## 2.1 Media

### 2.1.1 Yeast peptone adenine dextrose (YPAD)[a]

| | |
|---|---|
| Yeast extract | 20 g |
| Dextrose | 10 g |
| Peptone | 20 g |

### 2.1.2 Yeast malt extract medium (YM)[a]

| | |
|---|---|
| Yeast extract | 4 g |
| Malt extract | 10 g |
| Dextrose | 0.4 g |

[a] Double distilled water was added to 1 liter, adjusted to pH 6.5 and autoclaved (121 $^{0}$C, 15 min).

### 2.1.3 Luria Burtani (LB) Medium (pH 7.5) [b]

| | |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5 g |
| NaCl | 10 g |

### 2.1.4 M9 Minimal Medium (pH 7.4) [b] for *E. coli* strain expressing CHB1

| | |
|---|---|
| $Na_2HPO_4$ | 6 g |
| $KH_2PO_4$ | 3 g |
| NaCl | 0.5 g |
| $NH_4Cl$ | 14 g |

[b] Doubled distilled water was added to 1 liter and autoclaved (121 $^0$C, 15 min). After autoclaving, the following compounds were added (Per liter)

| | |
|---|---|
| $MgSO_4$ (1 M) | 2 ml |
| Glucose (20%) | 10 ml |
| $CaCl_2$ (1 M) | 0.1 ml |
| For *E. coli* strain (M15 pREP4), added following concentrations of antibiotics and/or vitamins | |
| Ampicillin | 1 µg |
| Thiamine | 5 µg |
| Kanamycin | 25 µg |

## 2.2 Buffers

### 2.2.1 Following standard buffers were prepared according to the lab protocols

Sodium phosphate buffer: $Na_2HPO_4/NaH_2PO_4$

Potassium phosphate buffer (CHS): $KH_2PO_4/K_2HPO_4$

Citrate phosphate buffer: Citric acid/$Na_2HPO_4$

### 2.2.2 Western transfer buffer

| | |
|---|---|
| CAPS | 10 mM |
| Methanol | 10% |
| Prepared in double distilled water and adjusted to pH 11.0 with 1 N NaOH | |

### 2.2.3 Acetonitrile (ACN) buffers for protein purification via HPLC

| | |
|---|---|
| Buffer A | Water/ACN/TFA (tri-fluoroacetic acid): 95% / 5% / 0.1% |
| Buffer B | Water/ACN/TFA : 20% / 80% / 0.08% |

**2.2.4 Buffers for blue native PAGE:**

| | |
|---|---|
| Anode buffer | 50 mM Bis-Tris/HCl, pH 7.0 |
| Cathode buffer | 50 mM tricine, 15 mM Bis-Tris/HCl, pH 7.0 (plus 0,02% Coomassie blue G-250) |
| Gel buffer (3 X) | 150 mM Bis-Tris/HCl, 1.5 M amino-caproic acid, pH 7.0 |

**2.2.5 4X sample loading buffer for SDS-PAGE**

| | |
|---|---|
| Tris/HCl, pH 6.8 | 0.25 M |
| SDS | 8% (w/v) |
| β-mercaptoethanol (β-Me) | 20% (v/v) |
| Glycerol | 40% (v/v) |
| Bromophenol blue (BPB) | 0.1% (w/v) |

**2.2.6 Coomassie brilliant blue (R-250) staining solution**

| |
|---|
| 0.2% Coomassie R-250 in 50% methanol and 10% acetic acid (prepared in double distilled water and filtered through 200 μm Whatman filter paper). |

### 2.2.6 Silver and zinc staining of the polyacrylamide gels

#### 2.2.6.1 Silver staining

**Solutions for silver staining**

| | |
|---|---|
| Solution I | 10% Tri-chloroacetic acid |
| Solution II | 1 g $Na_2B_4O_710H_2O$ (borax)<br>98 ml water and 2 ml glutraldehyde |
| Solution III | 0.4 g silver nitrate in 4 ml water |
| Solution IV | Solution III in 70 ml water containing 1.84 ml NaOH (1 M) and 1.96 ml ammonia |
| Solution V | 6 mg citric acid and 50 µl formaldehyde in 100 ml water |
| Solution VI | 10% acetic acid |

**A brief protocol for silver staining of SDS and/or native polyacrylamide gels**

**Step I** Soaking the gel in solution I for 20 min

**Step II** Washing with double distilled water x 2 times

**Step III** Fixing in solution II, 10 min

**Step IV** Washing in double distilled water x 3 times

**Step V** Incubation in solution IV, ca. 10-20 min (in a dark chamber and shaking)

**Step VI** Washing in double distilled water x 7 times

**Step VII** Incubation in solution V (30 sec to 20 min, until the protein bands appear)

**Step VIII** De-staining in 10% acetic acid

### 2.2.6.2 Zinc staining

Zinc staining of the gels was carried out as per the instructions of the manufacturer (Geno Tech).

## 2.3 *E. coli* and *S. cerevisiae* strains used in the present study

| **Name of the strain** | **Genotype** | **Source** |
|---|---|---|
| *E. coli* (CHB1) | M15 (pREP4) harboring recombinant plasmid pBA1-*chb1* | AGM |
| *S. cerevisiae* (wild type) | S288C | Entian, Frankfurt |
| *S. cerevisiae* (*chs1* mutant) | BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YNL192w::kanMX4 | Entian, Frankfurt |
| *S. cerevisiae* (*chs3* mutant) | BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YBR023c::kanMX4 | Entian, Frankfurt |
| *S. cerevisiae* (CSI-HA) | [*MAT***a** *leu2-Δ98cry1*$^{R}$/*MATα* leu2-Δ *98CRY1 ade2-101 HIS3/ade2-101 his3-Δ*200 *ura3-52 cani*$^{R}$/*ura3-52CAN1 lys2-801/lys2-801 CYH2/cyh2*$^{R}$ *trp1-1/TRP1 Cir*$^{0}$] carrying pGAL-*cre* (*amp, ori, CEN, LEU2*) | Open-Biosystems |

## 2.4 Antibodies used in this thesis

The antibodies used in the present study were hired from the following sources:

| **Name of the antibody** | **Source** | **Raised in host** | **Dilutions** | |
|---|---|---|---|---|
| | | | **Primary antibody** | **Secondary antibody** |
| Anti-CHB1 | AGM | Rabbit | 1:5000 | 1:10,000 |
| Anti-GAPDH (yeast) | Abcam Ltd 332 Cambridge Science Park, Milton Road Cambridge, UK | Do-- | 1:2000 | 1:10,000 |
| Anti-Enolase (*Trypanosoma cruzi*) | V Hannaert, Univ Catholique De Luvain, Bruxelles, Belgium | Do-- | 1:10,000 | 1:10,000 |
| Anti- pyruvate kinase 1 (Pyk1) (yeast) | J Thorner, University of California, Berkeley, CA, USA | Do-- | 1:2000 | 1:10,000 |
| Anti-HA (Haem-agglutinin) | Sigma | Do-- | 1:100 | 1:2000 |
| Anti- pyruvate decarboxylase (Pdc) (Yeast) | Stefan Koenig, Martin-Luther-Universitaet Halle-Wittenberg, Halle, Germany | Do-- | 1:2000 | 1:10,000 |

## 2.5 Biochemical methods

### 2.5.1 Cultivation of yeast

*S. cerevisiae* strains (wild type, *chs1* and *chs3* mutants respectively) were cultivated in yeast malt extract (YM) and CSI-HA strain in yeast peptone adenine dextrose (YPAD) media. A loopful of the cells from a freshly streaked strain from an agar plate was suspended in 500 ml of the respective medium and allowed to grow overnight. Overnight grown cells were diluted to $A_{600}$ of 0.4 in 1 liter media and were grown further to $A_{600}$ of 0.85-0.9 (log phase). Unless otherwise indicated, the cells were grown at 30 $^0$C at a speed of 120 rpm in 2 liter Erlenmeyer flasks in a standard YM medium.

### 2.5.2 Assays for chitin synthase activity in isolated chitosomes

1. WGA based assay
2. Chitin binding protein (CHB1) based assay

#### 2.5.2.1. WGA based assay

A stock solution of wheat germ agglutinnin (WGA, 10 mg/ml) was prepared in sterile water. The WGA stock solution was diluted 1/20 in water and 100 µl was added to the wells on a 96 well microtiter plate and allowed to bind for 16 hrs at room temperature. Plates were washed extensively to remove unbound WGA. To block the uncovered areas in the wells, 300 µl of 2% BSA (prepared in 50 mM Tris/HCl, pH 7.5) was added to the wells and incubated at room temperature for 3 hrs. The plates were then stored at minus 20 $^0$C until further use. Chitin synthase activity in the gradient purified fractions was

determined using the method of Lucero (Lucero *et al*., 2002), in 50 µl fraction volume. The substrates were used at a final concentration as follows:

| | |
|---|---|
| GlcNAc | 80 mM |
| UDP-GlcNAc | 4 mM |
| $MgCl_2$ | 10 mM |
| In 1X CHS buffer (50 mM, $K_2HPO_4/KH_2PO_4$, pH 6.5*) | |

* For assaying of three chitin synthase activities, separate conditions were used according to the following table

| **Enzyme** | **Ion requirements** | **Assay pH,** (1X CHS buffer) |
|---|---|---|
| CSI | $MgCl_2$, 10 mM | 6.5 |
| CSII | $CoCl_2$, 3.2 mM | 8.5 |
| CSIII | $CoCl_2$ (3.2 mM) and $NiCl_2$ (10 mM) | 8.5 |

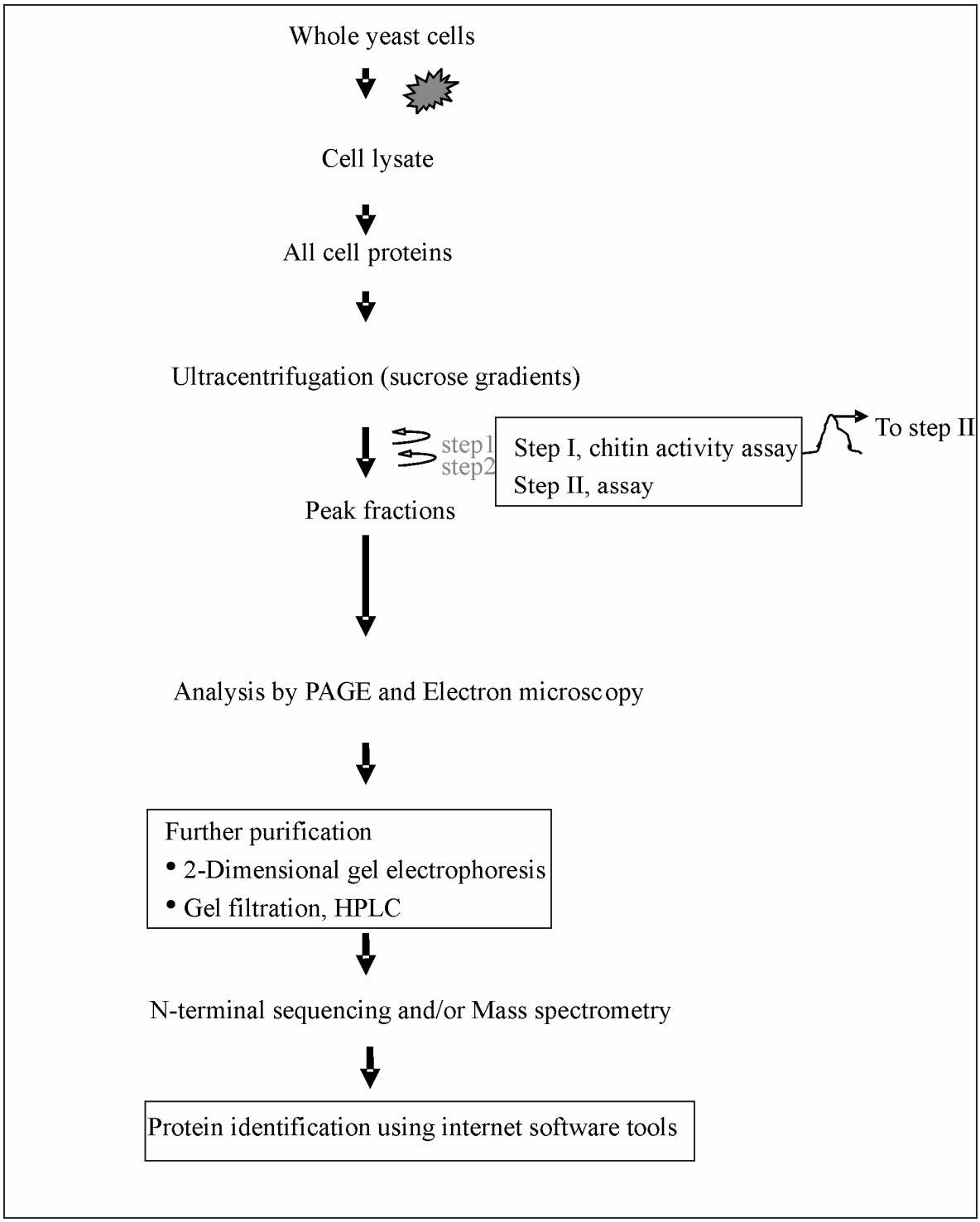
The chitin synthase reaction was allowed to take place for 2 hrs or overnight on WGA coated plates. After the reaction, plates were washed two times with distilled water and incubated with 1 µg/ml horseradish peroxidase (HRP) conjugated WGA (WGA-HRP, prepared in 2% BSA in Tris/HCl, pH 7.0) for 30 min at room temperature. Thereafter the plates were extensively washed with distilled water (five times) and the reaction was developed using substrates for HRP (0.8 mM TMB, prepared in DMSO), 2.15 mM $H_2O_2$ in 100 mM sodium citrate/acetate buffer, pH 3.7). The reaction was stopped by the

addition of 100 µl 1 N $H_2SO_4$ and yellow colored product was monitored spectrophotometrically at 450 nm (on a Tecan Sunrise microplate reader).

**2.5.2.2. Assay using chitin binding protein (CHB1)**

Chitin binding protein, CHB1 was purified from *E. coli* to homogeneity (for CHB1 purification see Section 2.9). Taking the advantage of a His-Tag fused onto CHB1 protein, it was coupled to Ni-NTA agarose beads and used to detect chitin. CHB1 protein (1 µg/ml) was allowed to bind 10 µl of pre-washed Ni-NTA agarose beads for 1 hr at room temperature while shaking. With the help of a magnet device unbound CHB1 was removed and the beads were washed further with 2 x 200 µl, 50 mM 1X CHS buffer (while keeping the eppendorf cup on a magnetic device to hold the beads in place during washing). Depending on the number of chitosomal fractions to be tested, the beads with bound CHB1 were diluted in the same buffer and distributed equally on 96 well Elisa plates. 50 µl chitosome fractions (with substrates; see WGA assay recipe for amounts) were incubated with the beads for variable time periods at room temperature (with shaking). After incubation, the plate was washed (while the plate seated on a magnetic device) two times with water. To detect bound chitin, 100 µl of 1 µg/ml WGA-HRP conjugate, that recognizes chitin, was incubated with beads for 30 min at room temperature while shaking. Plates were thereafter washed five times with water and the reaction was developed as described for WGA assay. Absorbance for the yellow color was recorded at 450 nm.

**2.6 Isolation and subsequent purification of chitosomes using sucrose gradients (flow diagram below shows a brief summary of the protocol)**

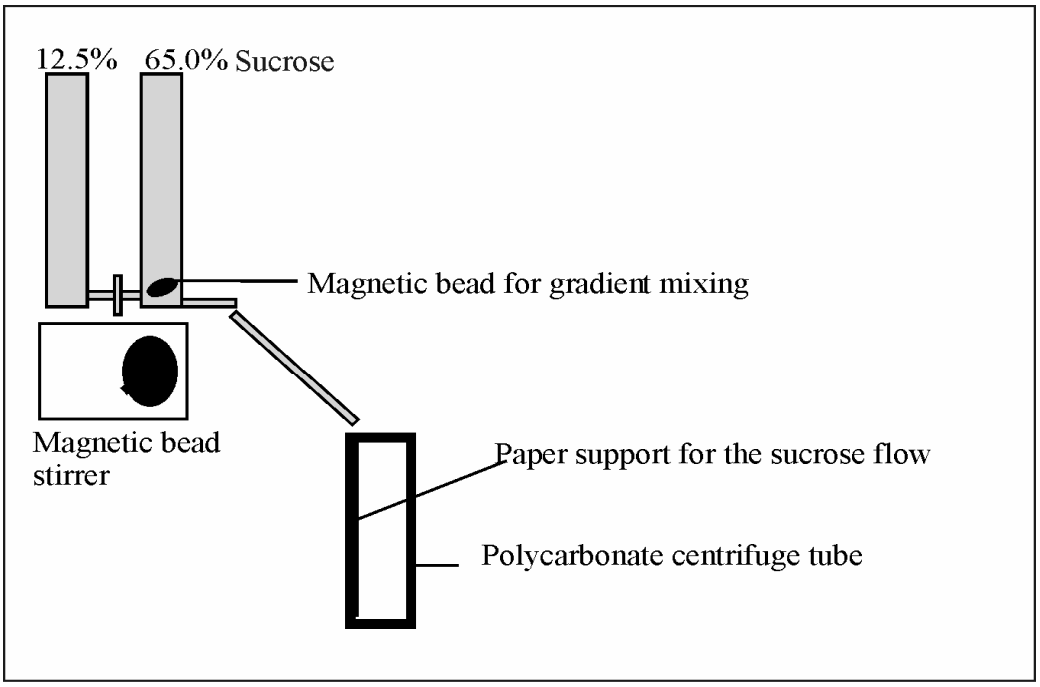


Log phase grown cultures ($A_{600}$: 0.85-0.90) were pelleted at 7000 rpm for 7 min using a JA10 rotor in Beckmann Coulter Avanti J25 centrifuge at 4 $^0$C in 500 ml polycarbonate bottles. Cells were resuspended and washed with 0.9% NaCl, two times at the same speed as above. A final wash was given with 1X CHS buffer (50 mM, $K_2HPO_4/KH_2PO_4$, pH 6.5, containing 10 mM $MgCl_2$). Finally the cells were resuspended in 10 ml 1X CHS buffer. For isolation of chitosomes, the cells were broken in a high pressure device (Constant Cell Disruption Systems) using three cycles of breakage at 1.35 K bar pressure. The cell extract was then centrifuged at a speed of 16000 rpm for 5 min to remove the cell debris. The supernatant from this step was subjected to a further centrifugation in a mini ultracentrifuge in 5 ml polycarbonate tubes at 38000 rpm for 45 min (to remove the membranous structures from the cellular extract). The supernatant from this step was filtered through 0.22 μm filter and the filtrate was subsequently loaded onto first linear sucrose gradients (20 ml gradient, 12.5 to 65% w/v, prepared in 1X CHS buffer containing 10 mM $MgCl_2$, see pictorial diagram for gradient making) and subjected to centrifugation at 40000 rpm for 4 hrs in a Ti70 rotor in Beckmann optima Ti ultracentrifuge at 4 $^0$C. After centrifugation, the gradients were fractionated using a peristaltic pump at a low speed from bottom to up. 1 ml fractions were collected and stored on ice. Chitin synthase activity in the fractions was determined using either WGA and/or CHB1 based assay (see previous section for CS activity assays). The peak fractions showing maximum chitin synthase activity were pooled together. The final sucrose concentration of the mixed pool was determined by a graphic method and was adjusted to 50% (w/v). This pooled mixture was taken at the bottom of a polycarbonate tube and a cushion of 48% sucrose (ca. 500 μl) was laid on it. A 20-46% (w/v) sucrose gradient was poured on the mixed pool gently. Gradients

were then centrifuged at a speed of 40000 rpm for 16 hrs in the Ti70 rotor and were fractionated as above.

CS activity was determined as described earlier. The fractions exhibiting optimal CS activity were taken for further analysis (Electron microscopy and SDS-PAGE).

**Gradient assembly**




## 2.7 Protein separation via native or SDS polyacrylamide gel electrophoresis (PAGE)

### 2.7.1 Separation of the proteins via SDS-PAGE

For the separation of proteins 25 µl protein sample was boiled for 5 min at 95 $^{0}$C in a protein denaturating buffer containing 8% SDS, 20% β-mercapto-ethanol (β-Me), 40% glycerol and 0.1% bromophenol blue (BPB). Proteins were

separated on 10 or 12% SDS-PAGE according to the standard recipe used in the lab (Molecular biology methods, Manniatis and Sambrook).

### 2.7.2 2-Dimensional blue native PAGE (see Cartoon for general scheme, page 21)

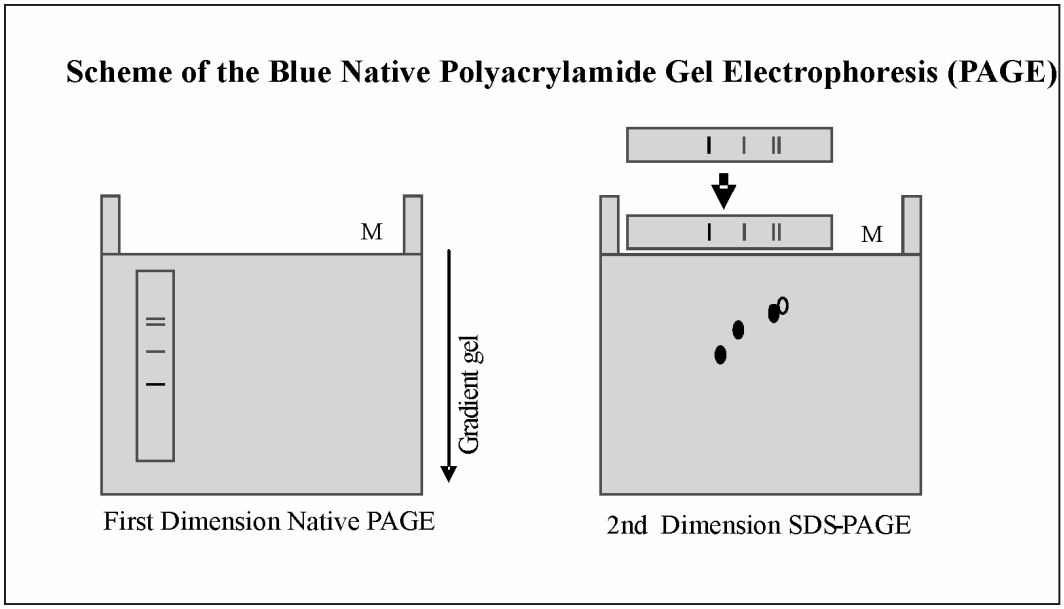
The polyacrylamide gels for blue native PAGE were prepared according to the method of Schaegger (Schaegger *et al.*, 1994). First dimension native gradient gel was prepared using a glass gradient maker according to the following recipe:

| | **6% gel** | **13% gel** |
|---|---|---|
| Water | 2.7 ml | 1.2 ml |
| Gel Buffer(3 X)* | 2 ml | 2 ml |
| Polyacrylamide | 1.2 ml | 2.6 ml |
| Glycerol | - | 1.2 ml |
| APS | 10 µl | 10 µl |
| TEMED | 2.5 µl | 2.5 µl |

*see buffers (Section 2.2.4)

6 ml of the 5% polyacrylamide mix according to the recipe was taken in the left arm of the gradient mixer and another 6 ml of 16% mix in the right arm. After addition of the indicated amounts of ammonium persulfate (APS) and TEMED, the gel solution was poured (while mixing) into the preassembled glass plates. The gel was allowed to polymerize for 20-30 min at room temperature. A 4% stacking gel was then overlaid on the stacking gradient gel. 2$^{nd}$ Dimension tricine-SDS gel (10%) was prepared as follows:

| | **Separating gel** | **Stacking gel** |
|---|---|---|
| Water | 1.194 ml | 3.276 ml |
| Buffer (3 M Tris/HCl pH 8.45, 0.4% SDS,) | 2.0 ml | 1.24 ml |
| Polyacrylamide | 2.172 ml | 0.48 ml |
| Glycerol | 0.8 ml | - |
| APS | 10 µl | 10 µl |
| TEMED | 3 µl | 3 µl |




The first dimension gel was run at a constant voltage of 60V for 1 hr and when the samples entered the separating gel, voltage was increased to 110V until the dye front reached 1 cm from the lower end of the gel. A gel lane containing the separated bands of interest was cut and soaked in a solution containing 15 mM β-Me and 0.4% SDS for 10 min. After incubation, the gel strip was freed of any mercapto-ethanol solution by slowly withdrawing the solution using filter paper. This strip was then transferred to a glass plate. A second plate was then placed above and both plates (having gel strip in between) were assembled.

Outer corners of the plates were sealed with 0.8% agarose. With the help of a 10 ml syringe 10% polyacrylamide gel (prepared according the recipe in the Table) was carefully poured in between the gel plates from one corner until the top keeping a space of 2 cm below the gel strip (obtained from the first dimension). The gel strip was then embedded in a 4% polyacrylamide stacking gel or alternatively 1% agarose. 2$^{nd}$ dimension SDS-tricine gel was run at a constant voltage 150V until the dye front reached the bottom end of the gel. Resolved proteins were visualized via staining the gel with Coomassie Brilliant Blue R-250 or alternatively with Zinc stain (Geno Tech) as required.

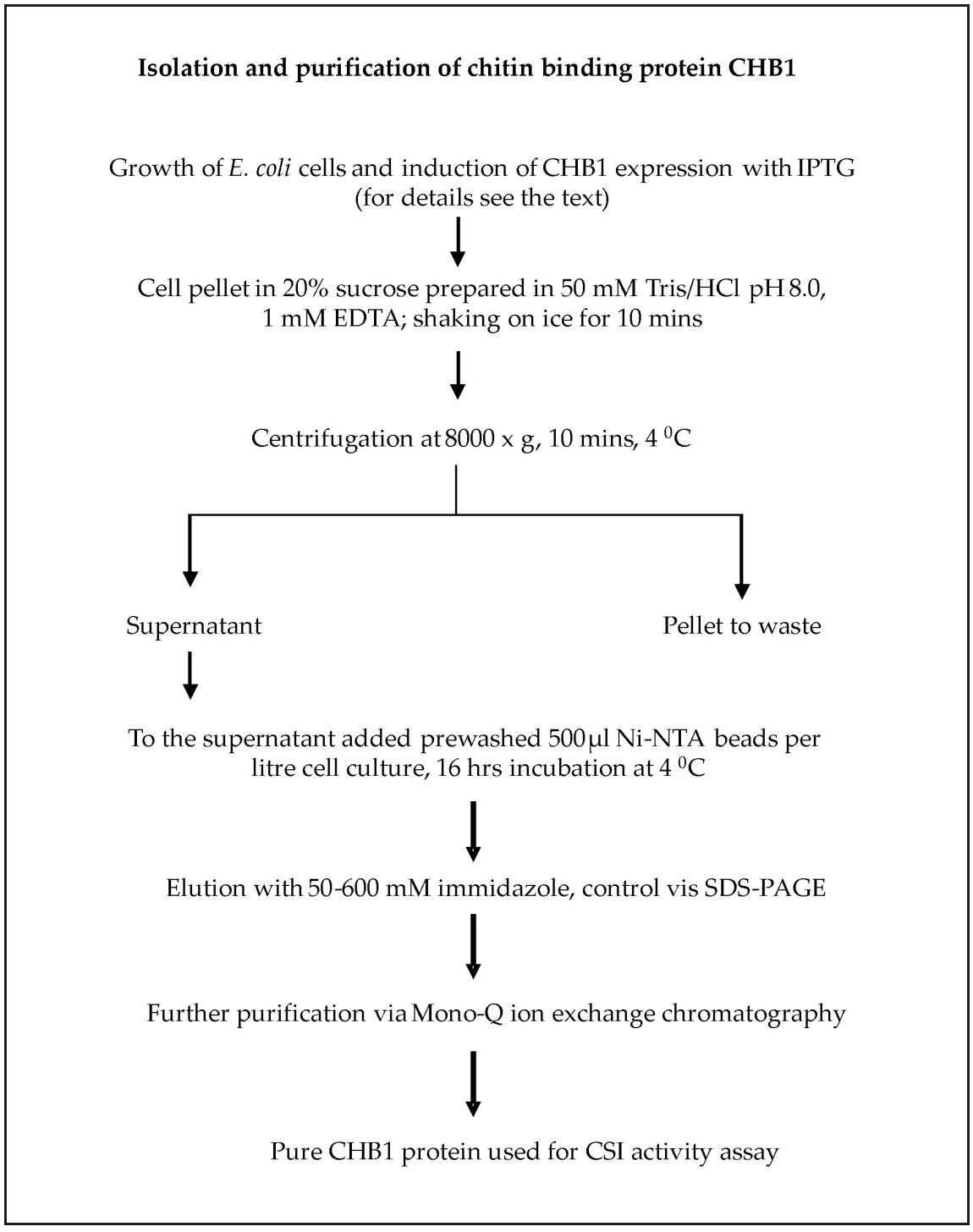
### 2.8 Western blotting

Transfer of proteins to the PVDF membrane was carried out as per the standard protocol. Proteins were separated via SDS-PAGE (10% or 12% gels) and transferred to the PVDF membrane (Fluorotrans membrane, PALL) via a semi dry method using CAPS buffer (for composition see buffers Section 2.2.2). The proteins were transferred using a power supply of 1 mA/cm$^2$ gel for 1.30 hrs. After the transfer, PVDF membrane was treated as per requirements. For detecting the proteins with antibodies, the membrane was blocked with 5% skimmed milk (prepared in 1X phosphate buffered saline, PBS) and further processed for antibody binding. For N-terminal sequencing via Edman degradation, the PVDF membrane was stained with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol for 15 sec and was subsequently destained with 50% methanol containing 10% acetic acid. Protein bands of interest were sequenced commercially (ChromaTech GmBH, Germany) and the obtained amino acid sequences were analyzed via protein-protein BLAST of NCBI. For alignment of amino acids sequences, Clustal W program was used.

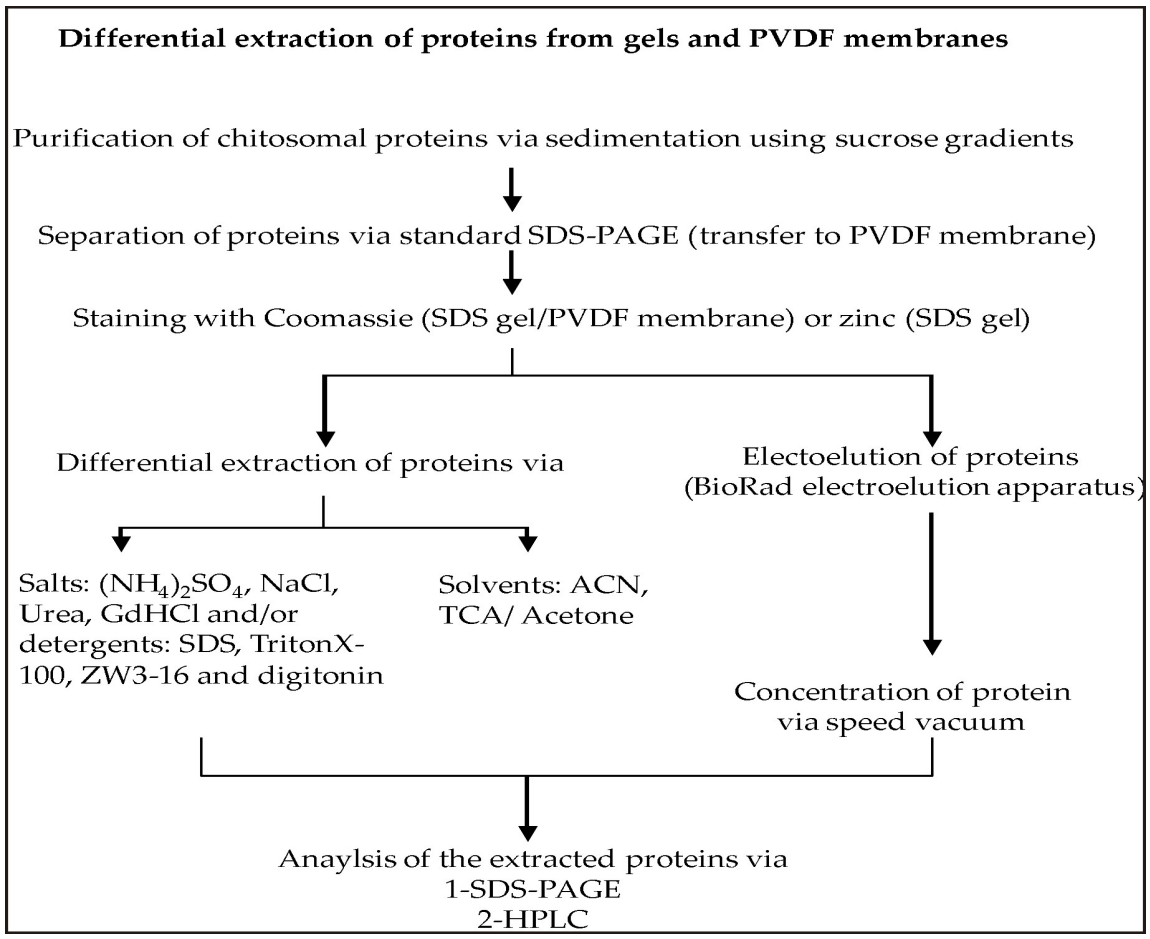
## 2.9 Isolation and purification of the chitin binding protein CHB1

An *E. coli* strain expressing chitin binding protein (CHB1) was grown in LB medium containing appropriate antibiotics overnight (in collaboration with K. Siemieniewicz). Saturated culture was diluted 1:25 in the LB medium and was allowed to grow to an $A_{600}$ of 0.6 at 37 $^0$C at 120 rpm. The culture was induced with 1 mM isopropyl thio-galactoside (IPTG) and further grown for 90 min. The cell pellet was collected by centrifuging the cultures at 10000 rpm for 7 min. Cell pellet was washed two times with 50 mM Tris/HCl-buffer, pH 8.0. The washed cell pellet was resuspended and incubated for 15 min in 20% sucrose prepared in 25 mM Tris/HCl, buffer pH 8.0. After centrifugation at 8000 x g for 10 min, the cell pellet thus obtained was resuspended in 5 mM magnesium sulphate and incubated on ice (shaking) for 10 min. Supernatant of this step was directly incubated overnight with Ni-NTA agarose beads (Qiagen). His-tagged CHB1 was then eluted from Ni-NTA matrix with 50-600 mM immidazole. For further purification, CHB1 containing solution from the above step was diluted 1:50 in 50 mM citrate/phosphate buffer, pH 5.2 and was loaded onto a Mono- Q ion exchange column (Pharmacia Biotech). The

pure CHB1 eluted as a single peak from this column (see results Section 4.2).



**3.0 Extraction of proteins from the gels and PVDF membranes (for a brief protocol see following flow diagram)**




For the separation of specific proteins from the mixture in chitosomal samples, several salts, detergents and solvents were tried. In each case the protein containing sample was incubated separately (different incubation times) with final concentrations of the respective chemicals viz: 30 µl sample plus 2 M NaCl, 3 M GdHCl, 1.5 M GdHCl, 2 and 4 M urea, 1% SDS, 1.8 and 0.9 M $(NH_4)_2SO_4$, different percentages of acetonitrile (ACN, 20-85% v/v) and acetone. In each case protein samples after incubation were analyzed either via HPLC or via SDS-PAGE.

Proteins transferred to PVDF membranes were extracted via incubating the membrane containing bound protein with various concentrations of acetonitrile. Extracted samples were analyzed via HPLC and/or SDS-PAGE.

### 3.1 Determination of protein concentration

Protein concentration in the samples was determined using standard Lowry's method (Lowry's *et al*., 1951). A sample containing protein (100 µl) was taken in a 1.5 ml tube, mixed with 1 ml of solution C and incubated at 37 $^0$C for 5 min. Solution D (100 µl) was then added to the sample and further incubated at 37 $^0$C for 20 min. The generation of the blue color via reduction of Folin-Ciocalteu (FC) reagent was monitored spectrophotometrically at 540 nm.

**Solutions**:

| | |
|---|---|
| Solution A | 2% $Na_2CO_3$ in 0.1 N NaOH 0.5% SDS. |
| Solution B1 | 1% $CuSO_4$ in $H_2O$ |
| Solution B2 | 2% Na-K tartarate in $H_2O$ |
| Solution D | FC reagent (1:2 in $H_2O$) |
| Solution C | 100 ml A + 1 ml B1 + 1 ml B2 |

### 3.2.1 Purification of proteins using HPLC or Gel filtration chromatography

The protein containing sample (100 µl) was applied to the C4 protein purification column (Pharmacia Biotech) which had been equilibrated with Buffer A (see buffers Section 2.2.3). A gradient with buffers A and B was programmed from 0-45 min scale (buffer gradient 25%-80%) and eluted protein

peaks were collected, concentrated via speed vacuum and were analyzed on SDS-PAGE.

In another procedure, the proteins were separated via SDS-PAGE and transferred to the PVDF membrane. The desired protein containing bands were cut and various extraction procedures with detergents and/or solvents were tried (for details see Section 3.0). Extracted samples were subsequently applied to HPLC as above.

### 3.2.2 Purification of proteins via gel filtration chromatography

The samples containing proteins were incubated either with detergent ZW 3-16 or digitonin up to a final concentration of 1% (v/v). The sample (100 μl) was applied to Superdex 200 gel filtration column (Pharmacia Biotech) and/or at times to a hand made 1 ml pipette column prepared using a matrix Bio Gel A. The columns were equilibrated and eluted with 1X CHS buffer containing selected detergent (0.1%). The peak fraction's protein concentration in the eluted samples was either determined via Lowry's method or taking absorbance at 280 nm and analyzed on SDS-PAGE.

## 3.3 Structural analysis of the chitosomes via electron microscopy

For EM analysis, the chitosomal samples were adhered to copper grids of 300 mesh size for 1-10 min. The grids were contrasted with 2% aqueous uranyl acetate and viewed under Zeiss 109 transmission electron microscope (TEM).

### 3.4 Additional enzymatic assays

**3.4.1 UDP-*N*-acetyl-glucosamine pyrophosphorylase assay:** Sucrose gradient purified samples were tested for the presence of UDP-*N*-acetyl-glucosamine pyrophorphorylase in 50 µ1 fractions. The reaction mixture contained 80 mM *N*-acetyl-glucosamine-1-phosphate, 4 mM uridine tri-phosphate (UTP), 10 mM $MgCl_2$ in 1 X CHS buffer.

**3.4.2 Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) assay:** GAPDH activity in the protein samples was determined spectrophotometrically (Delgado *et al*., 2001) in 0.1 M potassium phosphate buffer containing 100 µ1 proteins sample, 1.0 mM NAD, 10 mM EDTA, 0.1 mM DTT and 4 mM glyceraldehyde-3-phosphate in a final volume of 1.0 ml. The NADH formation was monitored at 340 nm for variable time periods from 0-4 hrs.

**3.4.3 Enolase assay:** For measuring enolase activity, 100 µl of the protein sample was incubated in a reaction mixture containing 2 mM D-glycerate-2-phosphate, 1 mM magnesium acetate and 0.01 mM EDTA in a final volume of 1 ml 50 mM Tris-acetate buffer (pH 7.8). Increase in the absorbance was monitored spectrophotometrically at 240 nm (Westhead, 1966).

**3.4.4 Determination of pyruvate kinase activity:** Pyruvate kinase activity in the samples was determined according to the method of Portela (Portela *et al*., 2002) in 50 mM immidazole buffer, pH 7.0, containing 60 mM $MgCl_2$ and 100 mM KCl. Under the standard assay conditions, 100 µ1 sample was incubated with 1.5 mM ADP, 1.5 mM FBP as activator and different amounts of phosphoenol pyruvate (PEP). The reaction was coupled to NADH oxidation by addition of 1U/ml lactate dehydrogenase and 0.22 mM NADH. The time course of the

reaction was monitored at 30 $^0$C by measuring the decrease in absorbance at 340 nm.

**3.4.5 Pyruvate decarboxylase activity:** Pyruvate kinase activity in the samples was determined in 50 mM immidazole buffer, pH 7.0, containing 10 mM $MgCl_2$ and 100 mM KCl. The reaction mixture contained 2 mM thiamine pyrophosphate, 0.2 mM NADH, 5 U/ml of alcohol dehydrogenase (ADH) and 30 mM pyruvic acid. The rate of NADH oxidation was followed at 30 $^0$C by measuring the absorbance at 340 nm (Schmitt and Zimmermann, 1982).

**3.5 Preparation of the protein samples for MALDI-MS and identification of peptide masses**

The peptide mass sequence of the desired proteins was got done via MALDI-MS commercially (ChromaTech GmbH, Germany). Proteins were separated via 1D or 2D gel electrophoresis and protein containing bands were cut from the gels and minced into small pieces. Gel pieces were incubated with ca. 50 µl of 25 mM $NH_4HCO_3$/50% ACN solution, mixed well. The supernatant was discarded and this step was repeated for a couple of times. Gel pieces were then dried in a speed vacuum device for ca. 20 min. 50 µl of Trypsin (12.5 ng/µl trypsin in 25 mM $NH_4HCO_3$, Promega sequencing grade V511A) solution was added to the gel pieces, allowed to rehydrate for 10 min on ice and further incubated overnight at 37 $^0$C. In the next step trypsin solution was removed and the gel pieces were treated with 50 µl peptide extraction solution containing 50% ACN/5% formic acid and sonicated. This step was repeated 2-3 times. The digested peptide solution was mixed with alpha-cyano-4-hydroxycinnamic acid and analyzed via MALDI-MS (Proteome-Analyzer 4700). The masses of the peptides thus obtained were blasted using the MS Fit program of UCSF

(http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Search criteria used for the peptide mass identification were kept standard for e.g. database for mass blast used was *S. cerevisiae.;* MW range, 1 D-250 kDa; pI, 3.0-10; Sample ID, magic bullet; minimum peptides required to match were kept as minimum as possible; Instrument, MALDI-TOF; digest, trypsin; search mode, identity; peptide masses, monoisotropic; mass tolerance was kept minimum (mostly 50 ppm).

# 4.0 Results

## 4.1 Identification and purification of chitosomal complexes from *S. cerevisiae*

For standardization of the isolation conditions for chitosomal complexes, initial experiments were carried out in a wild type strain of *S. cerevisiae* which harbors all the three chitin synthase genes. To isolate chitosomal complexes from other cellular structures, the cell free homogenate was fractionated via a two step isopycnic ultracentrifugation on linear sucrose gradients. Chitin synthase activity in the purified chitosomes was determined using a WGA assay method (Lucero *et al.,* 2002, see Methods). It has been reported in literature that chitosomes can be obtained by single step sedimentation through a sucrose gradient (Ruiz-Herrera *et al*., 1984). However in such a preparation a major portion of the chitosomes are still associated to macromolecular structures, contains ribosomes and other high molecular weight protein complexes. To reduce this contamination, a second step called as the floatation gradient was used (Lending *et al*., 1990; see Methods). Combined fractions exhibiting maximum chitin synthase activity from the first sucrose gradient (indicated via arrow, Fig. 4.1A) were further purified by floatation of the sample from bottom to up in a polycarbonate ultracentrifugation tube through the sucrose gradient (Fig. 4.1B). Based on the activity assay, electron microscopic analysis (see Chapter 4.2) and protein composition pattern (SDS-PAGE), following observations were further verified: (i) three chitin synthase activities from *S. cerevisiae* can be differentiated on the basis of different pH and ion requirements (Choi *et al*., 1994a); (ii) in the wild type strain, major CS activities were observed for CSI and CSIII. Contribution by CSII to the total CS activity in the wild type cells is negligible (Bulawa and Osmond, 1990).

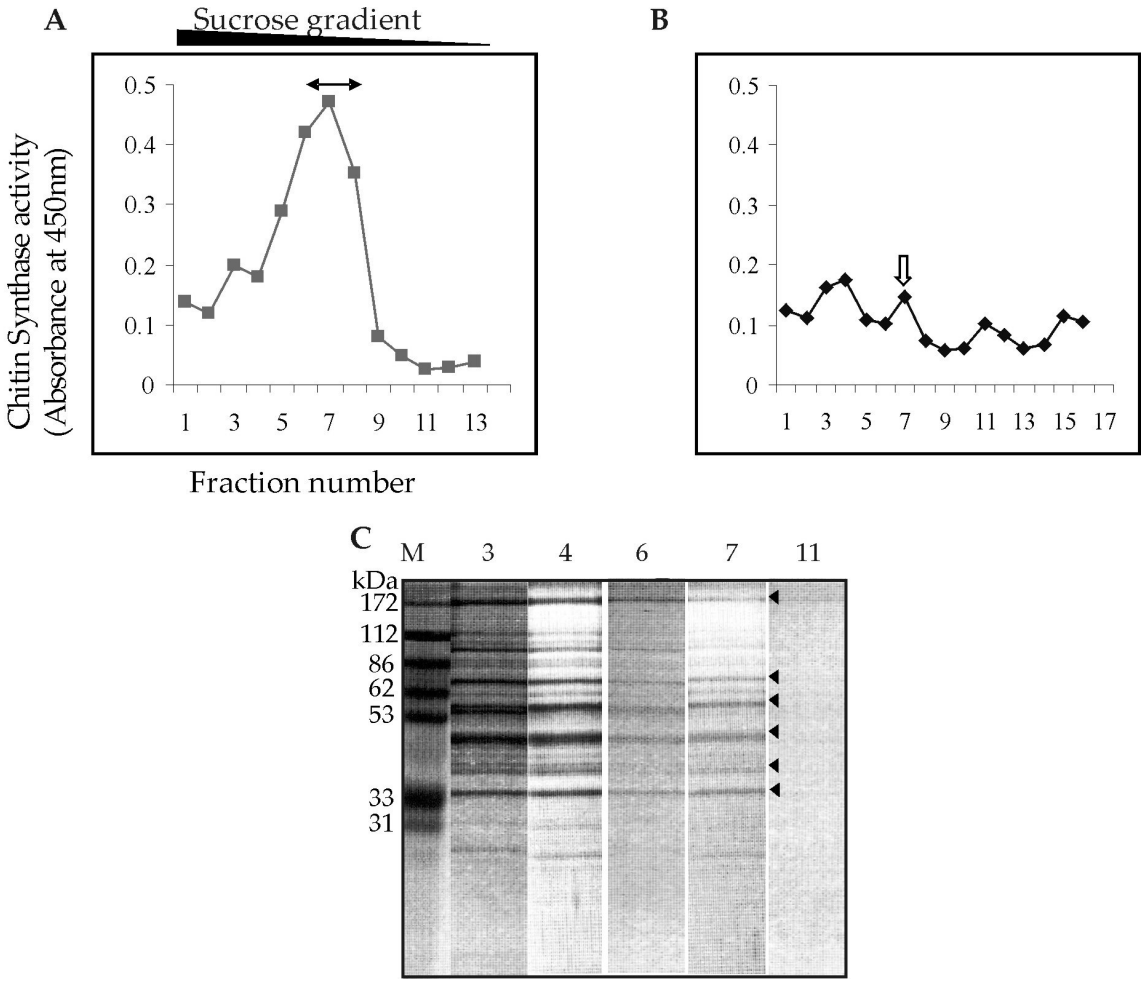



**Fig. 4.1 Purification of chitosomes from a wild type yeast strain**

Chitosomes were purified by a two step isopycnic ultracentrifugation on sucrose gradients. CSI activity (in the presence of $MgCl_2$ and pH 6.5, specific for optimal CSI activity) was determined via WGA assay. **(A)** CSI activity profile after first sedimentation in sucrose gradient (12.5 to 65% w/v). Fractions (indicated by double headed arrow) corresponding to similar activity and/or sucrose concentration were pooled and separated further on a **(B)** floatation sucrose gradient (20-46% w/v). **(C)** Proteins present in the fractions 3-11 of B were separated via SDS-PAGE and stained by Coomassie. Major protein bands are marked via arrow heads.

**Note**- The graph scales used in the results section are kept in a similar format. X-Axis denotes fraction number and represents a decrease in sucrose concentration from left to right and Y-axis shows chitin synthase activity represented via assay absorbance.

For protein composition analysis, the chitosome containing samples were boiled in a buffer containing β-mercaptoethanol (β-Me) and SDS and were separated via SDS-PAGE. The observed protein pattern in those fractions freed from other high molecular weight structures via a floatation gradient (Fig. 4.1B) consists of proteins of dominantly apparent molecular weight of about 37, 48, 55, 62, and a doublet protein band of >175 kDa (Fig. 4.1C, lane 7, indicated via arrow heads). In addition to these, a few other protein bands in trace amounts were also observed in the background. To gain in depth information about differences in the protein composition pattern in chitosomal preparations, yeast strains lacking CSI or CSIII were also investigated. In a *chs1* mutant harboring active CSII and CSIII enzymes, the major CS activity (as determined via WGA assay) is represented by CSIII. The observed protein pattern was almost similar to the wild type strain (data not shown).

It has been concluded earlier that CSI represents major *in vitro* CS activity (approximately 90%) in chitosomal preparations (Shaw *et al*., 1991). Because of this reason a *chs3* mutant, which has an active CSI enzyme, was chosen for further studies. A CSI activity profile as determined via WGA assay, showed a broad peak of the CS activity (Fig. 4.2A) in several preparations in the first step of sedimentation in a sucrose gradient. The fractions exhibiting peak CSI activity from the first step (Fig. 4.2A) from two or three gradients were pooled and subjected to sedimentation via flotation gradients. Activity assays were again conducted to determine the purity of chitosomal preparations (activity profile Fig. 4.2B). The protein profile in the relevant chitosomal fractions yielded five major bands (Fig. 4.2C). A decrease in CSI activity was observed in the presence of CS inhibitor nikkomycin Z (Fig. 4.2D) which further confirms that the fraction contain chitin synthase I activity.

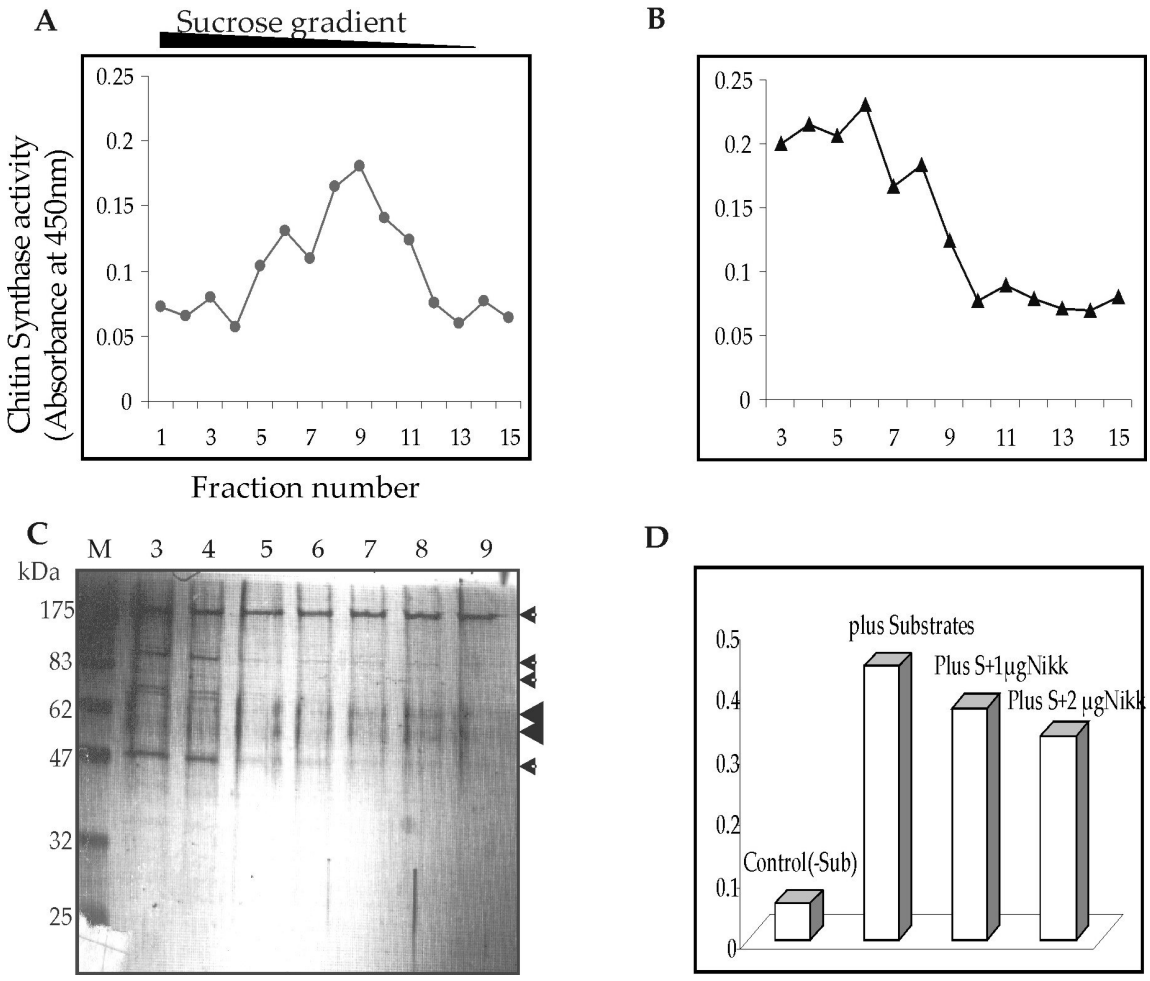



**Fig. 4.2 Purification of chitosomes from the *chs3* mutant strain**
Chitosomes were purified by a two step isopycnic ultracentrifugation as described in previous section. CSI activity profile (as determined via WGA assay) after first **(A)** and second **(B)** sedimentation steps. **(C)** Protein composition pattern in the purified chitosomal fractions after floatation gradient, Coomassie stained gel, (lanes 3 to 9). In addition to major proteins at the apparent molecular weight of about 180, 100, 70, 63 kDa, two shady areas (thick arrow heads) between 47 and 62 kDa could be some membrane proteins. **(D)** Assaying in the presence of different concentrations of nikkomycin Z resulted in a decrease in CSI activity in a peak fraction.

To make the assay more precise and to detect high molecular weight chitin, a novel assay procedure utilizing chitin binding protein (CHB1) was developed. Chitin binding protein from *Streptomyces olivaceoviridis* has been

shown to recognize chitin by the previous studies in the lab (Schnellmann *et al*., 1994). It was cloned in *E. coli* as a His-Tag fusion protein (Svergun *et al*., 2000) (see Chapter 4.2 for purification of CHB1). In a novel strategy, CHB1 His-tagged protein was coupled to Ni-NTA agarose beads. Immobilized CHB1 was used to detect chitin formed by chitosomes. Fractions which contain highly purified chitosomal particles (see Chapter 4.3) were found to exhibit a high CSI activity in presence of $Mg^{2+}$ ions and showed a broad peak in a WGA assay (Fig. 4.3A and B). The results obtained via the CHB1 assay showed variations depending on the quality of preparations. In a representative preparation, the CHB1 assay yielded peak of CSI activity in fractions having lower sedimentation values (Fig. 4.3C and E) for both the gradients as compared to WGA assay. The chitosomal peak that may represent high molecular weight chitin synthesizing population of particles was observed in fraction 1-8 and 1-9 (Fig. 4.3C). Notably these peak fractions contained a few proteins corresponding to the sizes of about 61, 63, 48 and 40 kDa (Fig. 4.4D, lanes 8, 9). Pure fractions in the second gradient although had a lower CSI activity (Fig. 4.3F, lanes 12-14) but contained individual chitosomal particles as viewed under EM. Dominantly four proteins were observed in these fractions: two proteins (doublet) of about 62, third at 48 and fourth protein at the size of 37 kDa (indicated via arrow heads). In addition some proteins in trace amounts are also present in the background. It is worthwhile to mention that preparation of chitosomes on different times did not represent the same profile of CSI activity and correspondingly protein composition also differed.

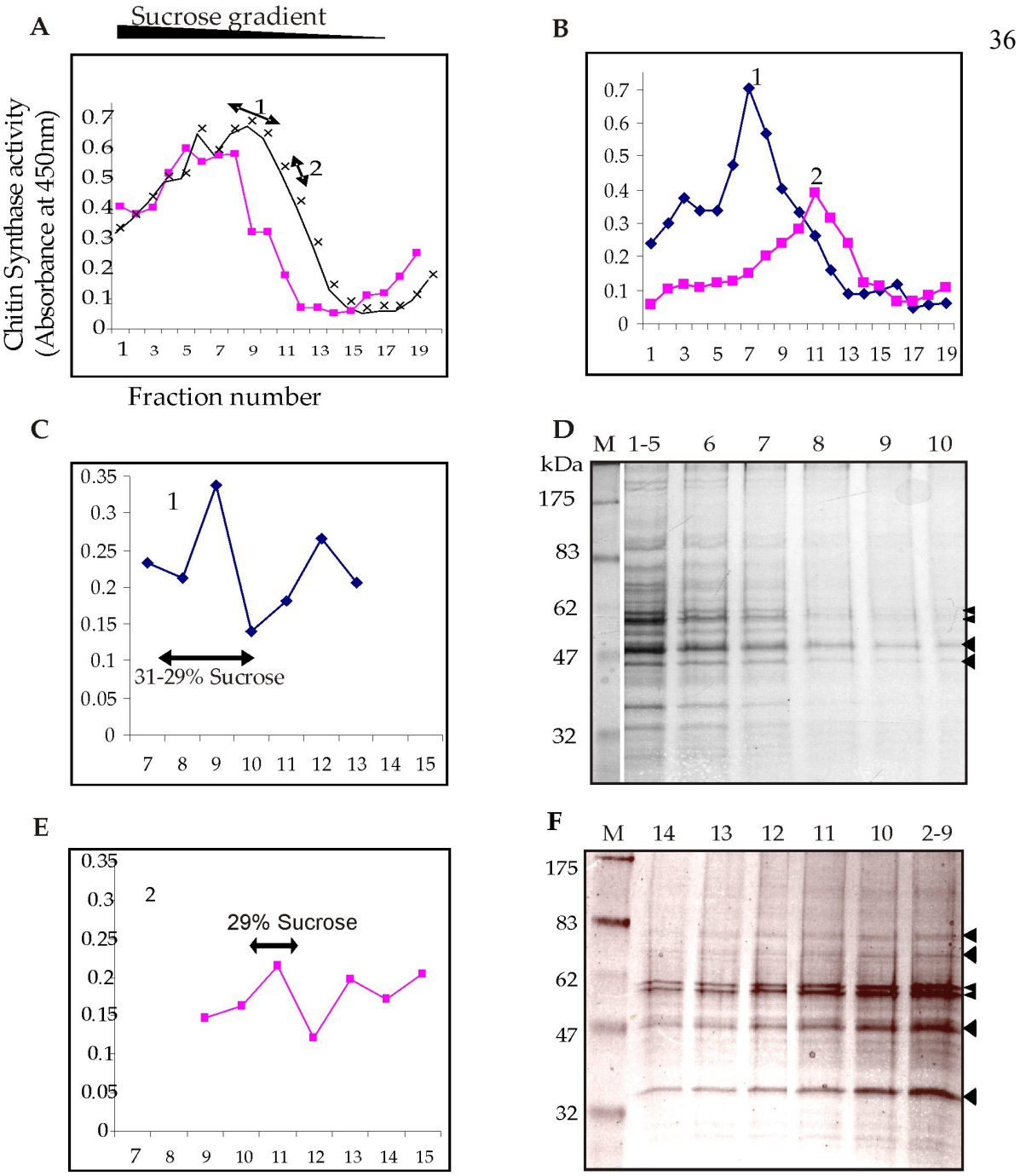



**Fig. 4.3 Comparison of CSI activity as determined via CHB1 and WGA assay in the chitosomal fractions from *chs3* mutant strain**

Chitosomes were purified via sucrose gradients as described in the previous sections. CSI activity was determined via WGA assay. **(A)** and **(B)** CSI activity profile after first and second purification steps. **(C)** CSI activity profile as assayed via CHB1 method, (lane 7-13 of gradient1) and **(D)** protein composition pattern in the first gradient. **(E)** CSI activity profile as assayed via CHB1 method, (lanes 9-15 of gradient 2) and **(F)** protein composition pattern in the second gradient. Major proteins (indicated via arrow heads) at the molecular weight of about 63, 61, 48 and 41 were observed in the first gradient **(D)** where as an additional band at about 37 kDa in second gradient is observed **(F)** and proteins between 70 and 80 kDa (indicated via arrows heads) were present in trace amounts. Arrow heads in C and E indicates concentration of sucrose in the respective fractions.

### 4.2 CHB1 as a tool for chitosomal quality control

Chitin binding protein (CHB1) from *Streptomyces olivaceoviridis* has been shown to recognize chitin by previous studies in the lab (Schnellmann *et al*., 1991). It is a 19 kDa extra cellular protein which is predicted to facilitate the interaction of Streptomycetes with the chitin containing substrates in natural environments (Schrempf, 1999). CHB1 was cloned and expressed (in cooperation with K. Siemieniewiecz) in *E. coli* as a His-Tag fusion protein. Using two steps of purification via Ni-NTA (Fig. 4.4A) and ion exchange chromatography on a Mono-Q column (Pharmacia Biotech) (Fig. 4.4B); it could be purified to homogeneity. In a novel strategy, CHB1-His tagged protein was coupled to Ni-NTA agarose beads. Immobilized CHB1 was used to detect chitin formed by the chitosomes. Thus the use of CHB1 was to distinguish high molecular weight chitin as compared to oligomers of *N*-acetylglucosamine and/or glycoproteins. Observation of the peak chitosomal fractions, tested by CHB1, leads to the conclusion that more pure, isolated chitosomal particles capable of synthesizing high molecular weight chitin were concentrated in fractions having a lower sedimentation values as compared to those tested via of WGA (see Chapter 4.1). Therefore the use of CHB1 proved to be a better option in addition to WGA or radioactivity method, in defining the quality and purity of chitosomal preparations.

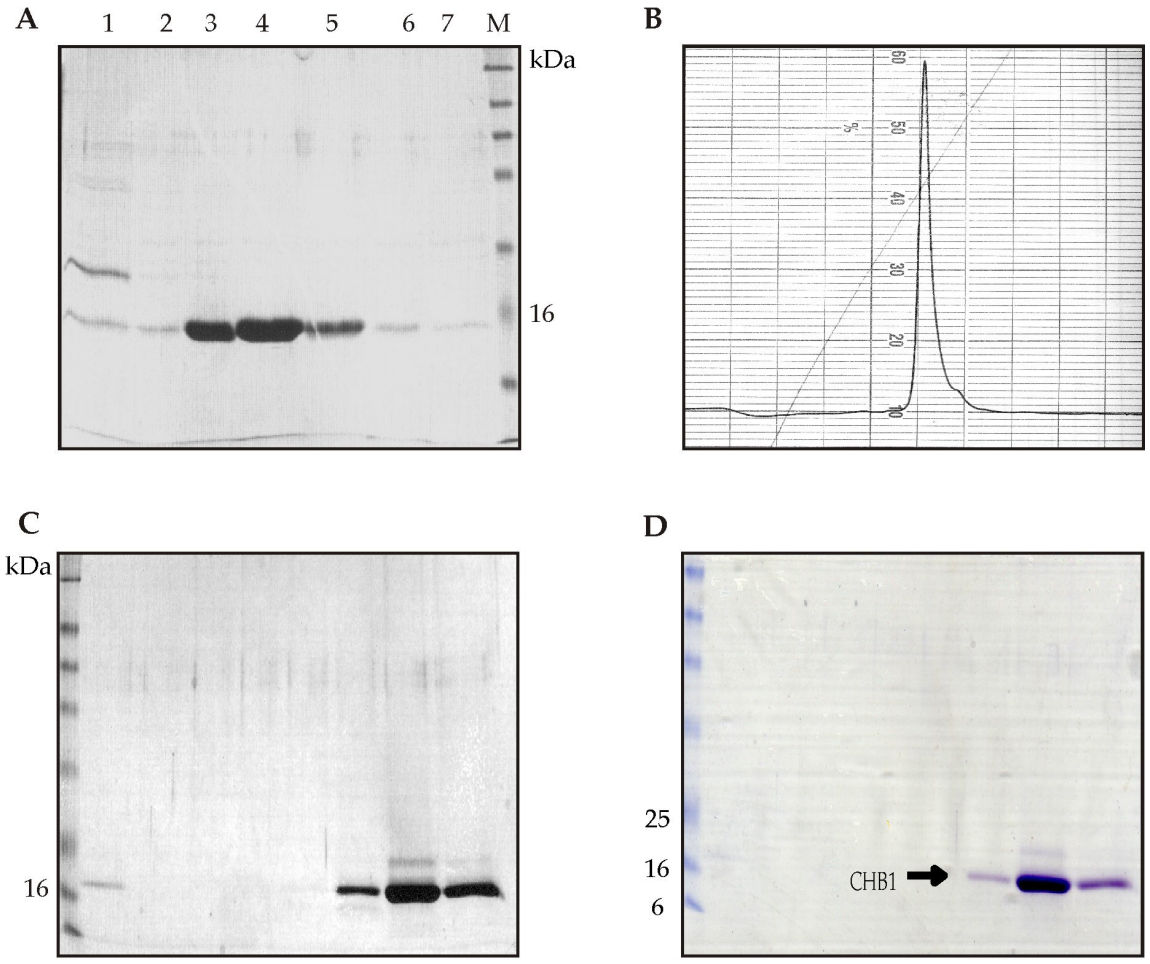



**Fig. 4.4 Purification of chitin binding protein (CHB1)**
The chitin binding protein was purified from *E. coli* using osmotic shock method. **(A)** Elution of CHB1 from Ni-NTA via different concentrations of immidazole. Lane 1 and 2, pre-washing steps with 50 mM immidazole; 3-5, elution with 400 mM and 6-7 with 500 mM. **(B)** Combined fractions 3-5 (from A) were subjected to Mono-Q purification. **(C)** Purified CHB1. **(D)** Detection of CHB1 with anti-CHB1 antibodies.

### 4.3 Electron microscopic examination of chitosomal complexes

The pure preparation of chitosomes was analyzed by electron microscopy (in cooperation with K. Siemieniewicz). Negatively stained chitosomal preparations contained a heterogeneous population of particles of the sizes ranging from 80-120 nm (Fig. 4.5A). After addition of the substrate UDP-GlcNAc and activator GlcNac, these particles synthesized fine chitin fibrils (Fig. 4.5B). By increasing the time of incubation of the particles with the substrates, an extensive network of chitin fibers could be observed (Fig. 4.5C). The synthesized product was indeed chitin was further confirmed in addition to CS activity by (i), incubation of the synthesized chitin with a chitinase isolated from *Streptomyces* led to degradation of the fibrils (Thesis, K. Siemieniewicz, 2004); and (ii), the interaction of fibrils with chitin binding protein CHB1 (Fig. 4.5D). Isolation of chitosomes though carried out under similar conditions, gave rise to a variety of structural differences under the electron microscope and also in protein pattern (SDS-PAGE). If preparations were not processed rapidly, the chitosomes had a collapsed appearance (Fig. 4.5E) and a number of protein bands appeared on SDS gels. Those fractions containing particles with a high sedimentation value (WGA assay, see Fr. 1-6, Fig. 4.3B, Chapter 4.1) also contained membranous and/or fibrillar structures (Fig. 4.5F). Such preparations were not processed further. Freshly prepared chitosomes processed immediately for EM as well as SDS-PAGE analysis gave better results than old preparation stored at cold temperature. The pure chitosomal particles obtained on a good day were processed for further analysis (for example those from Fig. 4.5A).

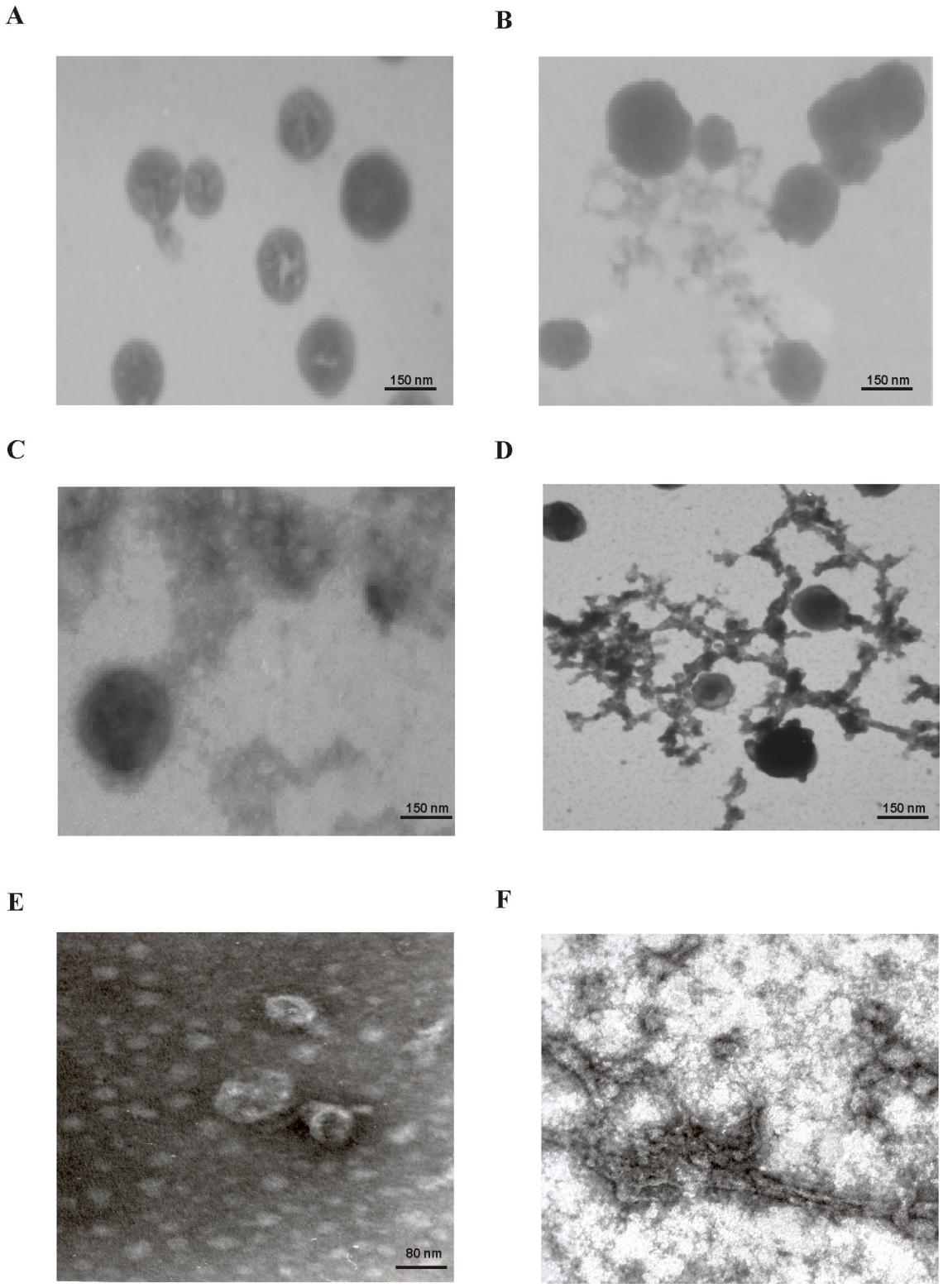



**Fig. 4.5 Electron microscopic examination of chitosomes and chitin fiber formation** Purified chitosomal complexes were viewed under transmission electron microscope (Zeiss TEM 109). **(A)** and **(B)** purified chitosomes without (left) and with (right) substrates after one hour incubation. Longer incubation led to an extensive network of fibers **(C). (D)** Interaction of fibers with CHB1. **(E)** Collapsed chitosomes. **(F)** Filamental structures observed at the peak of CSI activity when assayed with WGA. (Photos A-D were kindly provided by K. Siemieniewiecz)

### 4.4 Evaluation of different extraction conditions for the enrichment of specific chitosomal proteins

Several methods to recover proteins either from gel or from the blotted PDVF membranes were tried. Best extraction results were obtained via incubating the gel containing protein bands with a buffer containing SDS (25 mM Tris/HCl, pH 8.0, 0.1% SDS) and up to 90% of the original protein concentration could be recovered. However, several trials to digest the protein after extraction using this method yielded no satisfactory results. The presence of SDS and Coomassie in the extracted protein might hinder the protease activity. Similar trials to recover the proteins from a PVDF membrane either after incubation with a detergent or extraction via the solvents like acetonitrile failed. No proteins were detected after purifying such an extracted protein via HPLC. Poor recovery and trace amounts of the proteins gained via above methods were insufficient for sequencing purposes. More than 60% of the protein could be recovered via the electro-elution method but the presence of SDS and Coomassie dye in the eluted sample further hindered all protease digestion trials. Finally it was decided to electro-blot the protein and to analyze directly for N-terminal sequence by Edman degradation commercially. For two proteins, as described in the following section, this method worked well. Other proteins which were terminally blocked were cut either directly from the gel and/or further separated via 2D blue native-PAGE and were directly analyzed for sequence information via MALDI-MS (for details see following Section). Purification of the proteins by precipitation using salts or solvents has been used as a means to separate one protein from other or from the mixture of proteins. Several examples attest to the validity of such methods. Many proteins can be purified on the basis of selective solubility in different solvents. Often precipitation is used as a crude

purification step prior to the main purification steps using chromatographic separations. Alterations in pH, solubility preferences in different solvents and/or salting out could be used to differentially separate a single or set of proteins from the crude mixture. Trials were made to selectively separate some specific proteins in the chitosomal fractions using several salts, solvents or detergents like, NaCl, GdHCl, urea, $(NH_4)_2SO_4$, acetone and acetonitrile, SDS, Triton X-100 and ZW3-16, at different concentrations and time incubations. Many proteins were precipitated with 2 M NaCl. A supernatant fraction contained only a few proteins (Fig. 4.6B, lane 2). SDS and Triton did not enrich any proteins (Fig. 4.6B, lanes 3 and 4). The observed results indicated that two or three proteins remained in the supernatant fraction in the presence of 1.8 M $(NH_4)_2SO_4$ (Fig.4.6C, lane 1) whereas most of the proteins were precipitated in the pellet (Fig. 4.6C, lane2). Lowering of the $(NH_4)_2SO_4$ concentration to 0.9 M resulted in the enrichment of one major protein in the supernatant (Fig. 4.6C, lane 3) and two other minor proteins whereas further decrease in $(NH_4)_2SO_4$ gave rise to approximately 50:50 ratio of proteins in supernatant and precipitated pellet fraction (Fig. 4.6C, lane 5 and 6). GdHCl at a concentration of 3 M precipitated few proteins in trace amounts (Fig. 4.6D, lane 2), whereas using urea as precipitant, did not result in the enrichment of specifically desired proteins. None of the methods were found satisfactory in recovering the quantitative amount of respected proteins for identification purposes.

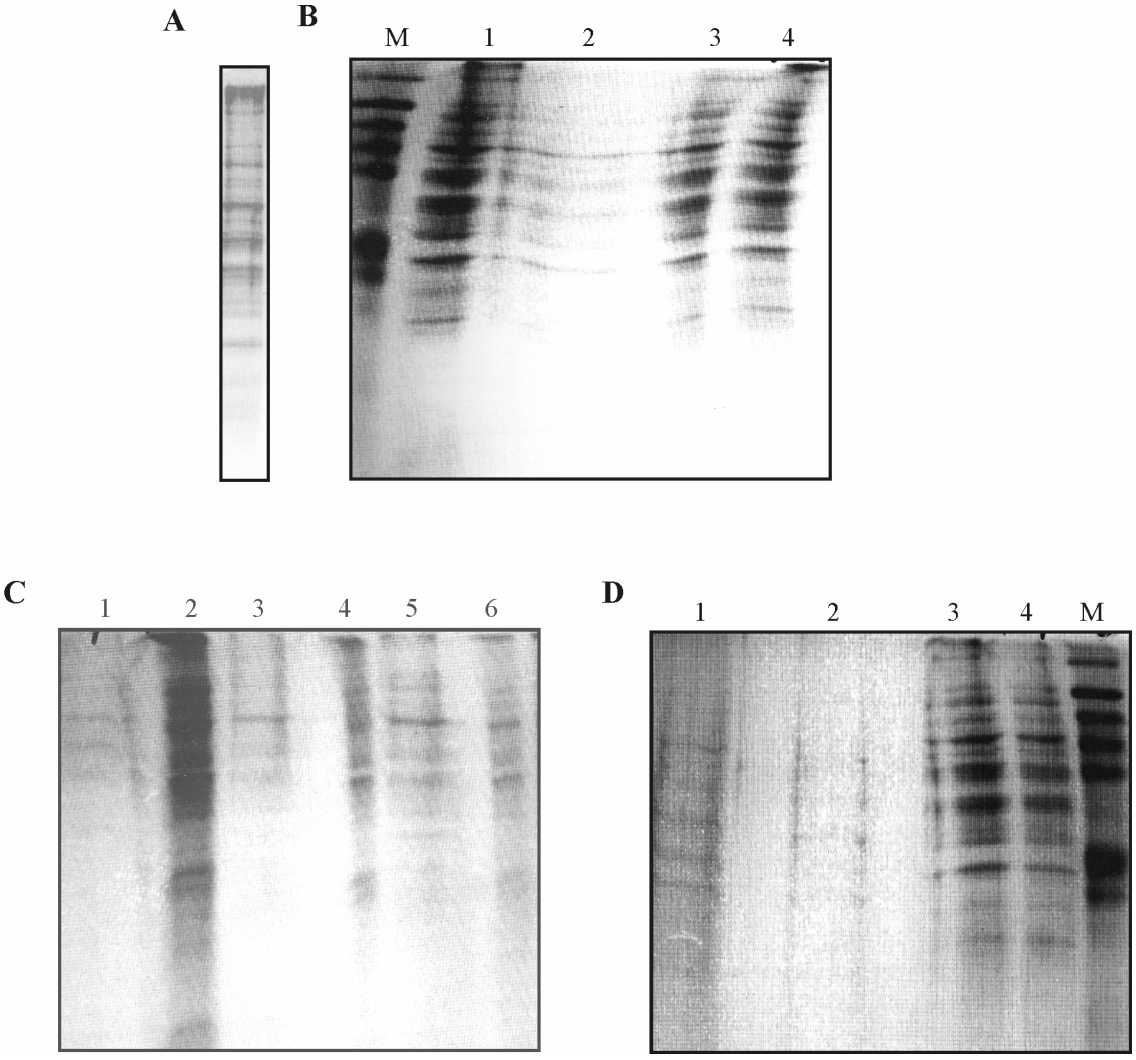



**Fig. 4.6 Enrichment of chitosomal proteins from wild type strain using different salts**

Chitosomes were purified via two step sedimentation as per standard protocol described earlier and were treated with different salts (see Methods) for 15 min. Protein samples were centrifuged at 14000 rpm and supernatant was analyzed on 12.5% SDS-PAGE. **(A)** Protein composition in purified chitosomal preparation with high CSI activity. **(B)** Control, lane 1; precipitation with 2 M NaCl, lane 2; with 1% SDS, lane 3 and 1% Triton, lane 4. **(C)** $(NH_4)_2SO_4$ precipitation; supernatant and pellet fraction after precipitation with 1.8 M, lane 1 and 2; with 0.9 M, lane 3 and 4 and with 0.45 M $(NH_4)_2SO_4$ respectively, lane 5 and 6. **(D)** Precipitation with 1.5 M GdHCl, lane 1; with 3 M GdHCl, lane 2; with 2 M Urea, lane 3 and with 4 M Urea, lane 4.

## 4.5 Identification of the major proteins in chitosomal fractions using 2-D gels and MALDI-MS

**4.5.1** Blue native (BN) PAGE has been used for the isolation of the native membrane protein complexes in recent years. It is a charge shift method in which the mobility of the proteins is mainly determined by the bound Coomassie Dye which is added to the protein sample to give it a charge. In combination with a 2nd dimension SDS-PAGE, this method provides information about the subunit composition and the degree of purity of the protein complexes (Schaegger *et al*., 1994). We used this method for further separating the chitosomal proteins with the same mobility on 1-D SDS-PAGE. The protein profile in the seemingly pure chitosomal fractions on SDS-PAGE showed four major protein bands (Fig. 4.7E, lanes 11, 13 and 14)**.** Two proteins of molecular weight sizes of about 55 and 62 kDa were of almost similar mobility on 1-D SDS PAGE. To further separate these proteins from Fr. 13/14 (Fig. 4.7E) were concentrated and further subjected to separation via 2-D PAGE. Well separated protein containing spots on the 2nd dimension of the blue native PAGE (Fig. 4.7G, proteins spots marked via arrow heads) were analyzed for the peptide mass information commercially via mass spectrometry (ChromaTech GmBH, Germany).

**4.5.2** Mass spectrometry is used to determine the mass of a molecule by transforming it into ions. This technique has been widely applied to get the mass/molecular weight information of a whole protein or a protein fragment generated by the proteolytic digestion or chemical cleavage. Various ionization techniques alone or in combination with the protein purification systems like HPLC or 2-D liquid chromatography for improving the detection limit of the system are currently in use. Two proteins of the apparent molecular weight of

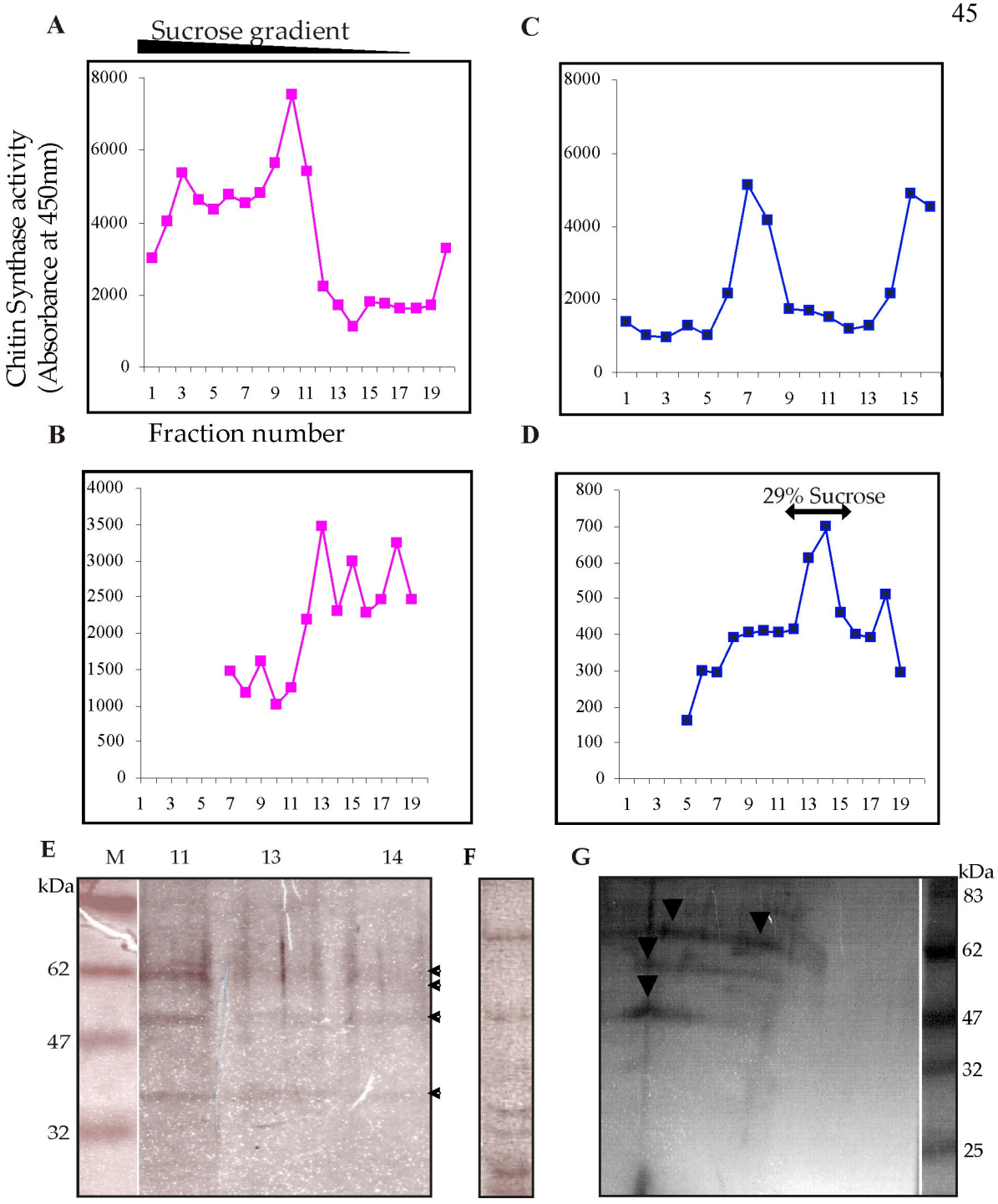



**Fig. 4.7 Separation of proteins via 2-D PAGE**

Chitosomes were purified as per standard protocol described in previous sections. CSI activity profile in the purified chitosomal preparations as determined using WGA **(A)** and via CHB1 method **(B)** in the first gradient. CSI activity profile via WGA assay **(C)** and CHB1 assay **(D)** after floatation gradient (arrow head indicates sucrose concentration in the fractions). Fractions 13 and 14 from (D) contained pure, individual chitosomal particles (as determined via electron microscopy). **(E)** Coomassie stained proteins in pure fractions and were further used for 2-D blue native PAGE. **(F)** Separated proteins in first dimension blue native PAGE and in second dimension tricine SDS-PAGE. **(G)** Protein spots, indicated via arrow heads were analyzed for protein identification via MALDI-MS.

55 and 62 kDa in the pure fractions (11-14) which were found to be N-terminally blocked and for which no sequence information using Edman degradation could be obtained, were processed for peptide mass identification via MALDI-MS commercially. In addition to these, a doublet protein band of about 220 kDa present in chitosomal fraction with high CSI activity (Fig 4.2C, lanes 7-9) was also analyzed for peptide mass sequence information. The masses {listed in Table. 4.9 (i) and (ii)} of the proteins obtained via MALDI-MS and MS/MS were analyzed through peptide mass analysis software programs available freely on internet. Most searches were done using MS-Fit program of UCSF. Search criteria for making a database search, were kept as stringent as possible (for details of the search parameters: see Section 3.5). Mono-isotropic masses for the above analyzed proteins were blasted against *S. cerevisiae* genome database (see next Section for the identification results).

### 4.6 Characterization of the identified proteins in chitosomal preparations via specific enzyme assays and immunological studies

On the basis of N-terminal sequencing and/or MALDI-MS analysis, six major proteins were identified in the chitosomal fractions from *chs3* mutant strain. The sequence of two proteins of the molecular weights of 37 and 48 kDa had the N-terminal sequence RVAINGFGRIGRLV and XVSKVYARSYX(D)X(P)X(R); letter X in the peptide sequence indicates unidentified amino acid. On a protein-protein BLAST search (NCBI), the first peptide sequence showed 100% identities to Tdh2 and Tdh3, glyceraldehyde-3-phosphate dehydrogenase isoforms. The second peptide sequence exhibited 84% identities to enolase isoforms Eno1 and Eno2, which almost indistinguishable from each other (see Fig. 4.8 for amino acid alignments). In addition to N-terminal sequencing these were also verified

via mass spectrometry. Peptide masses listed in Table 4.9 (i) and (ii) of the proteins corresponding to the apparent molecular weight of about 220 (doublet band), 55 and 62 kDa were used for database search analysis and showed identities to the fatty acid synthase (Fas1), glucan synthase (Fks1), Pyruvate kinase (Pyk1) and pyruvate decarboxylase (Pdc1) proteins of *S. cerevisiae* (see Table 4.10).


**Fig. 4.8 Clustal W alignment of N-terminal sequences of the two identified proteins**

N-terminal amino acid sequences of two analyzed proteins showing amino acid identities to GAPDH (Tdh2 and Tdh3) and enolase were aligned using Clustal W program. Eno1- enolase 1; Eno2-enolase 2; Query- N - terminal sequence of the 48 kDa protein. Tdh1, 2 and 3-Glyceraldehyde-3-phosphate dehydrogenase; Query – N - terminal sequence of 37 kDa protein.

A list of peptide masses of the desired proteins obtained via MALDI-MS:

| **Protein spot-A ca. 62 kDa** | **Protein spot-B ca. 55 kDa** | **Protein spot-C ca. 48 kDa** | **Protein spot-D ca. 37 kDa** |
|---|---|---|---|
| 986.53479 | 1012.4958 | 1012.5377 | 1045.55 |
| 1002.5231 | 1057.5914 | 1037.4869 | 1088.6222 |
| 1045.5245 | 1106.5121 | 1045.5304 | 1108.6208 |
| 1069.5355 | 1118.484 | 1106.5088 | 1126.546 |
| 1227.6422 | 1165.5695 | 1159.568 | 1179.6608 |
| 1315.7258 | 1179.5757 | 1179.5619 | 1296.63 |
| 1331.4451 | 1234.6627 | 1193.5751 | 1301.6135 |
| 1342.671 | 1277.6833 | 1288.6747 | 1329.6134 |
| 1344.7123 | 1383.6703 | 1383.6704 | 1374.7102 |
| 1393.6746 | 1390.6608 | 1387.6305 | 1470.8335 |
| 1493.7029 | 1434.7522 | 1430.7456 | 1492.8157 |
| 1500.8241 | 1475.7689 | 1460.7761 | 1703.8438 |
| 1515.606 | 1479.7366 | 1475.7589 | 1752.7802 |
| 1515.8195 | 1493.7181 | 1497.7789 | 1756.7759 |
| 1520.772 | 1501.8656 | 1519.7521 | 1768.7793 |
| 1748.9612 | 1596.8439 | 1557.7209 | 1784.7703 |
| 1760.8652 | 1618.8778 | 1578.7765 | 1882.9285 |
| 1782.8512 | 1707.7599 | 1716.8419 | 1940.9468 |
| 1816.9553 | 1716.8361 | 1821.9236 | 2011.0261 |
| 1872.991 | 1760.8784 | 1854.9806 | 2019.9976 |
| 1940.9258 | 1797.0005 | 1856.9451 | 2211.104 |
| 1993.9562 | 1940.9364 | 1866.8534 | 2225.1211 |
| 2001.0934 | 1993.9722 | 1894.8483 | 2239.1077 |
| 2211.104 | 2001.0922 | 1940.921 | 2246.1917 |
| 2225.1133 | 2096.9736 | 1961.0161 | 2263.2129 |
| 2239.126 | 2105.0933 | 1993.9939 | 2299.186 |
| 2246.2048 | 2211.104 | 2211.104 | 2313.2051 |
| 2266.1853 | 2225.123 | 2225.1223 | 2367.1968 |
| 2299.1746 | 2233.1252 | 2233.0942 | 2407.1941 |
| 2313.1848 | 2239.1367 | 2239.1345 | 2529.1067 |
| 2481.2871 | 2246.2075 | 2246.2039 | 2575.3042 |
| 2552.2087 | 2313.1997 | 2299.1768 | 2591.3052 |
| 2573.228 | 2383.96 | 2313.1846 | 2647.2935 |
| 2601.2036 | 2678.3926 | 2471.1807 | 2678.3811 |
| 2622.3274 | 2742.519 | 2559.2397 | 2811.3357 |
| 2678.3887 | 2811.3376 | 2678.4006 | 2914.4934 |
| 2811.3315 | 2839.3125 | 2811.3372 | 2946.4314 |
| 2838.3013 | 2914.511 | 2839.3354 | 3187.6235 |
| 2914.5293 | 3347.728 | 2914.5417 | 3265.6328 |
| 3353.8013 | 3353.7825 | 3353.7524 | 3269.6184 |

**Table 4.9 (i)**
Masses of four proteins corresponding to molecular weight of about 62, 55, 48 and 37 kDa (Fig. 4.7 E and G). The obtained peptide masses were analyzed via MS-Fit program. For results see Table 4.10.

| Peptide masses for the first of doublet protein of ca. 220 kDa | | | Peptide masses for the second of doublet protein of ca. 220 kDa | | | |
|---|---|---|---|---|---|---|
| 973.5247 | 1518.7618 | 2298.2283 | 922.5002 | 1447.7791 | 2254.0728 | 3337.7305 |
| 995.5064 | 1536.7814 | 2299.1794 | 930.4600 | 1463.6343 | 2263.2019 | 3348.6631 |
| 997.4891 | 1553.7816 | 2313.1838 | 947.5153 | 1475.7494 | 2274.0559 | 3353.6714 |
| 1005.5368 | 1569.7874 | 2344.2485 | 956.4835 | 1477.7529 | 2283.1804 | 3357.8152 |
| 1008.5085 | 1575.8096 | 2360.2549 | 959.4647 | 1483.6738 | 2286.1785 | 3361.6904 |
| 1036.5254 | 1599.8367 | 2383.9546 | 963.5001 | 1490.6831 | 2297.1960 | 3466.6443 |
| 1045.5557 | 1616.8130 | 2407.1025 | 971.5538 | 1518.7479 | 2299.1851 | 3488.6233 |
| 1055.5153 | 1639.8639 | 2510.1584 | 973.5608 | 1526.7488 | 2314.1895 | 3620.3027 |
| 1106.5392 | 1640.8759 | 2645.2642 | 979.4925 | 1535.7216 | 2344.2502 | 3794.6787 |
| 1107.5310 | 1657.8011 | 2663.3293 | 986.5362 | 1554.7577 | 2360.2458 | 3796.7927 |
| 1109.5115 | 1664.8474 | 2678.3113 | 1008.5163 | 1603.8385 | 2375.1033 | 3811.8152 |
| 1113.5565 | 1666.8630 | 2691.2766 | 1020.5810 | 1619.9036 | 2382.1697 | |
| 1126.5947 | 1699.8536 | 2695.2981 | 1029.6201 | 1625.8633 | 2383.9543 | |
| 1131.5833 | 1707.7784 | 2720.2966 | 1036.5712 | 1628.8518 | 2395.2107 | |
| 1141.5897 | 1716.8474 | 2724.2998 | 1045.5553 | 1641.8634 | 2402.2971 | |
| 1157.5848 | 1742.8969 | 2748.3081 | 1058.6086 | 1652.8909 | 2428.1804 | |
| 1165.5861 | 1744.9166 | 2753.3240 | 1089.5468 | 1664.8599 | 2451.1846 | |
| 1168.6278 | 1746.9064 | 2807.3208 | 1091.5753 | 1666.8654 | 2467.2004 | |
| 1169.6129 | 1928.9301 | 2825.3313 | 1092.5935 | 1853.8894 | 2497.2417 | |
| 1179.5870 | 1940.9296 | 2914.5020 | 1102.6168 | 1873.9755 | 2546.1858 | |
| 1203.6172 | 1993.9725 | 2921.3391 | 1116.5725 | 1890.9672 | 2620.2007 | |
| 1233.6273 | 1997.9664 | 3224.3445 | 1125.6295 | 1892.8773 | 2637.2253 | |
| 1234.6509 | 2003.0071 | 3232.6611 | 1138.6542 | 1893.8704 | 2641.2053 | |
| 1235.6427 | 2011.9750 | 3312.3401 | 1149.5969 | 1908.8630 | 2642.1970 | |
| 1265.6364 | 1567.7455 | 3324.7046 | 1176.6794 | 1940.9417 | 2646.1667 | |
| 1277.6931 | 2063.0366 | 3337.8105 | 1179.5978 | 1949.0262 | 2663.2979 | |
| 1293.6520 | 2083.0063 | 3341.7473 | 1192.6490 | 1965.0151 | 2678.3215 | |
| 1307.6736 | 2087.0447 | 3346.7029 | 1203.6232 | 1968.0067 | 2705.1848 | |
| 1308.6571 | 2089.0188 | 3349.6465 | 1233.6105 | 1985.9974 | 2708.2432 | |
| 1320.6139 | 2104.9983 | 3351.5967 | 1241.6023 | 1993.9738 | 2720.2817 | |
| 1331.7056 | 2118.0989 | 3353.7241 | 1265.6543 | 2001.9943 | 2748.3142 | |
| 1357.7018 | 2120.0374 | 3372.7075 | 1270.6019 | 2041.1401 | 2752.3223 | |
| 1365.6610 | 2121.0347 | 3381.6218 | 1292.6340 | 2063.0435 | 2773.4050 | |
| 1375.6792 | 2207.1165 | 3653.9470 | 1308.6378 | 2076.0979 | 2784.3464 | |
| 1383.6741 | 2211.1038 | 3799.8245 | 1312.6697 | 2083.0073 | 2807.3193 | |
| 1385.6930 | 2225.1184 | 3810.8320 | 1314.6683 | 2100.2048 | 2811.3132 | |
| 1427.8457 | 2230.1702 | 3830.9763 | 1325.6431 | 2109.0527 | 2870.3481 | |
| 1434.7498 | 2233.1028 | | 1344.6826 | 2141.9993 | 2914.5002 | |
| 1439.7623 | 2239.1355 | | 1352.6588 | 2151.2593 | 2921.3403 | |
| 1448.7567 | 2246.2024 | | 1354.6981 | 2174.0793 | 3035.4119 | |
| 1475.7562 | 2247.1697 | | 1374.6783 | 2211.1035 | 3041.5068 | |
| 1477.7528 | 2273.0325 | | 1398.6387 | 2225.1204 | 3232.6870 | |
| 1479.7737 | 2275.0361 | | 1402.7358 | 2230.1904 | 3265.5068 | |
| 1493.7277 | 2276.0537 | | 1425.7423 | 2233.0771 | 3312.2969 | |
| 1497.7402 | 2277.0715 | | 1427.7667 | 2239.1294 | 3325.6255 | |

**Table 4.9 (ii, continued from page 50)**
**A list of the peptide masses obtained via MALDI/MS.**
Peptide masses for two high molecular weight proteins (protein doublet) of about 220 kDa.

| Protein name | SGD identity | Mol wt. (kDa) | Identified by | Amino acid identity and/or Mass Sequence coverage | Location/Function |
|---|---|---|---|---|---|
| Fks1 | YLR342W | 214.8 | MALDI-MS | 19% | Involved in cell wall synthesis and maintenance; localizes to sites of cell wall remodeling (Inoe *et al*., 1995; Qadota *et al*., 1996; Douglas *et al*., 1994). |
| GAPDH (Tdh3) | YGR192C | 37.0 | Edman degradation & MALDI-MS | 100%/85% | Glyceraldehyde-3-phosphate dehydrogenase, isozyme 3, involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell-wall (McAlister *et al*., 1985a and b; Delgado *et al*., 2001). |
| Pyk1 | YAL038W | 54.5 | MALDI-MS | 74% | Pyruvate kinase, functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration (Pearce *et al*., 2001). |
| Pdc1 | YLR044C | 61.4 | MALDI-MS | 63% | Major of three pyruvate decarboxylase isozymes, key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subject to glucose-, ethanol-, and autoregulation; involved in amino acid catabolism (Kellermann *et al*., 1986; Hohmann, 1991; Liesen *et al*., 1996; Pronk *et al*., 1996; Dickinson *et al*., 2003). |
| Eno1 | YGR254W | 48.0 | Edman degradation & MALDI-MS | 84%/78% | Enolase I, catalyzes the first common step of glycolysis and gluconeogenesis; expression is repressed in response to glucose (McAlister *et al*., 1982; Entian *et al*., 1987). |
| Fas1 | YKL182W | 228.6 | MALDI-MS | 46% | Beta subunit of fatty acid synthetase, which catalyzes the synthesis of long-chain saturated fatty acids; contains acetyltransacylase, dehydratase, enoyl reductase, malonyl transacylase, and palmitoyl transacylase activities (Schweizer *et al*., 1986). |

**Table 4.10 Summary of the proteins identified via N-terminal sequencing and MALDI-MS in chitosomal fractions and their concluded functions in the cell.**
Fks1 - Glucan synthase; GAPDH (Tdh3) - Glyceraldehyde-3-phosphate dehydrogenase; Pyk1 - Pyruvate kinase1; Pdc1 - Pyruvate decarboxylase 1; Eno1 – Enolase 1; Fas1 - Fatty acid synthase 1. Protein identification results were obtained using peptide masses listed in Tables 4.9 (i) and (ii). Search was conducted using MS Fit program of UCSF. SGD identity: accession number of the gene in Saccharomyces Genome Database (www.yeastgenome.org).

To ascertain whether these newly identified proteins are the structural components or catalytically active enzymes or their homologues, two other experiments were conducted: (i) Catalytic activities of the four proteins were monitored via specific enzymes assays as per standard protocols (see Methods); (ii) Co-presence of some proteins in chitosomal fractions across the gradient were tested through cross reacting antibodies specific for each protein (see Fig. 4.13).

The enzymatic activities were tested in purified chitosomal fraction 3-7 (Fig. 4.11B). The chitosomal peak would be represented by fractions 4 and 5 of the floatation gradient (Fig. 4.11B). The GAPDH activity in the chitosomal samples was determined via the formation of NADH according to the following reaction.

$$\text{Glyceraldehyde-3-phosphate} + NAD^+ + P_i \longrightarrow \text{1-3, bisphosphoglycerate} + NADH + H^+$$

The data showed a high activity of GAPDH in rapidly sedimenting fractions which also contained several other cellular complexes. GAPDH is a constitutive cytosolic enzyme which might be associated to the other cytoskeletal structures. It is known that most of the glycolytic enzymes including phosphofructokinase, GAPDH and aldolase show a high affinity for microfilaments in the cell (Mejean *et al.*, 1989). Similar results were obtained with Pyk1 and Pdc1 activities (Fig. 4.11C, D and E). All the three enzyme activities showed a similar pattern of activity across the gradient. Interestingly fractions having lower CSI activity also showed reduced activity of the respective enzyme.

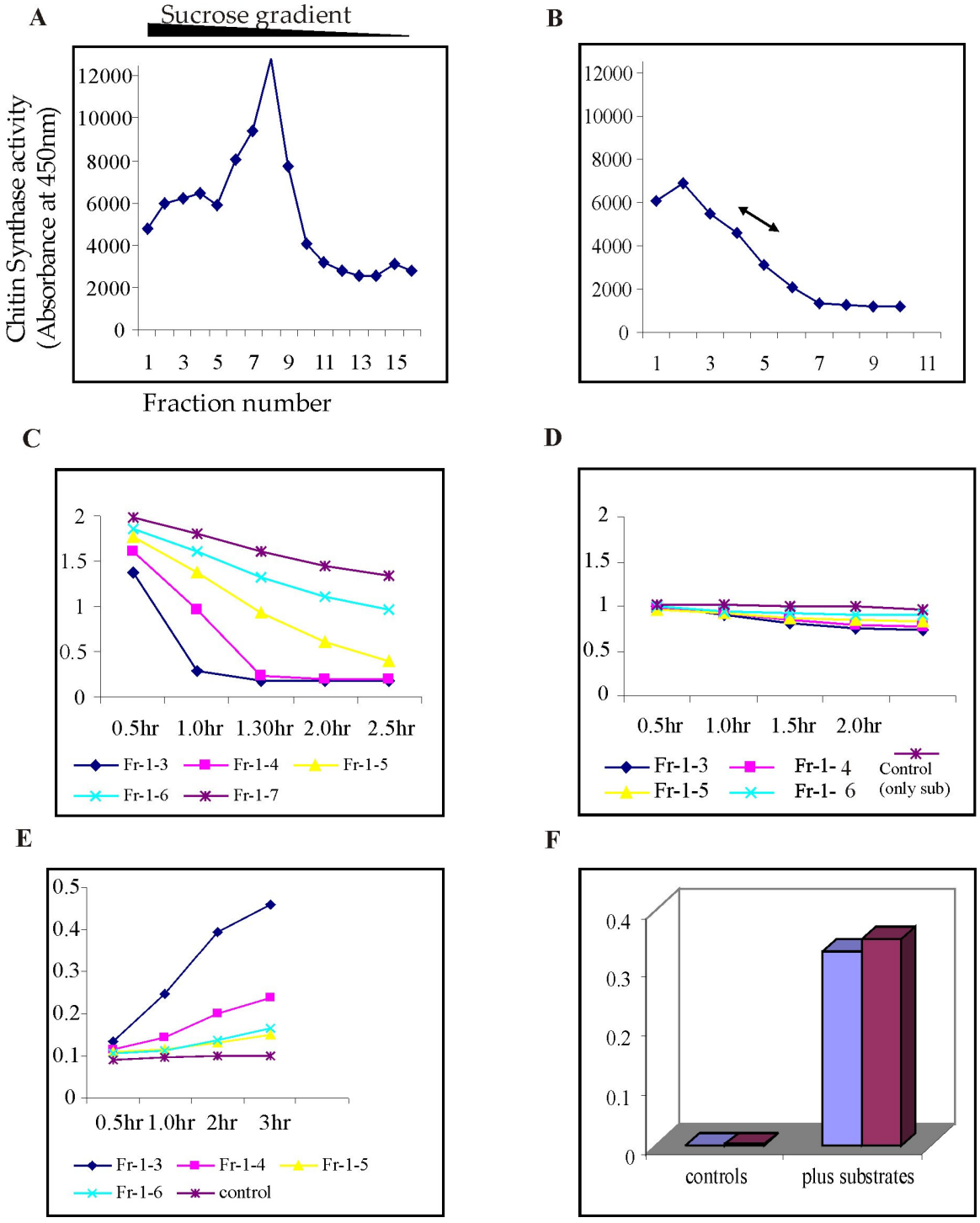



**Fig. 4.11 Evaluation of enzymatic activities across the purification gradient**
CSI activity profile as determined via WGA assay **(A)** after first sedimentation step, and **(B)** after floatation gradient (chitosomal peak would be marked via arrow). Separate enzyme assays were conducted according to the established assay protocols. (for details see Methods). Enzyme activities were monitored over a period of 2 to 3 hrs in fractions showing optimal CSI activity. Fractions tested were Fr. 3, 4, 5 and 6 in all three cases **(C** to **E)** from the floatation gradient (B). Enzyme activity profiles for **(C)** Pyruvate kinase1 (Pyk1); **(D)** Pyruvate decarboxylase (Pdc1) and **(E)** Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH). **(F)** Enolase activity in two different fractions from the floatation gradient.

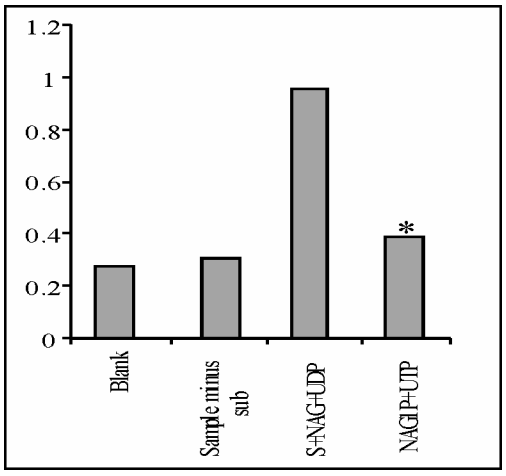



**Fig. 4.12 UDP-*N*-acetylglucosamine pyrophosphorylase assay in chitosomal preparations from *chs3* mutant**

Chitosomal fractions (having a peak CSI activity) were tested for the presence of UDP-*N*-acetylglucosamine pyrophosphorylase. The observed activity with the *N*-acetyl glucosamine-1-phosphate (NAG-1P) in the presence of uridine tri-phosphate (UTP) is only 10% (marked via star on the extreme right bar) as compared to CSI activity tested with normal substrates (*N*-acetyl- glucosamine and UDP, 3rd bar). S – Substrates.

Chitosomes from *chs3* mutant were tested for the presence of UDP-*N*-acetylglucosamine pyrophosphorylase, which leads to the final step of chitin synthesis *in vivo*. Testing in the presence of specific substrates (see assay Methods Section 3.4.1) yielded no significant activity for the above enzyme (Fig. 4.12, extreme right bar) and is only negligible as compared to the CSI activity (Fig. 4.12, 3rd bar).

The presence of newly identified proteins was further verified via immunological detection (Western blotting) experiments. Antibodies recognizing conserved domains within each of the following proteins GAPDH, enolase, pyruvate kinase and pyruvate decarboxylase, recognized proteins (immobilized to a PVDF membrane) of 37, 48, 55 and 62 kDa (Fig. 4. 13C, lane 2, 3, 4 and 5) in fraction representative of chitosomal proteins gained from

individual particles which were kept properly frozen (4.13C, lane 1). Whereas in a faster sedimenting fractions containing particles associated to high molecular weight structures (Fig. 4.13A, lane 1), cross reaction was only found dominantly for GAPDH and Pyk1 proteins (4.13B, lanes 2 and 4) and weakly for Pdc1 (Fig. 4.13B, lane 5). When scored through out the fractions of the purification gradient GAPDH and Pdc proteins were found to have a large distribution range (Fig. 4.14A and B). In addition, GAPDH antibodies also reacted with two proteins of about 175 kDa (4.14 A; indicated via arrow heads). Whether this is due to the unspecific reaction of the antibodies or indication that GAPDH exists in higher subunit isoforms or associated to other high molecular weight proteins is still to be investigated.

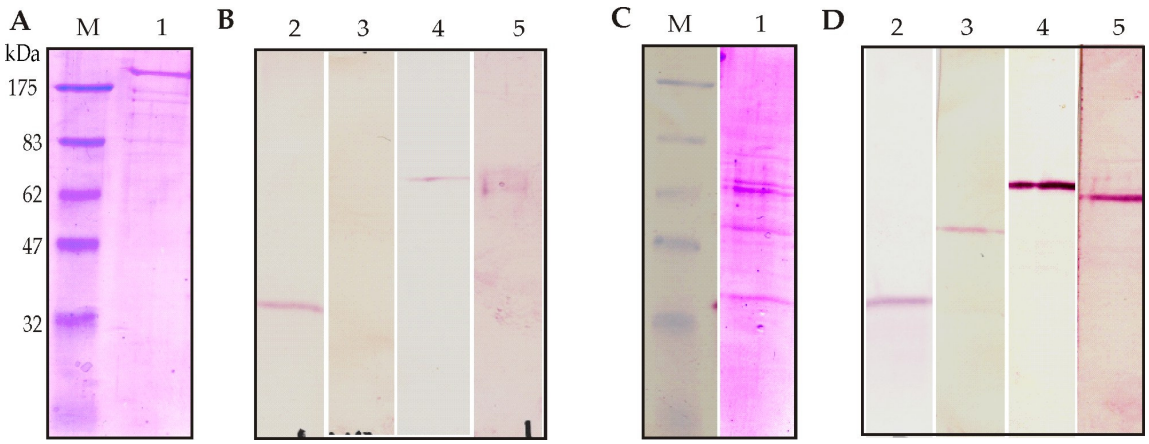



**Fig. 4.13 Immunodetection of newly identified proteins in chitosomal fractions**

Protein pattern in two chitosomal fractions (Coomassie stained gels). **(A)** Lane 1, high molecular weight proteins and **(C),** lane 1, pure chitosomes. Proteins were transferred to a PVDF membrane and were probed with cross reacting antibodies against four proteins. **(B)** Detection of GAPDH 37 kDa, lane 2; pyruvate kinase (Pyk1) 62kDa, lane 4; and pyruvate decarboxylase (Pdc1) 55 kDa, lane 5 (a weak and diffused signal). No cross reaction was observed with anti-enolase antibodies **(B),** lane 3. **(D)** A strong signal was obtained in pure chitosomal fraction for GAPDH, lane 2; Pyk1, lane 4 and Pdc1, lane 5. The signal for enolase (48 kDa) was however comparatively weak **(D),** lane 3.

All the data obtained via sequencing, MALDI-MS, enzymatic assays and the antibody studies together suggests that these proteins (GAPDH, Enolase, Pyk1, and Pdc1) are present in chitosomal fractions.

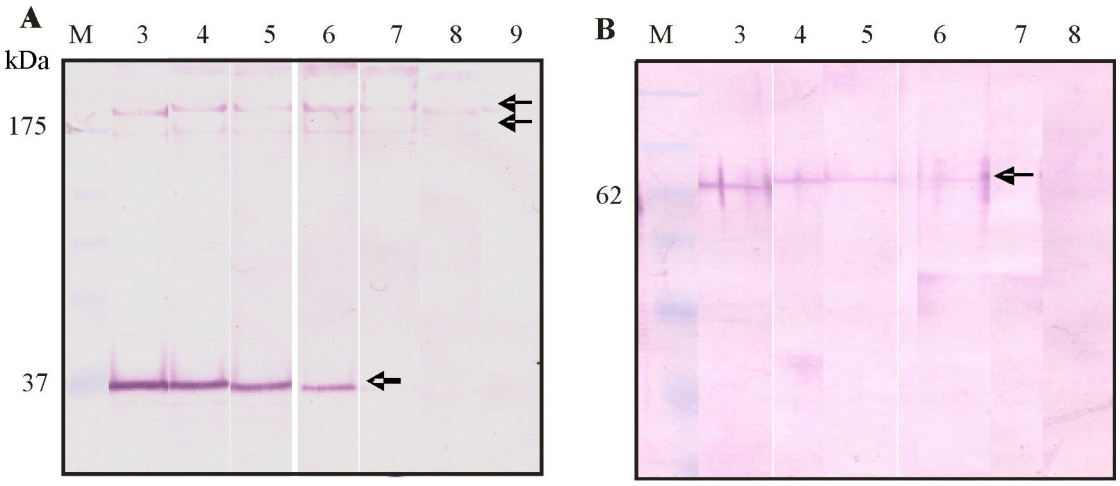



**Fig. 4.14 Immunodetection of GAPDH and Pdc1 in chitosomal fractions across the floatation gradient**
**(A)** Recognition of GAPDH at the size of 37 and **(B)** Pdc1 at 62 kDa by anti-GAPDH and anti-Pdc antibodies. In addition to protein at 37 kDa, anti-GAPDH antibodies recognized two or three bands at the apparent molecular weight of about 180 kDa.

The other two proteins showed maximum peptide mass (obtained via MALDI-MS) sequence coverage to fatty acid synthetase (Fas1) and β(1-3) glucan synthase (Fks1) respectively (see Table 4.10). However interpretation of these results need more in depth studies on their function in relation to the chitosomes and CS1. These results were obtained from the proteins gained from carefully selected fractions after intensive assays and EM examination of chitosomal preparations. Thus four proteins viz: GAPDH, enolase, Pdc1 and Pyk1 would be most interesting to investigate as a next step via further in depth studies.

**4.7 Studies on CSI-HA strain (isolation of chitosomes and investigation of HA-tagged CSI)**

To further investigate CSI in chitosomes, it was intended to use a tagged CSI protein. Main advantages of a tagged protein are; (i) it could be easily purified via affinity techniques and (ii) and could be traced via co-localization studies using fluorescent probes. Primary objective to use this strain was to get more information about the Chs1p protein in chitosomes. Interestingly microscopical examination of the exponentially growing cells exhibited slightly different cell morphology than a wild type yeast strain. At many places mother and the daughter separation was not complete or a clump of two or three cells was frequently observed. This may indicate that the introduction of HA-Tag (haem-agglutinin tag) into the *CHS1* gene has some altered phenotypic growth effects on the mother-daughter separations. Earlier studies have also indicated that the defects or mutations in CSI lead to small-lysed-bud phenotype (Cabib *et al.,* 1989; Valdivieso *et al*., 2000). In chitosomal preparations, tagged CS1 was observed to separate closely to the apparent molecular weight of 62 kDa (Fig. 4.15B). In an attempt to purify CSI, Kang *et al* have also reported the similar size of the Chs1p purified by chitin entrapment method (Kang *et al*., 1984). They observed two major protein bands at the molecular weight of 63 kDa and a minor protein band at 74 kDa after purification. In a mixed membrane fraction (MMF) however antibodies against CSI-HA tag recognized several proteins in addition to the major band at 63 kDa, indicating that either the protein is degraded or exist in multiple subunit form (Fig. 4.15C, lanes 5 and 6).

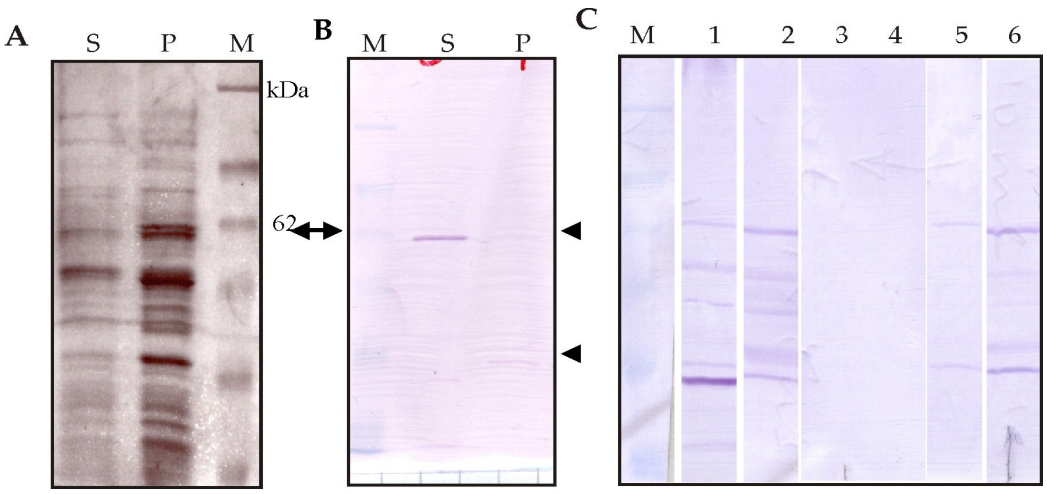



**Fig. 4.15 Analysis of CSI-HA tagged protein**
For detection of the HA-Tag, CSI-HA strain was grown to log phase and cells were broken via sonication **(A)**. Supernatant and pellet fractions (after a brief centrifugation) were separated via 10% SDS and stained with Coomassie. **(B)** Anti-HA antibodies recognized a protein of about 63 kDa in the soluble fraction and two minor protein bands (marked via arrows) at the molecular weight of about 40 and 27 kDa. A protein band of about 30 kDa is observed in the pellet fraction. **(C)** Supernatant was further subjected to purification in a small 5 ml sucrose gradient (12.5-65% w/v). CSI activity was determined via WGA assay and CSI-HA was traced across the gradient. Lane 1 - supernatant from 38K centrifugation step (this supernatant was applied to a sucrose gradient). Lane 2 - mixed membrane fraction (pellet from 38K centrifugation). Lanes 3-6 are gradient purified fractions. Notably lanes 5 and 6 showed increase in CSI activity (activity profile not shown here) and anti-HA antibodies recognized a number of protein bands.

CSI activity and protein profile in the chitosomal preparations from CSI-HA strain showed a similar profile as for *chs3* mutant strain (Fig. 4.16A, B and C). CSI-HA tagged protein could only be traced in the first step (Fig. 4.17A, lane-6) of the purification but it was not observed after the second step when tested across the gradient using anti-HA antibodies (Fig. 4.17C). This may be due to very low amount of protein or poor antibody performance.

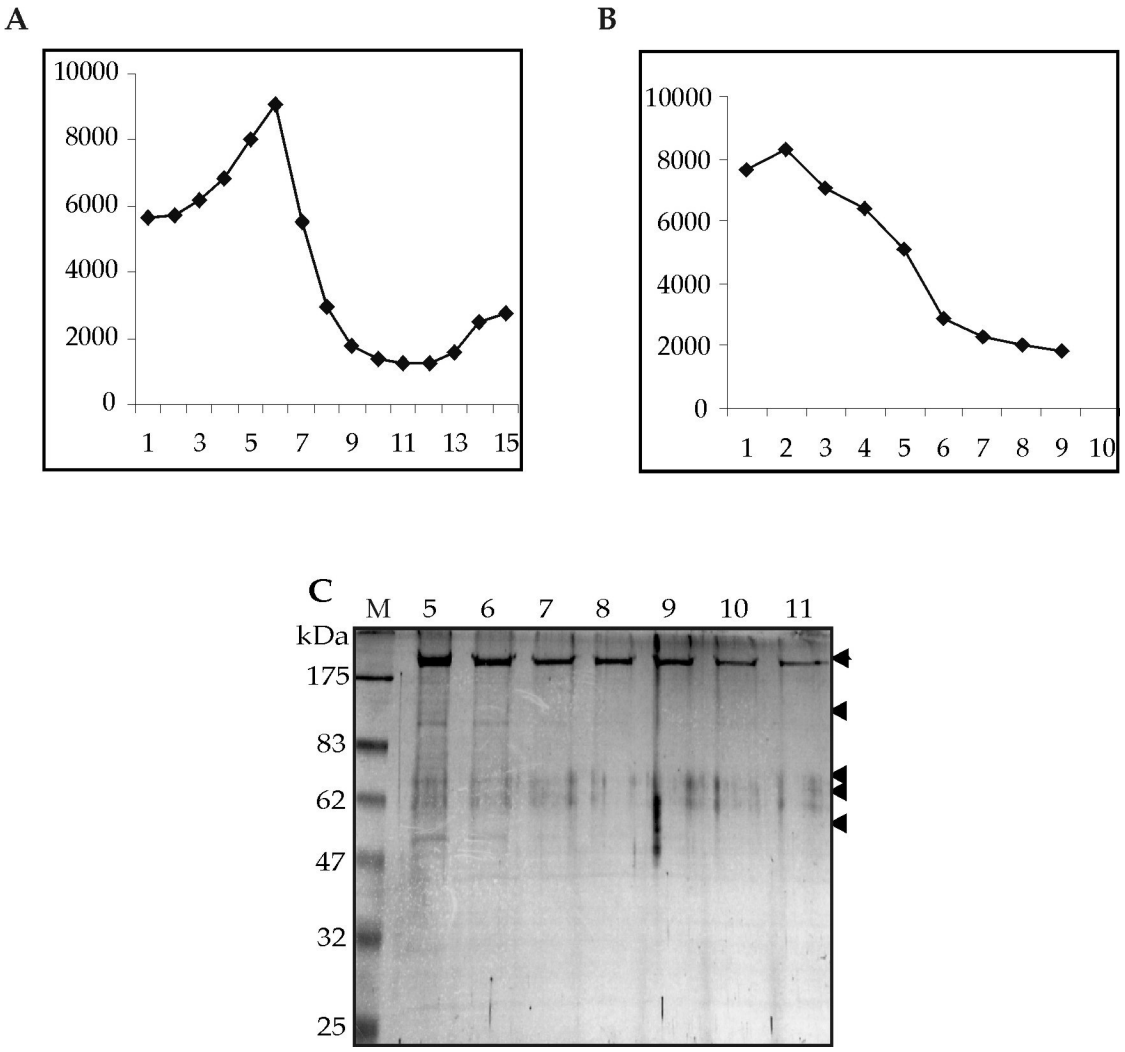



**Fig. 4.16 Purification of chitosomes from CSI-HA strain**

Chitosomes were purified and CSI activity (in the presence of $MgCl_2$ at pH 6.5, specific for optimal CSI activity) was determined via WGA assay as described earlier. **(A)** and **(B)** CSI activity profiles after first and second sedimentation steps **(C)** Major proteins present in chitosomal preparation across the floatation gradient are marked via arrow heads.

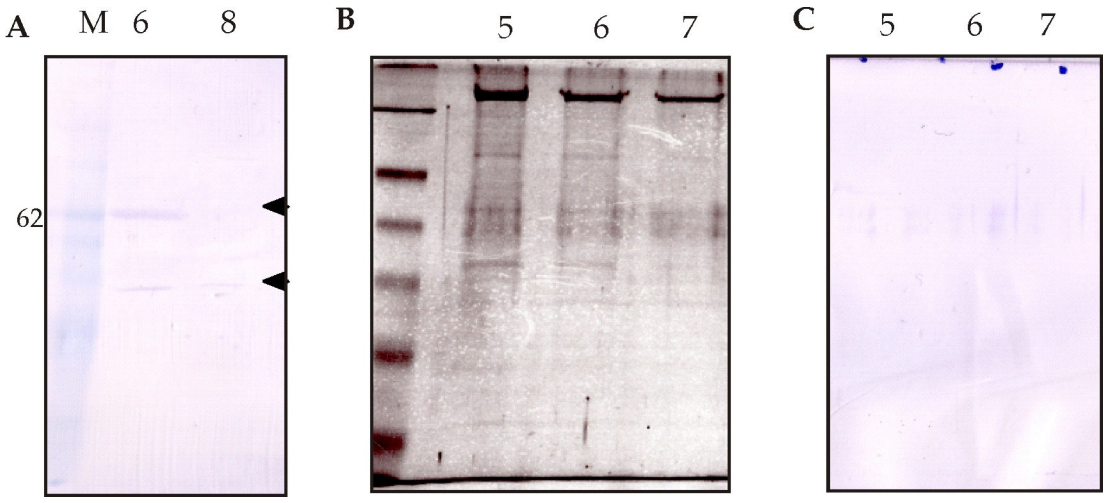



**Fig. 4.17 Detection of HA-Tag in gradient purified chitosomal fractions**
Proteins were separated and transferred to PVDF membrane as per standard protocol and CSI-HA tagged protein was detected using anti-HA antibodies. **(A)** Detection of protein band at the size of about 63 kDa in lane-6 (showing peak CSI activity in first purification step Fig. 4-13(A) and a minor protein at the size of 48 kDa. No protein was detected in lane-8 (which has a lower CSI activity) at the size of 63 but a minor band is seen at about 48 kDa. **(B)** Protein profile in the purified chitosomal fractions. **(C)** However no proteins were detected via anti-HA antibodies after the floatation gradient corresponding to Fig.4.13B.

## 5.0 Discussion

In the frame of this work it was planned to investigate whether CSI activity can be found within the vesicle like structures (chitosomes) in the absence of CSIII activity. Pre-studies were started in the wild type *S. cerevisiae* strain (used in the present studies) for the purpose of standardization of strain cultivation conditions, optimization of cell lysis and subsequent isolation conditions using sucrose gradient sedimentation for chitosomal complexes. Chitosomes were purified by modifying the two step method, described previously by Lending *et al*. (1990) [for details see Methods]. The results of the pre-studies in the wild type strain showed that CSI is the major CS activity in the chitosomal preparations (Fig. 4.1). A high CSI activity using a high through-put, WGA based method (Lucero *et al.,* 2002) was obtained. However, when peak fractions were analyzed under electron microscope, they were found to contain several other high molecular weight structures (see Section 4.3)**.** Whether they represent membrane and/or microtubular structures was not investigated further. It was concluded that WGA not only recognizes chitin but also oligomers of GlcNAc and other structures containing consecutive three GlcNAc residues (Raikhel *et al*., 1993). It needs to be mentioned that in the absence of sterilization, bacterial contamination was sometimes observed in chitosomal preparations; therefore they were routinely filter-sterilized. Taking all the precautions e.g. maintaining a temperature of 4 $^{0}$C during the whole preparation, processing for the steps in continuity and repeated sterilization steps, a fraction containing a relatively homogenous population of particles was obtained. Using substrates UDP-GlcNAc and GlcNAc, chitosomal preparations generated chitin fibers as

judged via assay and EM, which suggest that most of these particles represent CSI activity alone or in combination with other CS enzymes. Furthermore incubation of the chitin synthesized by chitosomes with an exochitinase ChiO1 isolated from *Streptomyces olivaceoviridis* (Blaak and Schrempf, 1995) led to the degradation of fibres (Thesis, K. Siemieniewicz, 2004). In addition, testing in the presence of nikkomycin Z, a strong inhibitor of CSI and CSII (Cabib, 1991; Choi *et al*., 1994a; Gaughran *et al*., 1994) and specific ions, reconfirmed the presence of CSI activity in the isolated fractions. Under EM the majority of the particles were in the size range of 80-120 nm (see Section 4.3). The different sizes of particles could represent intact or broken chitosomes or those which are not fully associated to other structures. Analysis of the chitosomal proteins on gel also showed a variable pattern in several preparations. Chitosomes from the investigated wild type *S. cerevisiae* and a mutant lacking CSI activity appeared to be relatively collapsed and were not used for further studies.

To test whether high molecular weight chitin fibers could be generated, it was intended to design an assay method which differentiates the detection of low molecular weight chitin oligomers (as documented earlier for WGA) or other structures containing GlcNAc residues (Raikhel *et al*., 1993) from high molecular weight chitin. Therefore, we decided to improve the CS assay procedure of Lucero *et al*. (2002) via using a chitin binding protein (CHB1) instead of WGA as chitin binding interphase. The principal idea to use CHB1 was to rapidly detect chitin during the assay procedure to select those fractions which comprises the corresponding chitin synthase activity. Previously CHB1, isolated from *Streptomyces olivaceoviridis*, the first protein of its kind, has been shown to specifically recognize high molecular weight chitin (Schnellmann *et al*., 1994, Schrempf, 1999).

Traditionally the CS activity has been determined using a radioactive method. The assay protocol includes incubation of the chitin synthase containing fractions with radiolabelled substrate $^{14}$C-UDP-GlcNAc and activator *N*-acetyl glucosamine, which results in the formation of radiolabelled chitin (Orlean, 1987 and Cabib, 1991). The reaction product is then precipitated and isolated by filtration. Radioactivity content via scintillation counting in the filter gives an indirect account of the CS activity. Limitation with respect to the maximum data points (that could be generated via scintillation counting) and the well documented biosafety hazards in working with the radiochemicals are the basic drawbacks of this method (Yeager and Finney, 2005). To circumvent these problems, another non-radioactive method based on natural binding affinity of WGA to the oligomers of chitin/chitin, was developed (Lucero *et al*., 2002). In this method chitin synthesized by the CS reaction is allowed to bind with WGA. The chitin is then detected via a second layer of WGA coupled to horse radish peroxidase (WGA-HRP) spectrophotometrically. This method has following advantages over the radioactive method: high sensitivity, high through put, compatibility with various assay conditions, low cost and being non hazardous. However the major drawback of this method lies in the fact that it is unspecific as to the recognition of chitin. WGA recognizes three consecutive NAG residues in oligomers and/or several glycoproteins (Raikhel *et al*., 1993). Thus the data obtained through this method does not only quantify the chitin but also other oligomers or proteins containing such sugars. The use of CHB1 has an advantage over the other two as it specifically recognizes high molecular weight chitin (Schrempf, 1999). Concomitant studies showed that CHB1 serve as a good tool to investigate nascent chitin fibers (Thesis, K. Siemieniewicz, 2004) by using very good chitosomal preparations described in results. It thus can be concluded that all the three CS assay methods has advantages and disadvantages. An ideal

chitin synthase assay method is still to be discovered. A recent trial to use a fluorescent analogue of UDP-GlcNAc as a substrate failed as it was found to be inhibitory for the CS (Yeager and Finney, 2005). For controlling the quality of chitin synthase preparations both assays viz: WGA based method and CHB1 magnetic bead based assay were simultaneously carried out. Both the methods, in combination, resulted in a good quality assessment in chitosomal preparations. In addition to the above controls and to exclude the interplay of other glycosyl transferases during the chitin synthesis reaction, CS specific inhibitor nikkomycin Z was included in the assays. Nikkomycins are nucleoside peptide antibiotics which has an inhibitory effect on fungal chitin synthases (Georgopapadakou and Walsh, 1996). Despite some practical difficulties to quantitatively determine stochiometric binding ratios of CHB1 with agarose beads, equal distribution of beads (containing bound CHB1), loss of beads/CHB1 complexes by extensive washing during assay, it could be concluded that the use of CHB1 based assay, in preference to WGA assay, was particularly useful in selecting those fractions which were freed from other high molecular weight structures (as determined based on the sedimentation characteristics and appearance under EM). Such a preparation was further processed for identifying protein components of the chitosomal complexes. The use of the CHB1 based assay in preference to and in combination with the WGA based assay proved to be a good tool to pre-select fractions comprising structures of high purity. However due to the ease of experimentation WGA based assay was mostly used for pre-selection of CSI containing fractions.

The observed results based on a combination of both assays mentioned above indicated that the peak of CSI activity has different sedimentation behaviour. More pure and individual chitosomal particles were obtained at a lower sucrose concentration in the range 29-31% w/v (Fig. 4.3 and 4.7) which

correspond to a density range of 1.1-1.13 g/ml, a value close to reported in literature. Earlier studies have demonstrated the presence of chitin synthase in chitosomes in several fungi namely *Mucor rouxii* (Ruiz-Herrera *et al*., 1984), *Nurospora crassa* (Sietsma *et al*., 1996), *Agaricus bisporus* (Hänseler *et al*., 1983) and *S. cerevisiae* (Leal-Morales 1988, 1994; Chuang and Schekman, 1996). The relatively pure quality of chitosomes has been gained mainly from *M. rouxii* and their structural properties differ as compared to those gained from *S. cerevisiae.*

After comparing the data obtained in present studies to previous investigations (Leal-Morales *et al.,* 1988, 1994), it can be concluded that we (in collaboration with K. Siemieniewicz) have obtained a population of highly pure and intact chitosomal particles, which under CSI favouring conditions synthesized fine fibrils which were found to degrade upon incubation with a chitinase (Thesis, K Siemieniewicz, 2004). Our studies thus are first to show that chitosomal particles of high integrity and principally representing CSI activity can be obtained. Earlier investigated chitosomes from *S. cerevisiae* strains (wild type as well as *chs1* mutant) appeared to have an irregular or collapsed appearence under EM and visible background impurities. Furthermore details of fiber biogenesis were not elucidated (Leal-Morales *et al.,* 1988, 1994).

Barring a few reports, there is not much known about the origin of chitosomes. It has been shown that Chs1p and Chs3p containing membranes do not correspond to endoplasmic reticulum (ER), Golgi, mitochondria or vacuole (Ziman *et al*., 1996). Chs3p containing chitosomes fractionate at a lower density than secretory vesicles and lack vesicle markers (like Pma1p). However chitosomes are absent when enodocytosis is blocked in (*end3-1* or *end4-1*) mutants (Raths *et al.,* 1993). Thus it is suggested that Chs3p containing chitosomes are endocytically derived. They may represent a general endosome or a specialized secretory organelle (Chuang and Schekman, 1996). Our focus

however was to know more about the presence of other accessory components present in chitosomes derived from *S. cerevisiae* lacking CSIII.

The *S. cerevisiae* wild type contains, in addition to CSI, CSII and CSIII activities respectively. Previously it has been shown that CSI represents 90% of the total measurable CS activity *in vitro* (Shaw *et al*., 1991). Earlier it was shown clearly that approximately 30-50% of the Chs3p resides in chitosomes. However due to the rapid turnover of the Chs2p in a cell cycle regulated manner, it could not be localized in the chitosomal vesicles (Chuang and Schekman, 1996). Although Chs1p has been found in chitosomes (Ziman *et al*., 1996), there are no thorough investigations on the presence of Chs1p in chitosomes. To avoid interference with Chs3p, we decided to use a mutant of *S. cerevisiae* lacking a functional CSIII enzyme to investigate the chitosomes and its components. This strain was chosen as double mutations in *chs2 chs3* are lethal to the cells (Shaw *et al*., 1991). As all chitin synthases can be differentiated on the basis of individual pH and ion requirements (Choi and Cabib, 1994a) it could be shown that in the purified chitosomal fractions, CSI was the dominant activity and barely any CSII activity could be detected (see Section 4.1). It is known that the levels of the CSI in the cell remain constant. CSII appears only at certain specific time periods during cell division and its contribution to the total CS activity either *in vitro* or *in vivo* is negligible as compared to CSI (Ziman *et al*., 1996; Leal Morales *et al*., 1994). Notably CSI activity in purified chitosomal preparations was found even without the proteolytic activation prior to assaying. However there have been conflicting reports in literature about the requirements of protease for optimal activity of CSI. It has been concluded earlier that CS (Leal-Morales *et al*., 1988; Sburlati and Cabib, 1996) is zymogenic and requires partial proteolysis for activation.

In course of the present studies a *S. cerevisiae* strain became available which harbor Chs1p tagged with haem-agglutinin (HA). Therefore it was

interesting to investigate this strain. It was intended to use the tagged protein to follow it during the preparation. In contrast to the wild type, cells of the CSI-HA strain appeared to have irregular shape and tended to clump together. It appeared likely that the introduction of the HA-Tag could interfere with the *in vivo* function and/or localization of Chs1p. Immunological analysis of the chitosomes purified like that from *chs3* mutant strain, indicated that HA-Tag was present on several proteins with apparent molecular weight sizes of about 63, 50, 45, 37 and 30 kDa respectively (Fig. 4.15) instead of the predicted full-sized CSI-HA protein of 130 kDa (including six amino acids of HA tag). Based on these results it had to be concluded that the HA-tagged Chs1p in the cell might be malfunctional. Thus further studies were not pursued with this strain.

*In vivo* the chitin biosynthesis pathway is a complex process and requires several steps leading to UDP-GlcNAc, which acts as a substrate for CS enzymes. An enzymatic test revealed that UDP-GlcNAc pyrophosphorylase which converts GlcNAc-1P to UDP-GlcNAc, the final step of UDP-GlcNAc formation**,** is absent within chitosomes from *chs3* mutant (Fig. 4.12). In contrast it was detected in the cell free extracts containing CS activity of *Mucor rouxii* (Ian McGurrough *et al*., 1971). However at that time no distinct vesicular compartments were investigated.

The proteins gained from highly purified chitosomes of the *S. cerevisiae chs3* mutant strain were concentrated via ammonium sulfate and/or preparative gel electrophoresis (1-D or 2-D). These were successfully used for N-terminal sequencing via Edman degradation. In parallel, protein containing bands were digested within the gel via trypsin and analyzed for peptide mass information via MALDI-MS. A combination of both methods yielded information about six proteins (see Table 4.10). A high molecular weight protein of about 200 kDa was identified as β-1, 3-glucan synthase (Fks1) known to synthesize glucan in the cell

wall (Douglas *et al*., 1994). It is known that *in vivo* chitin exists in a closely interwoven network with glucans and cell wall proteins. Chitin synthesized by CSIII in the cell wall and neck ring is attached to β(1-6) and β(1-3) glucans respectively. However some of the chitin in the primary septum is found attached to β(1-3) glucans (Cabib and Duran, 2005). Therefore a close coordination between chitin and glucan synthetic apparatus is expected to exist in the cell. Interactions among several genes involved in chitin metabolism were shown recently. With a large range of deletion mutants, many direct or indirect effects were scored on chitin synthesis in yeast. Many genes were found to interact with complex gene networks leading to chitin synthesis mediated by CSI or CSIII. A majority of *CHSI* interacting genes functions in maintaining the cell wall robustness and metabolism. The absence of Chs1p seems to be counterbalanced with β-1, 3-glucan synthesis pathway enzymes (*FKS1, GAS1* and *SMI1*, Lesage *et al*., 2005). These studies illustrate the complexity of genetic networks operating *in vivo* for chitin biosynthetic apparatus.

Interestingly four proteins were identified which showed high mass sequence coverage and/or N-terminal amino acid identities to glyceraldehyde phosphate dehydrogenase (Tdh3), Enolase (Eno1), pyruvate kinase (Pyk1) and pyruvate decarboxylase (Pdc1) of *S. cerevisiae* (for details see Table 4.10). Immunological studies verified these proteins (GAPDH, enolase, Pyk1 and Pdc1) in fraction representative of pure chitosomes. The relative levels of the three proteins, namely GAPDH, Pyk1 and Pdc1 were found to be high in comparison to enolase in pure fractions (Fig. 4.13). In addition GAPDH and Pyk1 were also found to be present in fraction comprising high molecular weight proteins which suggest that they might be associated to other cellular structures. When tested across the fraction purification gradient, GAPDH and Pdc1 proteins showed a wide distribution range. The amount of these two respective proteins was

found to be highest in the rapidly sedimenting aggregates and lower in the relatively pure chitosomal fractions. Finding of GAPDH in the chitosomal fractions was surprising. As it was also found in neighboring fractions around peak of CSI activity therefore its presence in chitosomes needs to be investigated further. Association of GAPDH with chitosomes (if proved via further *in vivo* elaborate experiments) will be an interesting finding as several reports have unequivocally confirmed that GAPDH displays diverse activities exclusive to its glycolytic function. A number of genes are already known that can encode proteins exhibiting strong similarities to GAPDH. Among new functions ascribed to GAPDH-isoforms includes cell wall associated multifunctional protein (especially Tdh2 and Tdh3; Delgado *et al*., 2001), role in cytoskeleton modulation, membrane fusion, ER to Golgi trafficking of proteins, and binding to G proteins (Doucet and Tuana, 1991). In concerted action with PGK (phospho glycerate kinase), GAPDH functions in the energy generating process for the import of neurotransmitter glutamate into presynaptic vesicles and plays an important role in the normal synaptic transmission (Ikemoto *et al*., 2003). The identified isoform of the GAPDH could possibly be involved in the transport of CS proteins between endoplasmic reticulum (ER) and vesicles and/or in adhering the vesicles to the plasma membrane where they finally deliver CS proteins. A function depicting the fusion of neutrophils to the plasma membrane mediated via GAPDH has been earlier reported (Hesseler *et al*., 1998).

Peptide mass analysis and immunological studies suggests the presence of pyruvate decarboxylase (Pdc1) in chitosomal fractions. Notably Pdc1, one among the three pyruvate decarboxylase isozymes in yeast, was also predicted to interact with CHSI in the above mentioned high through-put analysis (Tong *et al*., 2004; Lesage *et al*., 2005)**.** However direct evidence to prove its functional

associations with respect to chitin synthesis machinery awaits further investigations.

A database search of the N-terminal amino acid sequence of another investigated protein exhibited a high level of identity with the enolases, Eno1p and Eno2p. Both enolase isoforms in yeast are highly identical (93%). In addition, the identified protein reacted with anti-enolase antibodies (Fig. 4.13). An enolase catalyzes conversion of 2-phosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis. In *S. cerevisiae,* in addition to cytosolic form, enolase is also present as a non-covalently attached, detergent soluble cell wall protein (Edwards *et al*., 1999). In the cell wall of *Candida albicans*, enolase is found associated to glucans and is a member of glucans associated proteins and is a major surface antigen (Edwards *et al*., 1999; Angiolella *et al*., 1996, 2002; Chaffin *et al*., 1998). Enolase has also been found in RNA degradosome in *E. coli* where it exists in a multiprotein complex with RNAseE as the major endonucleolytic component along with other associated proteins. It was found that in the absence of enolase in the complex, mRNA degradation does not take place (Morita *et al*., 2004). Interestingly high amount of enolase was secreted by yeast cells having a mutation in *COP1*, a part of vesicle coatomer complex which surrounds transport vesicles in the early secretory pathway between ER and Golgi (Le Borgne and Hoflack, 1998; Rothman *et al*., 1996; Schekman and Orci, 1996). Importantly Cop1 has been implicated to play a role in the recognition and translocation of glucan synthase complex and other cell wall proteins (Lee *et al*., 1999). Excessive secretion of enolase linked to absence of Cop1 function could be a sign for defects in cell wall integrity and biogenesis in yeast (Kim and Park, 2004). Whether enolase is indeed a part of chitosomes needs to be investigated further.

With the use of MALDI-MS, a protein with high peptide mass sequence coverage (for details see Table 4.10) to a pyruvate kinase (Cdc19/Pyk1) was also

identified. This was verified via cross reaction with antibodies against yeast Pyk1 (Fig. 4.13). *CDC19* is one of two *S. cerevisiae* genes encoding pyruvate kinase which catalyzes the final step in glycolysis i.e., the conversion of phosphoenolpyruvate to pyruvate which is a precursor for aerobic (TCA cycle) or anaerobic respiration (Burke *et al.*, 1983).

A protein corresponding to β-subunit of fatty acid synthetase (Fas1) was also identified via MALDI-MS in the chitosomal fractions (see Table 4.10). Fatty acid synthase complex catalyzes the synthesis of long-chain saturated fatty acids and contains acetyltransacylase, dehydratase, enoyl reductase, malonyl transacylase, and palmitoyl trans-acylase activities. Interestingly *FAS1* was also found to interact with *PDC1*, in a high through-put systematic analysis of protein complexes in *S. cerevisiae* (Anne-Claude Gavin *et al*., 2002).

On the basis of the available resources, the data obtained in the present studies on chitosomes opens a way for further interesting questions. Based on MS analysis, immunological detection and enzymatic assays, four proteins of the glycolytic pathway (GAPDH, enolase, Pdc1 and Pyk1) are suggested to be the ideal candidates for further investigations to ascertain their inter-relationships among various components acting together in the chitosomal complex which is a interesting nanomachinery.

Further studies should contribute to elucidate the underlying mechanisms leading to the formation of chitosan from chitin *in vivo.* Chitosan is found in many marine organisms and some fungi. Biosynthesis of chitosan requires simultaneous deacetylation of acetyl-moieties from the nascently synthesized chains of chitin by an enzyme called chitin deacetylase.

Currently chitosan is produced via harsh chemical methods. As mentioned in the introduction, chitosan is widely used as valuable compound in food industry and has many medical as well as biotechnological applications. It

is expected to have a large potential in nanotechnology. Therefore further studies on its biogenesis are interesting and important.

## 6.0 Summary

In the presented studies, chitin synthase containing nanoparticles (chitosomes) from the yeast *Saccharomyces cerevisiae* lacking the *chs3* gene encoding for chitin synthase III were investigated. Two step centrifugations using sucrose gradients led to considerable purity of the chitosomal complexes. Chitin synthase I activity was determined via a previously described ELISA based WGA assay and a novel assay using the *Streptomyces* chitin binding protein CHB1, which provided good tools to follow the purification procedure. In collaboration, it could be shown that the complexes produce fibers in the presence of the substrates uridine-diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) and *N*-acetylglucosamine (GlcNAc) and this reaction was inhibited by addition of chitin synthase inhibitor nikkomycin Z. These results demonstrate for the first time that CSI containing chitosomes can be gained. Investigation of the purified nanocomplexes with CSI activity led to the additional conclusion that proteins of the glycolytic pathway such as glyceraldehyde-3-phosphate (GAPDH isoform Tdh3), enolase (Eno1), pyruvate decarboxylase (Pdc1) and pyruvate kinase (Pyk1) are also concentrated around the peak of CSI activity. The presence of these proteins in the pure chitosomes was further verified via testing for their individual enzymatic activities and by antibody studies. The relative levels of GAPDH, Pdc1 and Pyk1 were found to be higher in comparison to enolase; however GAPDH and Pdc1 proteins had a broad distribution across the purification gradient and were also found in neighboring fractions of peak of CSI activity. In addition to these, two high molecular weight proteins showing similarity to glucan synthase and fatty acid synthase were also found in such fractions as analyzed via MALDI-MS. As chitin exists *in vivo* within a network of glucans and proteins, it is likely that glucan synthase might be associated to the chitin synthesis machinery. In future it will be worthwhile to ascertain the active functional relationships among the different proteins found in chitosomal preparations using immuno fluorescence co-localization studies.

## 7.0 Bibliography

**Angiolella, L., Facchin, M., Stringaro, A., Maras, B., Simonetti, N. & Cassone, A. (1996).** Identification of a glucan-associated enolase as a main cell wall protein of *Candida albicans* and an indirect target of lipopeptide antimycotics. *J Infect Dis* **173,** 684-690.

**Angiolella, L., Micocci, M. M., D'Alessio, S., Girolamo, A., Maras, B. & Cassone, A. (2002).** Identification of major glucan-associated cell wall proteins of *Candida albicans* and their role in fluconazole resistance. *Antimicrob Agents Chemother* **46,** 1688-1694.

**Annie Claude, G. *et al.* (2002).** Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415,** 41-47.

**Bartnicki-Garcia, S. & Nickerson, W. J. (1962).** Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochim Biophys Acta* **58,** 102-119.

**Bartnicki-Garcia, S., Bracker, C. E., Lippman, E. & Ruiz-Herrera, J. (1984).** Chitosomes from the wall-less "slime" mutant of *Neurospora crassa*. *Arch Microbiol* **139,** 105-112.

**Blaak, H. & Schrempf, H. (1995).** Binding and substrate specificities of a *Streptomyces olivaceoviridis* chitinase in comparison with its proteolytically processed form. *Eur J Biochem* **229,** 132-139.

**Bowen, A. R., Chen-Wu, J. L., Momany, M., Young, R., Szaniszlo, P. J. & Robbins, P. W. (1992).** Classification of fungal chitin synthases**.** *Proc Natl Acad Sci U S A* **89,** 519–523.

**Briza, P., Ellinger, A., Winkler, G. & Breitenbach, M. (1988).** Chemical composition of the yeast ascospore wall. The second outer layer consists of chitosan. *J Biol Chem* **263,** 11569–11574.

**Bulawa, C. E. & Osmond, B. C. (1990).** Chitin synthase I and chitin synthase II are not required for chitin synthesis in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **87,** 7424–7428.

**Bulawa, C. E. & Wasco, W**. **(1991).** Chitin and nodulation. *Nature* **353,** 710.

**Bulawa, C. E. (1992).** *CSD2, CSD3,* and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. *Mol Cell Biol* **12,** 1764-1776.

**Bulawa, C. E. (1993).** Genetics and molecular biology of chitin synthesis in fungi. *Annu Rev Microbiol* **47,** 505-534. Review.

**Burke, R. L., Tekamp-Olson, P. & Najarian, R. (1983).** The isolation, characterization, and sequence of the pyruvate kinase gene of *Saccharomyces cerevisiae*. *J Biol Chem* **258,** 2193-2201.

**Cabib, E. & Farkas, V. (1971).** The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. *Proc Natl Acad Sci U S A* **68,** 2052-2056.

**Cabib, E., Roberts, R. & Bowers, B. (1982).** Synthesis of the yeast cell wall and its regulation. *Annu Rev Biochem* **51,** 763-793. Review.

**Cabib, E., Sburlati, A., Bowers, B. & Silverman, S. J. (1989).** Chitin synthase 1, an auxiliary enzyme for chitin synthesis in *Saccharomyces cerevisiae*. *J Cell Biol* **108,** 1665-1672.

**Cabib, E. (1991).** Differential inhibition of chitin synthetases 1 and 2 from *Saccharomyces cerevisiae* by polyoxin D and nikkomycins. *Antimicrob Agents Chemother* **35,** 170-173.

**Cabib, E., Roh, D. H., Schmidt, M., Crotti, L. B. & Varma, A. (2001).** The yeast cell wall and septum as paradigms of cell growth and morphogenesis. *J Biol Chem* **276,** 19679–19682.

**Cabib, E. & Duran, A. (2005).** Synthase III-dependent chitin is bound to different acceptors depending on location on the cell wall of budding yeast. *J Biol Chem* **280,** 9170-9179.

**Chaffin, W. L., López-Ribot, J. L., Casanova, M., Gozalbo, D. & Martinez, J. P. (1998).** Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol Mol Biol Rev* **62,** 130-180.

**Choi, W. J. & Cabib, E. (1994a).** The use of divalent cations and pH for the determination of specific yeast chitin synthases. *Anal Biochem* **219,** 368–372.

**Choi, W. J., Sburlati, A. & Cabib, E. (1994b).** Chitin synthase 3 from yeast has zymogenic properties that depend on both the *CAL1* and the *CAL3* genes. *Proc Natl Acad Sci U S A* **91,** 4727–4730.

**Chuang, J. S. & Schekman, R. W. (1996).** Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. *J Cell Biol* **135,** 597-610.

**Davis, L. L. & Bartnicki-Garcia, S. (1984).** The co-ordination of chitosan and chitin synthesis in *Mucor rouxii*. *J Gen Microbiol* **130,** 2095-2102.

**Delgado, M. L., O'Connor, J. E., Azorin, I., Renau-Piqueras, J., Gil, M. L. & Gozalbo, D. (2001).** The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the *Saccharomyces cerevisiae TDH1, TDH2* and *TDH3* genes are also cell wall proteins. *Microbiology* **147,** 411-417.

**DeMarini, D. J., Adams, A. E. M., Fares, H., De Virgilio, C., Valle, G., Chuang, J. S. & Pringle, J. R. (1997).** A septin-based hierarchy of proteins required for localized deposition of chitin in the *Saccharomyces cerevisiae* cell wall. *J Cell Biol* **139,** 75–93.

**Dickinson, J. R., Salgado, L. E. & Hewlins, M. J. (2003).** The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *J Biol Chem* **278,** 8028-8034.

**Doucet, J. P. & Tuana, B. S. (1991).** Identification of low molecular weight GTP-binding proteins and their sites of interaction in subcellular fractions from skeletal muscle. *J Biol Chem* **266,** 17613-17620.

**Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W., El-Sherbeini, M., Clemas, J. A., Mandala, S. M., Frommer, B. R. & Kurtz, M. B. (1994).** The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-glucan synthase. *Proc Natl Acad Sci U S A* **91,** 12907-12911.

**Duran, A. & Cabib, E. (1978).** Solubilization and partial purification of yeast chitin synthetase. Confirmation of the zymogenic nature of the enzyme. *J Biol Chem* **253,** 4419-4425.

**Edwards, S. R., Braley, R. & Chaffin, W. L. (1999).** Enolase is present in the cell wall of *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **177,** 211-216.

**Entian, K. D, Meurer, B., Kohler, H., Mann, K. H. & Mecke, D. (1987).** Studies on the regulation of enolases and compartmentation of cytosolic enzymes in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **923,** 214-221.

**Flores-Martinez, A. & Schwencke, J. (1988).** Chitin synthetase activity is bound to chitosomes and to the plasma membrane in protoplasts of *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **946,** 328-336.

**Gagou, M. E., Kapsetaki, M., Turberg, A. & Kafetzopoulos, D. (2002).** Stage-specific expression of the chitin synthase *DmeChSA* and *DmeChSB* genes during the onset of Drosophila metamorphosis. *Insect Biochem Mol Biol* **32,** 141-146.

**Gaughran, J. P., Lai, M. H., Kirsch, D. R. & Silverman, S. J. (1994).** Nikkomycin Z is a specific inhibitor of *Saccharomyces cerevisiae* chitin synthase isozyme Chs3 *in vitro* and *in vivo*. *J Bacteriol* **176,** 5857-5860.

**Georgopapadakou, N. H. & Walsh, T. J. (1996).** Antifungal agents: chemotherapeutic targets and immunologic strategies. *Antimicrob Agents Chemother* **40,** 279-291.

**Goldstein, I. J. & Poretz, R. D. (1986).** Isolation and chemcial properties of lectins. In *The Lectins*: Properties, Functions and applications in Biology and Medicine, ed. I. E. Liener, N. Sharon, I. J. Goldstein, pp. 103-115. Orlando: academic.

**Hessler, R. J., Blackwood, R. A., Brock, T. G., Francis, J. W., Harsh, D. M. & Smolen, J. E. (1998).** Identification of glyceraldehyde-3-phosphate dehydrogenase as a $Ca^{2+}$ - dependent fusogen in human neutrophil cytosol. *J Leukoc Biol* **63,** 331-336.

**Hänseler, E., Nyhlen, L. E. & Rast, D. M. (1983).** Dissociation and reconstitution of chitosomes. *Biochimica et Biophysica Acta* **745,** 121-123.

**Hohmann, S. (1991).** Characterization of *PDC6*, a third structural gene for pyruvate decarboxylase in *Saccharomyces cerevisiae*. *J Bacteriol* **173,** 7963-7969.

**Ikemoto, A., Bole, D. G. & Ueda, T. (2003).** Glycolysis and glutamate accumulation into synaptic vesicles. Role of glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate kinase. *J Biol Chem* **278,** 5929-5940.

**Inoue, S. B., Takewaki, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y. & Watanabe, T. (1995).** Characterization and gene cloning of 1, 3-beta-D-glucan synthase from *Saccharomyces cerevisiae*. *Eur J Biochem* **231,** 845-854

**Kamada, T., Bracker, C. E. & Bartnicki-Garcia, S. (1991).** Chitosomes and chitin synthetase in the asexual life cycle of *Mucor rouxii*: spores, mycelium and yeast cells. *J Gen Microbiol* **137,** 1241-1252.

**Kang, M. S., Elango, N., Mattia, E., Au-Young, J., Robbins, P. W. & Cabib, E. (1984).** Isolation of chitin synthetase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. *J Biol Chem* **259,** 14966-14972.

**Kapteyn, J. C., Van Den Ende, H. & Klis, F. M. (1999).** The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys Acta* **1426,** 373-383.

**Kellermann, E., Seeboth, P. G. & Hollenberg, C. P. (1986).** Analysis of the primary structure and promoter function of a pyruvate decarboxylase gene (*PDC1*) from *Saccharomyces cerevisiae*. *Nucleic Acids Res* **14,** 8963-8977.

**Kim, K. H. & Park, H. M. (2004).** Enhanced secretion of cell wall bound enolase into culture medium by the *soo1-1* mutation of *Saccharomyces cerevisiae*. *J Microbiol* **42,** 248-252.

**Leal-Morales, C. A., Bracker, C. E. & Bartnicki-Garcia, S. (1988).** Localization of chitin synthetase in cell-free homogenates of *Saccharomyces cerevisiae*: chitosomes and plasma membrane. *Proc Natl Acad Sci U S A* **85,** 8516-8520.

**Leal-Morales, C. A., Bracker, C. E. & Bartnicki-Garcia, S. (1994).** Subcellular localization, abundance and stability of chitin synthetases 1 and 2 from *Saccharomyces cerevisiae*. *Microbiology* **140,** 2207-2216.

**Le Borgne, R. & Hoflack, B. (1998).** Mechanisms of protein sorting and coat assembly: insights from the clathrin-coated vesicle pathway. *Curr Opin Cell Biol* **10,** 499-503.

**Lee, D. W., Ahn, G. W., Kang, H. G. & Park, H. M. (1999).** Identification of a gene, *SOO1,* which complements osmo-sensitivity and defect in *in vitro* beta 1,3-glucan synthase activity in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1450,** 145-154.

**Lending, C. R., Lippman, E., Bracker, C. E. & Bartnicki-Garcia, S. (1990).** An efficient preparative isopycnic flotation method for the isolation of chitosomes. *Protoplasma* **159,** 16-25.

**Lesage, G., Shapiro, J., Specht, C. A., Sdicu, A. M., Menard, P., Hussein, S., Tong, A. H., Boone, C. & Bussey, H. (2005).** An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae. BMC Gene* **6,** 8.

**Liesen, T., Hollenberg, C. P. & Heinisch, J. J. (1996).** ERA, a novel cis-acting element required for autoregulation and ethanol repression of *PDC1* transcription in *Saccharomyces cerevisiae*. *Mol Microbiol* **21,** 621-632.

**Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951).** Protein measurement with the Folin-Phenol reagents. *J Biol Chem* **193**, 265-275.

**Lucero, H. A., Kuranda, M. J. & Bulik, D. A. (2002).** A nonradioactive, high throughput assay for chitin synthase activity. *Anal Biochem* **305,** 97-105.

**McAlister, L. & Holland, M. J. (1982).** Targeted deletion of a yeast enolase structural gene. Identification and isolation of yeast enolase isozymes. *J Biol Chem* **257,** 7181-7188.

**McAlister, L. & Holland, M. J. (1985).** Isolation and characterization of yeast strains carrying mutations in the glyceraldehyde-3-phosphate dehydrogenase genes. *J Biol Chem* **260,** 15013-15018.

**McMurrough, I., Flores-Carreon, A. & Bartnicki-Garcia, S. (1971).** Pathway of chitin synthesis and cellular localization of chitin synthetase in *Mucor rouxii*. *J Biol Chem* **246,** 3999-4007.

**Mejean, C., Pons, F., Benyamin, Y. & Roustan, C. (1989).** Antigenic probes locate binding sites for the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase, aldolase and phosphofructokinase on the actin monomer in microfilaments. *Biochem J* **264,** 671-677.

**Morita, T., Kawamoto, H., Mizota, T., Inada, T. & Aiba, H. (2004).** Enolase in the RNA degradosome plays a crucial role in the rapid decay of glucose transporter mRNA in the response to phosphosugar stress in *Escherichia coli*. *Mol Microbiol* **54,** 1063-1075.

**Novick, P., Field, C. & Schekman, R. (1980).** Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21,** 205-215.

**Orlean, P. (1987).** Two chitin synthases in *Saccharomyces cerevisiae*. *J Biol Chem* **262,** 5732–5739.

**Orlean, P. (1997).** Biogenesis of yeast wall and surface components. In *The Molecular and Cellular Biology of the Yeast Saccharomyces*, vol. III, pp. 229–362. Edited by E. W. Jones, J. R. Pringle, & J. R. Broach. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Pearce, A. K., Crimmins, K., Groussac, E., Hewlins, M. J., Dickinson, J. R., Francois, J., Booth, I. R. & Brown, A. J. (2001).** Pyruvate kinase (Pyk1) levels influence both the rate and direction of carbon flux in yeast under fermentative conditions. *Microbiology* **147,** 391-401.

**Portela, P., Howell, S., Moreno, S. & Rossi, S. (2002).** *In vivo* and *in vitro* phosphorylation of two isoforms of yeast pyruvate kinase by protein kinase A. *J Biol Chem* **277,** 30477-30487.

**Pronk, J. T., Yde-Steensma, H. & Van Dijken, J. P. (1996).** Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12,** 1607-1633.

**Qadota, H., Python C, P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E. & Ohya, Y. (1996).** Identification of yeast Rho1p GTPase as a regulatory subunit of 1, 3-beta-glucan synthase. *Science* **272,** 279-281.

**Raikhel, N. V., Lee, H. I. & Broekaert, W. F. (1993).** Structure and function of chitin-binding proteins. *Annu Rev Plant Physiol Plant Mol Bio* **44,** 591-615.

**Raths, S., Rohrer, J., Crausaz, F. & Riezman, H. (1993).** *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J Cell Biol* **120,** 55-65.

**Roncero, C. (2002).** The genetic complexity of chitin synthesis in fungi. *Curr Genet* **41,** 367–378.

**Rothman, J. E. & Wieland, F. T. (1996).** Protein sorting by transport vesicles. *Science* **272,** 227-234.

**Ruiz-Herrera, J. & Bartnicki-Garcia, S. (1974).** Synthesis of cell wall microfibrils in vitro by a "soluble" chitin synthetase from *Mucor rouxii. Science* **186,** 357–359.

**Ruiz-Herrera, J., Sing, V. O., Van der Woude, W. J. & Bartnicki-Garcia, S. (1975).** Microfibril assembly by granules of chitin synthetase *Proc Natl Acad Sci U S A* **72,** 2706–2710.

**Ruiz-Herrera, J. & Bartnicki-Garcia, S. (1976).** Proteolytic activation and inactivation of chitin synthetase from *Mucor rouxii. J Gen Microbiol* **97,** 241–249.

**Ruiz-Herrera, J., Bartnicki-Garcia, S. & Bracker, C. E. (1980).** Dissociation of chitosomes by digitonin into 16 S subunits with chitin synthetase activity. *Biochim Biophys Acta* **629,** 201-206.

**Ruiz-Herrera, J., Bracker, C. E. & Bartnicki-Garcia, S. (1984).** Sedimentation properties chitosomes from *Mucor rouxii. Protoplasma* **122,** 178-190.

**Ruiz-Herrera, J., Gonzalez-Prieto, J. M. & Ruiz-Medrano, R. (2002).** Evolution and phylogenetic relationships of chitin synthases from yeasts and fungi. *FEMS Yeast Res* **1,** 47-56.

**Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Santos, B., & Snyder, M. (1997).** Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J Cell Biol* **136,** 95-110.

**Sburlati, A. & Cabib, E. (1986).** Chitin synthetase 2, a presumptive participant in septum formation in *Saccharomyces cerevisiae. J Biol Chem* **261,** 15147–15152.

**Schaegger, H., Cramer, W. A. & von Jagow, G. (1994).** Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Anal Biochem* **217,** 220-230.

**Schekman, R. & Orci, L. (1996).** Coat proteins and vesicle budding. *Science* **271,** 1526-1533.

**Schmitt, H. D. & Zimmermann, F. K. (1982).** Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis. *J Bacteriol* **151,** 1146-1152.

**Schnellmann, J., Zeltins, A., Blaak, H. & Schrempf, H. (1994).** The novel lectin-like protein CHB1 is encoded by a chitin-inducible *Streptomyces olivaceoviridis* gene and binds specifically to crystalline alpha-chitin of fungi and other organisms. *Mol Microbiol* **13,** 807-819.

**Schörr, M., Then, A., Tahirovic, S., Hug, N. & Mayinger, P. (2001).** The phosphoinositide phosphatase Sac1p controls trafficking of the yeast Chs3p chitin synthase. *Curr Biol* **11,** 1421-1426.

**Schrempf, H. (1999).** Characteristics of chitin-binding proteins from Streptomycetes. *EXS* **87,** 99-108.

**Schweizer, M., Roberts, L. M., Holtke, H. J., Takabayashi, K., Hollerer, E., Hoffmann, B., Muller, G., Kottig, H. & Schweizer, E. (1986).** The pentafunctional *FAS1* gene of yeast: its nucleotide sequence and order of the catalytic domains. *Mol Gen Genet* **203,** 479-486.

**Semino, C. E., Specht, C. A., Raimondi, A. & Robbins, P. W. (1996).** Homologs of the Xenopus developmental gene *DG42* are present in zebrafish and mouse and are involved in the synthesis of Nod-like chitin oligosaccharides during early embryogenesis. *Proc Natl Acad Sci U S A* **93,** 4548-4553.

**Shaw, J. A., Mol, P. C., Bowers, B., Silverman, S. J., Valdivieso, M. H., Duran, A. & Cabib, E. (1991).** The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. *J Cell Biol* **114,** 111–123.

**Sietsma, J. H., Beth-Din, A., Ziv, V., Sjollema, K. A. & Yarden O. (1996).** The localization of chitin synthase in membranous vesicles (chitosomes) in *Neurospora crassa*. *Microbiology* **142,** 1591-1596.

**Siemieniewicz, K. W. (2004).** Studien zur Erkennung und Biogenese von Chitin mit Hilfe des *Streptomyces olivaceoviridis* chitinbindenden Proteins CHB1.

**Smits, G. J., van den Ende, H. & Klis, F. M. (2001**). Differential regulation of cell wall biogenesis during growth and development in yeast. *Microbiology* **147,** 781-794.

**Svergun, D. I., Becirevic, A., Schrempf, H., Koch, M. H. & Gruber, G. (2000).** Solution structure and conformational changes of the *Streptomyces* chitin-binding protein (CHB1). *Biochemistry* **39,** 10677-10683.

**Tong, A. H., Lesage, G., Bader, G. D. & 47 other authors (2004).** Global mapping of the yeast genetic interaction network. *Science* **303,** 808–813.

**Trilla, J. A., Cos, T., Duran, A. & Roncero, C. (1997).** Characterization of *CHS4 (CAL2),* a gene of *Saccharomyces cerevisiae* involved in chitin biosynthesis and allelic to *SKT5* and *CSD4*. *Yeast* **13,** 795-807.

**Trilla, J. A., Duran, A. & Roncero, C. (1999).** Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in *Saccharomyces cerevisiae*. *J Cell Biol* **145,** 1153-1163.

**Valdivieso, M. H., Mol, P. C., Shaw, J. A., Cabib, E. & Durán, A. (1991).** *CAL1,* a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. *J Cell Biol* **114,** 1101–1109.

**Valdivieso, M. H., Ferrario, L., Vai, M., Duran, A. & Popolo, L. (2000).** Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **182,** 4752-4757.

**Westhead, E. W. (1966).** Enolase from yeast and rabbit muscle. In Methods in Enzymology, Vol. 8: Complex Carbohydrates, E.F. Neufeld and V. Ginsburg, eds (New York: Academic Press), pp. 670-679.

**Yeager, A. R. & Finney, N. S. (2005).** Synthesis of fluorescently labeled UDP-GlcNAc analogues and their evaluation as chitin synthase substrates. *J Org Chem* **70,** 1269-1275.

**Ziman, M., Chuang, J. S. & Schekman, R. W. (1996).** Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the *Saccharomyces cerevisiae* endocytic pathway. *Mol Biol Cell* **7,** 1909-1919.

**Ziman, M., Chuang, J. S., Tsung, M., Hamamoto, S. & Schekman, R. (1998).** Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Mol Biol Cell* **9,** 1565-1576.

## Affidavit

I declare that I wrote this thesis myself. I did not use other auxiliary material than indicated. Other work is always cited.

I have not tried to get a Ph. D. before.

Osnabrück, September, 2005.

Kajla, Mayur Kumar

# Acknowledgements

I wish to express my heartfelt gratitude to Prof. Dr. H. Schrempf for giving me this opportunity to work in this lab under her able guidance, ever encouraging and constant support through the tenure of my stay here. The special care she took to arrange for the things at various occasions in the last three years is highly appreciated. I could not have learned German if she had not forced me and for which I extend my heartiest thanks.

I extend my sincere thanks to Prof. Dr. Hofemeister, Prof. Betzler and Dr. Dario for kindly accepting to review my thesis.

I take this opportunity to thank all the people in the lab who contributed in one way or the other to my thesis and helpful discussions at various times.

My special thanks to Mr. Holger Hippe (ChromaTech GmBH) for his kind help and understanding.

I duly acknowledge the support of International Graduate School of Osnabrueck University and express my thanks to Dr. Schnack and Dr. Mikosch for their kind helps during these three years.

I extend a million thanks to my parents who have contributed a lot for the cause of my education during last few years. Dear, Mom and Dad, I dedicate with this thesis with my sincere gratitude to you for supporting me throughout the years emotionally and financially, mostly caring least about your own comfort. Thanks. Loving support from, my brothers, sister and bhabiji are duly acknowledged.

Lastly I take this opportunity to thank my beloved Master without whose hidden support, care and helping hand I could not have survived the hardships of life …….. no words of thanks will ever be suffice to convey my deep sense of gratitude to u dear Master for everything you did/do/will do, visible and invisible……..Thanks.

**Mayur Kumar Kajla**

**Private address**
Jahnstrasse 32/09, 49080 Osnabrück.

**Permanent address**
VPO- Jawal, Teh-Amb, Distt-Una, HP-177110, India.
Telephone- +91 1976 256213
Email-mayurkkajla@rediffmail.com

**Personal details**

| | | |
|---|---|---|
| Date of Birth | : | 13th January 1976 |
| Gender | : | Male |
| Marital Status | : | Single |
| Nationality | : | Indian |

**Academic Qualifications**

**August 2002 till present:** ***Pursuing*** Ph. D. thesis at the Dept. of Applied Genetics of Microorganisms, Osnabrueck University, Germany.

**1997-1999: Master of Science, Biotechnology**, Himachal Pradesh University Shimla, India.

**1996-1997: Bachelor of Education,** Teaching certificate for High School standard**,** Himachal Pradesh University Shimla, India.

**1993-1996: Bachelor of Science** (Botany, Zoology and Chemistry), Himachal Pradesh University Shimla, India.

**1991-1993**: **Senior School Examination** (Physics, Chemistry and Biology), Himachal Pradesh Board of School Education Dharamshala, India.

**Publications/Posters**

1. Krzysztof Siemieniewicz, **Mayur K. Kajla**, Hildgund Schrempf. Visualizing the biogenesis of chitin fibres mediated by *Saccharomyces cerevisiae* chitin synthase I using *Streptomyces* proteins as tools. (Submitted)
2. Jannet Rodrigues, Anil Sharma, Neema Agrawal, **Mayur K. Kajla**, Deepali Singh, T. Adak and Raj K. Bhatnagar. Role of *Anopheles culicifacies* serine protease and proPhenol oxidase in imparting strain refractoriness (Submitted to Molecular and Biochemical Parasitology).

3. Poster - K. Siemieniewicz, A. Becirevic, P. Stamouli-Walder, **Mayur K. Kajla,** Z. Chen and H. Schrempf. Characteristics of the secreted *Streptomyces* chitin-binding protein CHB1 - presented at Symposium: Biology of *Streptomyces* and related Actinomycetes, February 2003, Münster, Germany.

4. Poster - K. Siemieniewicz, P. Stamouli-Walder, **Mayur K. Kajla,** Z. Chen and H. Schrempf. Characteristics of secreted chitin-binding proteins - presented at annual meeting of VAAM, March 2003, Berlin, Germany.

**Conferences attended**

- VAAM Annual Meeting, Berlin, 2003, Germany.
- Workshop on Immuno-Electron Microscopy, September, 2003, MPI Dresden, Germany.
- International Meeting on Biology of *Streptomyces* and related Actinomycetes, March, 2003, Munster, Germany.
- International meeting on Topogenesis of Organellar proteins, October 2004 Bochum, Germany.
- First Annual BioMalPar Conference on the Biology and Pathology of the Malaria Parasite, March 2005, EMBL, Heidelberg, Germany.

**Scholarships**

- **Stipend (Lichtenberg-Stipendium)** from International Graduate School (IGS), Osnabrueck University, Germany, for doctoral thesis.
- **Senior research fellowship (SRF),** Malaria Research Center, Indian Council of Malaria Research, Govt. of India.
- **UGC-CSIR-NET- N**ational **E**ntrance **T**est for eligibility of lecturership, **U**niversity **G**rants **C**ommission and **C**ouncil for **S**cientific & **I**ndustrial **R**esearch (CSIR), Govt. of India.

*Be merciful unto me, O God, be merciful unto me:*
*For my soul trusteth in thee: yeh, in the shadow of thy wings will I make my refuge,*
*until these calamities be overpast……*

*Pslams*