Glucan synthase of *Phytophthora sojae*. Characterization, purification and attempts of molecular cloning

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List of abbreviations

2D electrophoresis	two-dimensional electrophoresis
Abs	absorbance
APS	ammonium peroxydisulfate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propansulfonate
CMC	critical micelle concentration
COSY	correlation spectroscopy
cpm	counts per minute
CTAB	hexadecyltrimethylammonium
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DMSO-d_6	deuterated dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DP	degree of polymerization
DTT	dithiothreitol
EDTA	ethylendiamintetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethylether)-tetraacetic acid
EST	expressed sequenced tag
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
GTPase	guanosine triphosphatase
HH-COSY	^{1}H-^{1}H correlation spectroscopy
HMQC	heteronuclear correlation through multiple quantum coherence
HMW	high molecular weight
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
IEF	isoelectric focusing
IPTG	isopropyl-β-D-thiogalactopyranoside

K_m	Michaelis constant
LysC	Lysine C endopeptidase
NMR	nuclear magnetic resonance
p. a.	pro analyse
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylenglycol
pfu	plaque forming unit
PPi	inorganic pyrophosphate
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SA-PMPs	Streptavidin-Paramagnetics Particles
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylendiamine
TLC	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UDP	uridine diphosphate
UDP-Glucose	uridine diphosphate glucose
UV light	ultraviolet light
V_{max}	maximal velocity
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. Introduction

During 1845 and 1846 the late blight caused by *Phytophthora infestans* totally destroyed Ireland's staple potato crop. The most immediate effects were poverty and mass starvation, which led to profound social and economic changes in that country and to the emigration of large numbers of people to the United States and elsewhere. It is estimated that Ireland lost one fourth of its eight million inhabitants to starvation and emigration. Perhaps no other single plant disease has resulted in such widespread human suffering and sociological impacts (Erwin and Ribeiro, 1996). Still nowadays world-wide losses due to late blight are estimated to exceed two billion US-dollars annually (Kamoun et al., 1999). Another economically important member of the family that caused the disaster in Ireland, is *Phytophthora sojae*, which causes the root and stem rot in soybean (Erwin and Ribeiro, 1996). In Ohio (United States) alone, *Phytophthora* root rot of soybean causes a 50 million US-dollars annual loss to growers (McBlain et al., 1991a, b).

A better understanding of the pathogen characteristics, the plant host defense mechanisms and the plant-pathogen interactions should result in the development of devices for controlling or minimizing the pernicious effects of these plagues.

1.1. Plant pathogens and plant defense

Plants are resistant to most potential pathogens in their environment. In fact, relatively few true host-pathogen pairs exist in which the plant is susceptible and the pathogen virulent (Ebel and Scheel, 1997). Plant disease resistance can be defined as the ability of the plant to prevent or restrict pathogen growth and multiplication (Benhamou, 1996). In functional terms, the chain of molecular events constituting critical steps of plant-pathogen interactions, can be divided into three steps (Ebel, 1998):

1. Generation and recognition of signal compounds
2. Inter- and intracellular signal conversion and transduction
3. Activation of signal-specific responses of target cells

The extracellular signal compounds involved in triggering defense reactions are commonly known as elicitors. In a general model, elicitors of plant defense responses fall into two categories depending on their source: exogenous and endogenous signals. Exogenous elicitors can be considered the primary signals in plant-pathogen interactions, while endogenous

elicitors are of plant origin and arise as a result of the interaction with the aggressor (Ebel and Cosio, 1994).

Several facts suggest the involvement of receptors in elicitor recognition (Ebel and Scheel, 1997):

- cultivar or species specificity of purified elicitors
- the diverse chemical nature of elicitors
- the ability of elicitors to induce various plant responses at low (nanomolar) concentrations
- the elicitor dose/response relationships
- high degree of signal specificity observed for elicitor activity.

Following pathogen recognition, a subsequent intracellular signal propagation occurs. As in animal cells, different classes of membrane-localized receptors may operate as activators of ion channels, protein kinases, or pathways generating intracellular signal compounds. There are, nevertheless, cases of intracellular receptors for bacterial (Sessa and Martin, 2000) and fungal (Jia et al., 2000) elicitors.

Typical elements of the multicomponent defense response include the hypersensitive reaction, the production of reactive oxygen species, the activation of defense-related genes, structural changes of the cell wall, and the synthesis of phytoalexins. In addition to these localized defenses, systemic acquired resistance, which increases the plant's resistance systemically to subsequent pathogen attack, is activated in many plants and can be induced by certain elicitors (Ebel, 1998).

Two types of plant resistance response to potential pathogens can be distinguished: the non-host resistance response (frequent), and the race/cultivar-specific resistance response (comparatively rare). The latter is genetically defined by the direct or indirect interaction between the product of a dominant or semi-dominant major plant resistance gene (R) and the complementary product of the corresponding dominant pathogen avirulence (avr) gene. Non-host resistance (basic incompatibility) is exhibited by all plant species that respond to potential pathogens without apparent R/avr gene combinations. Apart from this distinction, however, the biochemical processes occurring in host and non-host resistance are very similar (Somssich and Hahlbrock, 1998).

1.1.1. Types of elicitors

In terms of its chemical characteristics elicitors can be classified into two main groups (Ebel and Mithöfer, 1998):

1. (glyco)peptides and proteins
2. oligosaccharides

The first group includes the elicitins, small extracellular proteins that are secreted by most *Phytophthora* species. A 42-kDa glycoprotein secreted by *P. sojae* elicited phytoalexin production in parsley; a 13 amino acid-long part of its sequence was shown to be necessary and sufficient for elicitor activity (Ebel and Scheel, 1997).

Four major classes of elicitor-active oligosaccharides have been identified (Ebel, 1998):

- oligoglucan
- oligochitin
- oligochitosan
- oligogalacturonide (plant origin)

Glucans with the ability to stimulate the production of phytoalexins were initially detected in culture filtrates of *P. sojae* (Ayers et al., 1976).

1.2. *Phytophthora sojae*

1.2.1. The genus

The name of the genus *Phytophthora* is derived from the Greek that literally means *phyto* (plant) and *phthora* (destroyer) (Webster, 1980). *Phytophthora* is a genus that is mainly, if not entirely, parasitic on various plant hosts. Some species are host specific and others have broad host ranges. It occupies a small but pathologically significant niche in Oomycota.

The phylum Oomycota had been previously classified in Myceteae (fungi), but studies of metabolism, cell wall composition and rRNA sequence (Figure 1) indicate that oomycetes are more properly classified together with chrysophytes, diatoms, and brown algae (Judelson, 1997) and should be included in the Chromista kingdom (Erwin and Ribeiro, 1996) or in the Stramenopiles (Qutob et al., 2000; taxonomy browser on NIH).

The thallus of *Phytophthora*, as in real fungi, is called mycelium (Greek *mykes*, a fungus; *-lium*, after epithelium). It is composed of hyaline, branched, coenocytic (nonseptate) filaments, except in old cultures where septa can sometimes be seen. In young cultures, the cytoplasm flows freely within the mycelium. The diameter of the mycelium (5-8 µm) is

variable and dependent of the physical and chemical nature of the medium and on whether the mycelium is on the surface, aerial, submerged, or within host cells. Hyphae (single branches of the mycelium) branch at nearly right angles. Some species (*P. sojae* among them) show hyphal swellings in-chains or clusters, noted most readily in liquid cultures.

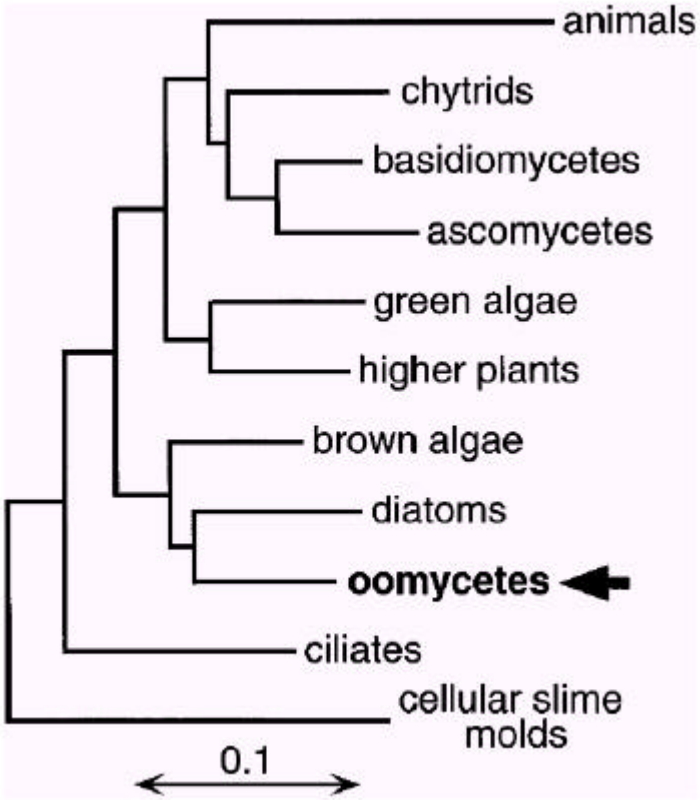


Figure 1: Phylogeny based on small-subunit rRNA sequence similarities. Incorporates data from (top to bottom) *Homo sapiens*, *Blastocladiella emersonii*, *Aureobasidium pullulans*, *Neurospora crassa*, *Clamydomonas reinhardtii*, *Zea mays*, *Ochromonas danica*, *Skeletonema costatum*, *Phytophthora sojae*, *Paramecium tetraurelia*, and *Dictyostelium discoideum*. The horizontal bar indicates the distance representing 10 changes per 100 nucleotides (figure taken from Judelson, 1997)

Asexual sporangium development (Figure 2) is the means of most rapid reproduction. Sporangia (Greek *spora*, a seed; *angeion*, a vessel) are asexual spores that are produced on stalks called sporangiophores, which differ slightly or not at all from vegetative hyphae.

Biflagellate zoospores are reniform to pyriform in shape; the anterior end is tapered, and there is a deep longitudinal groove on one side. Two flagella of unequal length emerge from the groove, one of the whiplash type, and the other of the tinsel type.

The sexual process in the *Phytophthora* life cycle involves the production of the female oogonium (the sac in which the oospore is formed) and the antheridium (the male structure), both of which emanate from mycelial tips that make contact. Oospores are thick-walled sexual structures formed after the antheridium and oogonium fuse, nuclei from the antheridium flow into the oogonium, and the antheridial and oogonial nuclei fuse in the oogonium. The oospores of homothallic (self-fertile) species (*P. sojae* among them) form in agar or liquid media and in infected plant tissue.

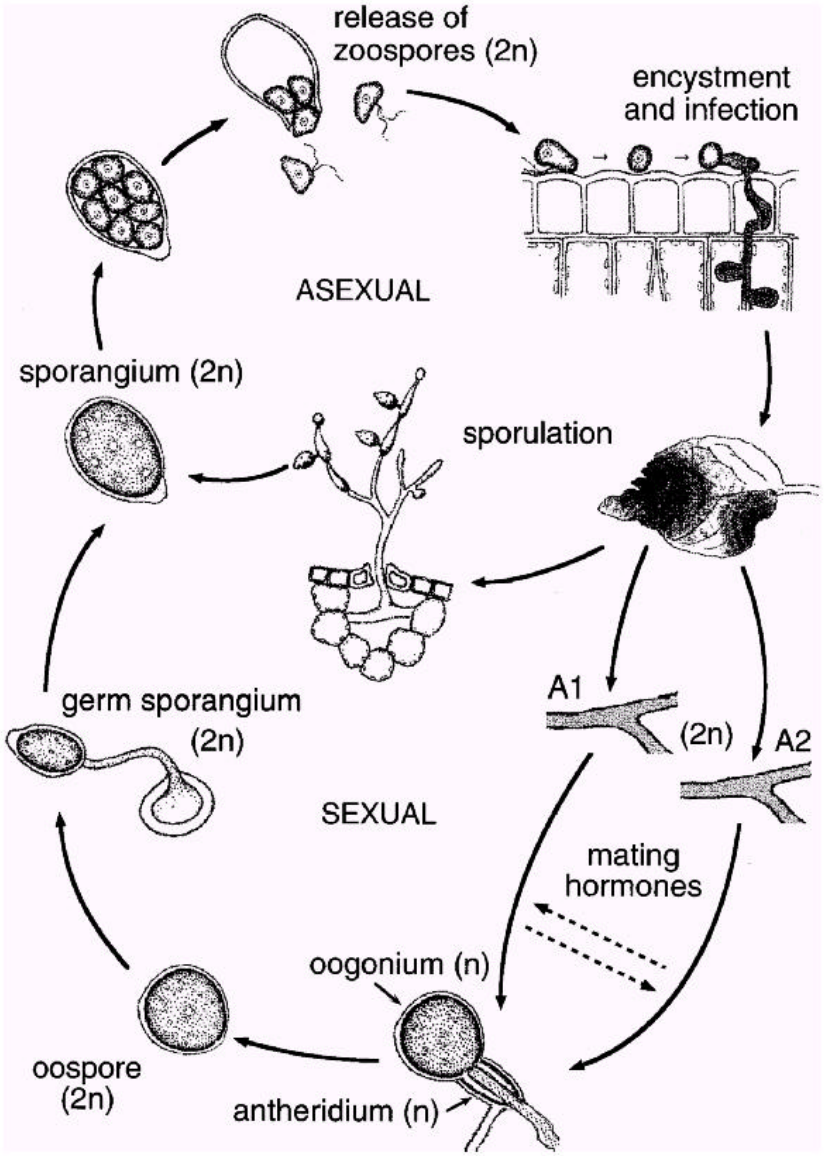


Figure 2: Disease (asexual) and sexual cycles of *Phytophthora*. Figure taken from Judelson 1997. *P. sojae* is homothalic, thus, no mating event occurs.

1.2.2. *Phytophthora sojae*

Formerly known as *Phytophthora megasperma* var. *sojae* or *P. megasperma* f. sp *glycinea*, *Phytophthora sojae* is the causal agent of root and stem rot of soybean (*Glycine max* L.). The host range is relatively narrow; soybean is the major host (Erwin and Ribeiro, 1996).

P. sojae is homothallic, and thus may reproduce sexually by the development of oospores trough self-fertilization or by outcrosses between different strains.

Zoospores encyst and germinate on the root or hypocotyl surface, and the resulting germ tube may swell to form an appressorium-like structure at the point of penetration into host tissues. In susceptible interactions, large, water-soaked lesions develop as the pathogen rapidly invades the host. *P. sojae* spreads through the intercellular matrix of the plant and forms haustoria for intimate contact with host cells.

Phytophthora root and stem rot develops rapidly at temperatures between 25 and 30°C, particularly in areas with poorly drained, heavy soils and after heavy rainfall. *P. sojae* infects lateral roots and progresses into the taproots and hypocotyls in susceptible cultivars, but in resistant cultivars the fungus is limited to the cortex and stele of lateral roots. Sporangia and zoospores form on infected roots, and zoospores are transported in free water.

Cultivars with race-specific resistance are not affected by *P. sojae* until a new pathologic (physiologic) race develops in high enough population to cause disease. Fortunately, plant breeders and plant pathologists have been able to select a wide array of resistant genes that have been incorporated into agronomically adapted soybean cultivars.

Estimations of genome size of *P. sojae* vary from 62 to 97 Mb. The average G + C content is 58% (Qutob et al., 2000).

1.3. The cell wall of *Phytophthora*

Cell walls have two major roles: protection and morphogenesis. In addition to allowing an organism to safely generate high metabolite concentrations in its cytoplasm to sustain high growth rates, the wall can be moulded into the diversity of shapes that characterize the vegetative and reproductive structures found in the life cycle of *P. sojae*. This morphological plasticity is a key part of an adaptive capacity with which an organism can successfully meet a wide assortment of ecological challenges.

The cell walls of *Phytophthora* are made principally of β-linked glucose polymers. These β-glucans comprise about 80-90% of the wall dry weight (Bartnicki-Garcia and Wang, 1983). The cell walls also contain small amounts of protein, lipid, and other polysaccharides, in

which mannose and glucosamine appear to be main sugar components, and are probably part of surface glycoproteins (Ayers et al., 1976; Bartnicki-Garcia, 1966).

Cell wall β-glucans are highly insoluble polymers. Two different types of glucans can be distinguished in the cell wall of *Phytophthora*: cellulose and noncellulosic glucan. The latter is much more abundant, about two to tenfold more than the cellulosic glucan. The cellulosic glucan contains the typical β-1,4-linked chains of glucose, but this cellulose is poorly crystalline and does not give any sharp reflections upon X-ray diffraction (Bartnicki-Garcia, 1966). The noncellulosic glucan is mainly a highly branched (1→3)-β-glucan with (1→6)-β-linkages at the branching residues. In addition, about 10% of the glucose units in the noncellulosic glucan were found to be joined by (1→4)-β-linkages (Bartnicki-Garcia and Wang, 1983)

1.4. The b-glucans

Glucans, with the (1→3)-β-glucosidic linkage as major feature, are present in most of the higher plants, in many lower plants as well as in micro-organisms (Stone and Clarke, 1992).

Prokaryotic organisms, such as the bacteria and blue-green algae, synthesize a variety of polysaccharides as components of capsules and other extracellular products. These polysaccharides include (1→3)-β-glucans, (1→2)-β-glucans, and cellulose (Stone and Clarke, 1992).

Glucans containing (1→3)- and (1→6)-β-glucosidic linkages are important components of cell walls and secretions of fungi in classes within the *Ascomycotina*, *Basidiomycotina* and *Deuteromycotina*. The (1→3),(1→6)-β-glucans are usually highly branched. They are often present as an inner wall layer and sometimes associated covalently with other wall polymers, particularly polysaccharides such as chitin. As major wall components, they and the appropriate (1→3)-β-glucan hydrolases and synthases are involved in those wall modifications which occur during growth and morphogenesis. The (1→3)-β-glucan hydrolases which depolymerize their fungal substrates are used commercially in fermentation and food processing (Stone and Clarke, 1992).

Callose, the (1→3)-β-glucan present in plants, occurs in low amounts in cell walls of intact tissues in a limited number of locations such as sieve plates, cell plates of newly divided cells and plasmodesmata (Northcote et al., 1989). It is synthesized rapidly, however, in response to wounding, pathogen attack, and mechanical pressure and it is deposited locally to form a protective barrier in association with other components. It is also deposited in pollen mother

cells and in pollen tubes during incompatible interactions (McCormack et al., 1997). Callose constitutes 75% (w/w) of the total wall carbohydrates in pollen tubes of *Nicotiana alata* (Rae et al., 1985)

The (1→3),(1→4)-β-glucans are apparently restricted to members of the *Gramineae*, among the Angiosperms, where they are a major component of endosperm walls of cereals like barley, rye, sorghum, rice and wheat (Carpita, 1996).

X-ray diffraction studies have shown that linear (1→3)-β-glucans obtained *in vivo* or synthesized *in vitro* are arranged in a triple helix. The individual strands are extensively hydrogen bonded; essentially each hydroxyl oxygen atom participates in at least one hydrogen bond (Stone and Clarke, 1991).

1.5. Glucan synthases

The synthesis of (1→3)-β-glucan *in vivo* is catalyzed by the enzyme (1→3)-β-glucan synthase (EC.2.4.1.34, UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyl transferase) using UDP-glucose as substrate (Figure 3).

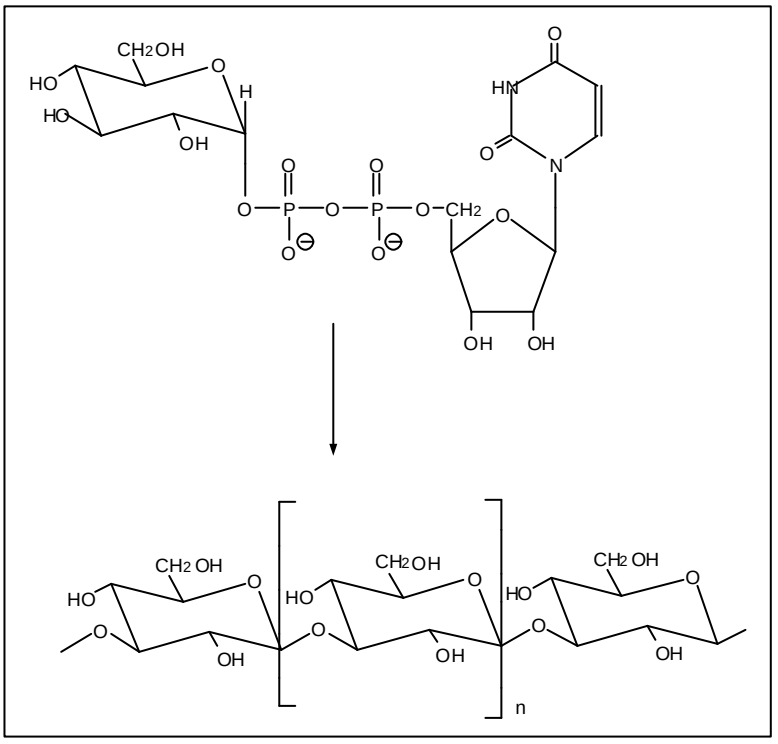


Figure 3: Diagrammatic representation of the reaction catalyzed by (1→3)-β-glucan synthase.

The earliest investigations of *in vitro* β-glucan synthesis were directed toward understanding the mechanism of cellulose synthesis in plants; however, protein preparations from a number of different plants catalyzed the incorporation of labeled glucose from UDP-glucose into water insoluble polymers, identified later as (1→3)-β-glucan (Delmer, 1987, 1999). Almost all attempts at *in vitro* synthesis of cellulose with plant preparations have resulted in the formation of exclusively (1→3)-β-glucans (Carpita and Delmer, 1980; Delmer, 1987, 1999).

The (1→3)-β-glucan synthase (it will be named glucan synthase in the rest of this work) was characterized in a number of fungi and plants, but not very much work was done with oomycetes (Stone and Clarke, 1992) even though one of the earliest successful *in vitro* assays for glucan synthase activity was achieved using *Phytophthora cinnamomi* (Wang and Bartnicki-Garcia, 1976; Selitrennikoff, 1995).

Glucan synthase is, apparently, an integral transmembrane enzyme, which makes purification difficult. This fact, coupled with the observation that in many cases the enzyme is not a single polypeptide but rather is composed by several components, has made purification by monitoring enzyme activity even more challenging (Selitrennikoff, 1995). Genetic approaches led to the discovery of genes necessary for glucan synthesis but not coding for catalytic enzymes, but for e.g. nuclear factors (Enderlin and Selitrennikoff, 1994) or post-translational modifying enzymes (Díaz et al., 1993; Inoue et al., 1999).

A putative glucan synthase gene of *Saccharomyces cerevisiae* was cloned independently by several groups using different strategies. In the first of the reports (Douglas et al., 1994), it was shown that in *Saccharomyces cerevisiae*, mutations in FKS1 conferred hypersensitivity to the immunosuppressants FK506 and cyclosporin A, while mutations in ETG1 conferred resistance to the cell-wall-active echinocandins (inhibitors of glucan synthase) and, in some cases, concomitant hypersensitivity to the chitin synthase inhibitor nikkomycin Z. The FKS1 and ETG1 genes were cloned by complementation of these phenotypes and were found to be identical. Disruption of the gene resulted in:

- pronounced slow growth phenotype
- hypersensitivity to FK506 and cyclosporin A
- slight increase in sensitivity to echinocandin
- significant reduction in (1→3)-β-glucan synthase activity *in vitro*.

Simultaneously, the gene CWH53 was cloned (Ram et al., 1995). This gene complemented the calcoflour white-hypersensitive mutant cwh53 and is identical to FKS1 or ETG1.

Parallel work by Inoue et al. (1995) resulted in the cloning of the same gene by using a biochemical approach rather than a genetic one. The β-glucan synthase from *S. cerevisiae* was purified up to 700-fold by product entrapment. A 200 kDa protein was clearly observed by SDS-PAGE of the purified fraction. This protein was digested and microsequenced, and this information was used for cloning two genes: GSC1, which is identical to ETG1/FSC1/CWH53, and GSC2, which shares 88% similarity at the amino acid level with GSC1. Hydropathy profiles of both proteins suggest that these genes encode integral membrane proteins which can be assumed to have approximately 16 transmembrane domains.

Simultaneous disruption of FKS1 and FKS2 is lethal (Inoue et al., 1995; Mazur et al., 1995), suggesting that Fks1p and Fks2p are alternative subunits with essential overlapping function. Expression of FKS1 predominates during growth under optimal condition. In contrast, FKS2 expression is induced by mating pheromone, high extracellular [Ca^{2+}], growth on poor carbon sources, or in a fsk1 mutant. FKS2 expression is induced as cells enter stationary phase through a SNF1-, calcineurin-, and cell integrity signaling-independent pathway (Zhao et al., 1998).

A GTP-binding protein is required for glucan synthase activity in yeast (Mol et al., 1994) and other fungi (Wang and Bartnicki-Garcia, 1982; Kang and Cabib, 1986). This protein was identified as Rho1, a small GTPase (Mazur and Baginsky, 1996) and it was demonstrated to form part of the glucan synthase complex, interacting directly with Fks1p (Drgonová et al., 1996; Qadota et al., 1996).

FKS1 homologues were identified in other fungi like *Aspergillus nidulans* (Kelly et al., 1996), *Candida albicans* (Douglas et al., 1997), *Cryptococcus neoformans* (Thompson et al., 1999) or *Paracoccidioides brasiliensis* (Pereira et al., 2000). Recently, a FSK1 homologue was identified in *Pneumocystis carinii* (Kottom and Limper, 2000) with a predicted molecular mass correlating well with a peptide that immunoprecipitates with the glucan synthase activity, if an antibody raised against a synthetic peptide derived from the gene sequence is used.

Until now, no functional heterologous expression of any of these putative glucan synthase genes has been reported.

1.6. Objectives

The aim of this work was to identify and isolate the (1→3)-β-glucan synthase of *Phytophthora sojae* and to clone the genes or cDNAs coding for the protein or proteins

responsible for glucan synthesis. This required the characterization of the enzyme's biochemical and kinetic properties as well as the identification of possible cofactors or inhibitors. This knowledge should then be used for optimizing culture conditions and purification methods. As the glucan synthase activity is membrane-bound, a solubilization method should be established.

After isolation of one or several proteins involved in glucan synthesis, their polypeptides should be microsequenced. With the information contained in the peptide sequences derived from the proteins, degenerated oligonucleotides should be designed, in order to obtain PCR products, using cDNA of *P. sojae* as template. These PCR products should be used as a probe for screening a cDNA phage library of *P. sojae*. Resulting cDNAs, possibly coding for proteins involved in glucan synthesis, should be identified and sequenced.

2. Materials

2.1. Organisms

2.1.1. *Phytophthora sojae*

Phytophthora sojae, race 1 and 3 (previously named *Phytophthora megasperma* f. sp. *glycinea*), was obtained from E. Ziegler, Aachen.

2.1.2. *Escherichia coli* strains

JM109: Allows blue-white screening, cloning and amplification.
Genotype: recA1, endA1, gyrA96, thi, hsdR17 (r_{K-},m_{K+}), relA1, supE44, Δ(lac-proAB), [F´, traD36, proAB, $lacI^qZ\Delta M15$]; Promega.

DH5a : Allows blue-white screening, cloning and amplification.
Genotype: recA1, endA1, gyrA96, thi, hsdR17 , relA1, supE44, lac[F´, proAB, $lacI^qZ\Delta M15$, TN, $(Tet^r)]^c$; Gibco BRL

Y1090(ZL): Used for phage infection
Genotype: mcrB hsdR; Gibco BRL.

DH10B(ZIP): Used for *in vivo* excision
Genotype: F^-*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ^-*rps*L *nup*G; Gibco BRL.

2.1.3. *Saccharomyces cerevisiae*

INVSc2: Used for heterologous expression, ideal for pYES2 vector.
Genotype: MATα, his3Δ1, leu2, trp1-289, ura3-52; Invitrogene.

2.2. Chemicals

Table 1: List of chemicals

Name	Quality	Company	Catalogue no.
α-Amylase		Sigma	A-6255
α-D-Glucose monohydrate		Roth	6887.1
β-Sitosterol	60%	Fluka	85451
2-Propanol	p.a.	Roth	6752.1
Acetic acid	p.a.	Roth	3738.1
Acrylamide	30% acrylamide, 0.8% bisacrylamide	Roth	3029.1
Adenine	99%	Sigma	A8026
Agar-agar	<13.9%	Roth	5210.2
Agarose	peqGold Universal	Serva peqlab	86556 35-1020
Aldolase	p.a.	Serva	400006
Amonium peroxydisulphate (APS)	p.a.	Roth	9592.2
Ampicillin, sodium salt	99%	Appligene	69-52-3
Amylase	p.a.	Sigma	A-8781
Anthrone	p.a.	Merck	3125985
Apoferritin	p.a.	Sigma	A-3660
Bacto-peptone		Difco	0118-17-0
Bacto-tryptone		Difco	0145-6-0
Bacto-yeast extract		Difco	0127-17-9
BioRad protein assay	Dye reagent concentrate	BioRad	500-0006
Bromophenol blue		Roth	A512.1
BSA	>96% p.a.	Roth Sigma	3452 A-8531
$CaCO_3$	p.a.	Merck	1544
Calcium chloride ($CaCl_2 \cdot 2\ H_2O$)	p.a.	Merck	2382

Name	Quality	Company	Catalogue no.
Casamino acids		Difco	0230-15-5
CHAPS	99%	Calbiochem	220201
Chloroform	p.a.	Roth	7342.1
Citric acid	Ph. Eur.	Roth	6490.1
$CuSO_4 \cdot 5\ H_2O$	p.a.	Roth	8175
DEPC		Roth	k028.1
D-Galactose	p.a.	Serva	22020
DMSO	puriss. Molec. Biol	Fluka Sigma	41648 D-8418
DTT	p.a.	Roth	6908.2
EDTA-disodium	p.a.	Serva	11280
EGTA	p.a.	Roth	3054.3
Ethanol	p.a.	Roth	9065.4
Ethidium bromide	10 mg/ml	Roth	2208.1
Ficoll	type 400	Sigma	F-4375
Fish sperm DNA		Appligene	161641
Formaldehyde	35%	Roth	4980
Formamide	p.a.	Roth	6749.1
Gelatine		BioRad	9000-70-8
Gentiobiose		Sigma	G-3000
Glass beads	0.45-0.50 µm	B. Braun	8541701
Glycerine	99.5%	Zefa	Z.7530.5
Glycine	p.a.	Roth	3908.3
H_2SO_4	p.a.	Roth	4623.1
HCl		Roth	3633.1
Hexadecyltrimethylammonium (CTAB)	99%	Sigma	H-5882
HPTLC plates		Merck	1.05547.0001
IPTG		peqlab	37-2020
Iron sulfate ($FeSO_4 \cdot 7H_2O$)	p.a.	Merck	9018173
Isoamyl alcohol	p.a.	J.T. Baker	8010
K_2HPO_4	p.a.	Roth	6875.1

Name	Quality	Company	Catalogue no.
KAc	99%	Roth	4986.1
KCl	p.a.	Roth	6781.1
KH_2PO_4	p.a.	Roth	3904.1
Laminaribiose		Sigma	L-9384
Laminarinase		Sigma	L-9259
L-arginine	USP grade	GibcoBRL	21008-032
L-asparagine	p.a.	Merck	23431
LiAc	p.a.	Roth	5447.1
Lima bean agar		Difco	0117-17-1
L-Tryptophan	cell culture	Sigma	T-0271
Methanol	HPLC	Roth	7342.1
Methylene Blue	82%	Sigma	M-9140
$MgCl_2 \cdot 6H_2O$	p.a.	Roth	2189.1
$MgCl_2 \cdot 6H_2O$	p.a.	Roth	2189.1
$MgSO_4 \cdot 7H_2O$	p.a.	Roth	P027.2
$MnCl_2 \cdot 2H_2O$	p.a.	Merck	6854
Myoglobin equine	p.a.	Serva	29895
Na_2CO_3	99%	Roth	8563.1
$Na_2MoO_4 \cdot 2H_2O$	p.a.	Merck	135793
NaAc	p.a.	Roth	3742.1
NaCl	p.a.	Roth	3957
NaF	p.a.	Sigma	S-7920
NaOH	99%	Roth	6771.1
NH_4OH	25%	Roth	6774
Octyl glucoside	98%	Sigma	O8001
Orange G	60%	Sigma	O-1625
Ovalbumin	p.a.	Serva	11840
PEG 4000	p.a.	Roth	0156.1
PEG 6000	p.a.	Roth	0158.1

Name	Quality	Company	Catalogue no.
Phenol	p.a.	Roth	0038.1
Phenol:chlorophorm: isoamilalcohol	25:24:1, TE saturated	Roth	A156.1
Polivinylpyrrolidone (PVP)		Sigma	P-5477
Scintillation cocktail		Roth	4355.2
Silver nitrate	99%	Sigma	S-8157
Sodium citrate	p.a.	Roth	1264-1
Sodium dodecyl sulphate (SDS)	99%	Roth	4360.2
Spermidine	mol. biol.	Sigma	S-0266
ß-Mercaptoethanol	p.a.	Roth	4227.1
Sucrose	Ph. Eur	Roth	4661.2
TEMED	p.a.	Roth	2367.3
Thiamine-HCl		Merck	8181
Thiourea	ACS	Sigma	T-8656
Thyroglobulin	p.a.	Sigma	T-9145
Tinopal		Sigma	F-3543
Tris-(hydroxymethyl)-aminomethane	p.a.	Roth	4855.2
Tryptophane	98%	Sigma	T8659
UDP-glucose	98%	Sigma	U-4625
X-Gal	99%	Roth	2315.3
Yeast nitrogen base		Difco	0919-15
$ZnSO_4 \cdot 7H_2O$	p.a.	Merck	8883
Zwittergent 3-12	99%	Calbiochem	693015

Table 2: Radiochemicals

	Activity	Company
Uridine diphospho-D-[U-^{14}C]glucose	7.4 GBq/mmol	Amersham-Pharmacia
Deoxycytidine 5`-[α-^{32}P]triphosphate	110 TBq/mmol	Amersham-Pharmacia

Table 3: Enzymes for molecular biology

Enzyme	units/µl	company	Cat. no
Taq polymerase	5	Appligene	120187
T4 ligase	1	MBI	EL0015
Pst I	10	MBI	ER0611
Sma I	10	MBI	ER0661
Ecl 136 II	12	MBI	ER0251
Sal I	10	MBI	ER0641
Eco 52 I	10	MBI	ER0331
Aat II	5	MBI	ER0991
Bgl II	15	Pharmacia	E1021Y

Kits

- Clean-A-Gene DNA purification kit; Renner GmbH, Dannstadt.
- The QIAexpressionist; Qiagen GmbH, Hilden
- PolyATtract mRNA isolation systems; Promega, Mannheim
- Packagene Lambda DNA Packaging System; Promega, Mannheim
- SuperScript lambda systemGibcoBRL; Life Technologies GmbH, Karlsruhe
- Prime-A-Gene; Promega, Mannheim
- Jetstar; Genomed GmbH, Bad Oeynhausen

2.3. Plasmids and oligonucleotides

2.3.1. Plasmids

pYES2: 5.9 kb shuttle vector designed for inducible expression of genes in *Saccharomyces cerevisiae*. The plasmid contains a GAL1 promoter for high level inducible protein expression in yeast by galactose and repression by glucose, a CYC1 transcription termination signal, a pMB1 origin for maintenance and high copy replication in *E. coli*, an ampicillin resistance gene for selection in *E. coli*, an URA3 gene for selection of yeast transformants in uracil-deficient medium, and a 2 µ origin for maintenance and high copy replication in yeast. Source: Invitrogen, Catalogue no. V825-20

pQE30, pQE31, pQE32: Vectors for expression in *E. coli* of recombinant proteins with a 6×His-tag at the N-terminus. Includes an ampicillin resistance gene for selection. Three different open reading frames. Source: Quiagen, catalogue no. 32149

pGEM-T: Vector for cloning PCR products in *E. coli*. It is a linearized plasmid with an added 3´ terminal thymidine to both ends, includes an ampicillin resistance gene, a multiple cloning site within the gene for β-galactosidase and an origin of replication. Source: Promega, catalogue no. A362A

2.3.2. Oligonucleotides

Table 4: Non-degenerated oligonucleotides

name	sequence 5´→3´
56up_ac_pmg	TTCCGCAATTTGCTGCCAAGCC
891_acanti_pmg	ATGGTAGAGCCACCGGAGAGTA
iy377fw	CATGAACAACCTGGACAC
iy277rev	GTGTCCAGGTTGTTCATG
iy736fw	GGCAACTACTGGTTCTCA
iy736rev	TGAGAACCAGTAGTTGCC
iy1081fw	GAGATGTACAACGAGCTG

name	sequence 5´→3´
iy1081rev	CAGCTCGTTGTACATCTC
iy1434fw	CTGCTGTAAGCGTGTGTA
iy1434rev	TACACACGCTTACAGCAG

Table 5: Degenerated oligonucleotides

name	sequence 5´→3´
litsense	GCNCTBGARTAYACBYTBTAYYT
litanti	ARRTAVARVGTRTAYTCVARNGC
tndsense	TAYACBAAYGAYGTNGGNCARAT
tndanti	ATYTGDCCNACRTCRTTVGTRTA
vigsense	GTNATYGGNGAYACBGTNGGNGA
viganti	TCNCCNACVGTRTCNCCRATNAC
vaassense	GTNGCNGCNWCNYTNGARMGN
vaasanti	GTNCKYTCNARNGWNGCNGCNAC
dafsense	ATHGTNMGNGAYGCNTTYGAYCA
dafanti	TGRTCRAANGCRTCNCKNACDAT
raisense	ATHCCNYTNGAYGTNAAYGARGG
raianti	CCYTCRTTNACRTCNARNGGDAT
evesense	AAYACBGGNGTNGGNATYGARGT
eveanti	ACYTCRATNCCNACNCCVGTRTT
yensense	TAYTAYGARAAYCAYAAYCCNYT
yenanti	ARNGGRTTRTGRTTYTCRTARTA
iqarsense	ATHCARGCNMGNGTNCARGGNGA
iqaranti	TCNCCYTGNACNCKNGCYTGDAT

2.3.3. Equipment and other material

- FPLC, with pumps P-500, fraction collector Frac-100, Superloop, LKV UV-MII, conductivity monitor, controller LCC-501 Plus; Pharmacia LKB, Freiburg (D)
- Spectrophotometer Ultrospec 3000; Pharmacia LKB, Freiburg (D)

- Centricon-30, Millipore GmbH; Eschborn (D)
- Ultracentrifuge, rotors 45Ti, SW40, SW28; Beckmann, Palo Alto (USA)
- Tabletop ultracentrifuge Beckman Optima TLX rotors 100.3 and 100.4; Beckmann, Palo Alto (USA)
- Centrifuge Sorvall RC-5B, Rotor SS34, GSA; Kendro Laboratory Products GmbH, Berlin (D)
- Centrifuge 3k12, SIGMA Laborzentrifugen GmbH; Osterode am Harz (D)
- Microcentrifuge, Eppendorf; Hamburg (D)
- Peristaltic pump Minipuls 2; Gilson, Villiers Le Bel (F)
- Electrophoresis apparatus, BioRad minigel; Bio-Rad Laboratories GmbH, München (D)
  agagel Mini, Biometra, Göttingen (D)
- Hand-held UV lamp; Renner GmbH, Dannstadt (D)
- Ultraviolet crosslinker; Amersham Pharmacia Biotech, Freiburg (D)
- Phosphorescence Imager; Fujifilm BAS-1500
- Video imaging: Geldocumentation system; MWG Biotech, Ebersberg (D); Gel electrophoresis Photosystem QuickShooter QSP, Kodak IBI, New Haven, CT (USA)
- Thermocycler Uno II; Biometra, Göttingen (D)
- Electroporation unit, Gene Pulser II electroporator; Bio-Rad Laboratories GmbH, München (D)
- Electroporation cuvettes, 0.1 and 0.2 cm; Bio-Rad Laboratories GmbH, München (D)
- Nylon membranes: Hybon-N+, 0.45 µm; Amersham Pharmacia Biotech, Freiburg (D)
- Nitrocellulose membrane: Protran BA 85, 0.45 µm; Schleicher & Schuell
- Spin columns, S-200, Amersham Pharmacia Biotech, Freiburg (D)

2.4. Software and Internet resources

2.4.1. Computer software

- SigmaPlot 5.0; 1986-1999, SPSS Inc.
- Windows98; Microsoft Corporation
- Microsoft word; Microsoft Corporation
- Tina 2.10g; 1993 raytest Isotopenmeßgeräte GmbH
- Netscape Navigator 4.0; 1994-1998 Netscape Communications Corporation
- Chromas 1.5; 1998 Technelysium Pty Ltd

2.4.2. Web resources

Blast: http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1

Blast two sequences: http://www.ncbi.nlm.nih.gov/gorf/bl2.html

Taxonomy browser: http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/

Phytophthora initiative: http://www.ncgr.org/research/pgi/

3. Methods

3.1. *Phytophthora* cultures

3.1.1. Growth on solid medium

Phytophthora sojae (previously known as *Phytophthora megasperma* f. sp. *glycinea*), race 1 and race 3, was grown on Lima bean agar (Recipe 1, 20-25 ml per plate) in plastic 9 cm Petri dishes. For inoculation of new plates, approximately 1 cm^2 of the growing edge of a previous culture was cut and transferred aseptically to a fresh plate with the mycelium in contact with the agar. Plates were incubated at 26ºC in the dark for 1 week when used for maintaining the line, or 10 days or longer when used for inoculation of liquid medium. Cultures on solid medium could be stored up to 8 weeks at 4°C.

Recipe 1: Lima bean agar

23 g/l Lima bean agar
autoclave

3.1.2. Growth in liquid medium

Culture in liquid medium was performed mainly as described by Ayers et al. (1976) based on the methods of Erwin and Katznelson (1961) and Keen (1975). The medium was optimized by doubling the concentration of β-sitosterol and K_2HPO_4, and reducing that of KH_2PO_4 to one half.

The following components were poured into a 1.8 l Fernbach flask in this order: 2.5 g $CaCO_3$, 400 ml of liquid medium (Recipe 2) and 1 ml of the stock solution of β-sitosterol, followed by autoclaving. One fourth to one third of a 10 days old culture plate was used for inoculation. Incubation was carried out at 26ºC in the dark without stirring. A single gentle shake was given to the flasks after one week of growth to break slightly the mycelium and thereby stimulate further growth. Usually, 3 weeks were sufficient for maximum yield.

Recipe 2: Modified Erwin synthetic medium

	Stock concentration	Amount per liter	Final concentration
D (+)-Sucrose	-	15 g	15 g×l^{-1}
L-Asparagin	-	2 g	2 g×l^{-1}
$FeSO_4 \cdot 7H_2O$	-	1 mg	1 g×l^{-1}
$MgSO_4 \cdot 7H_2O$	100 g×l^{-1}	2 ml	0.2 g×l^{-1}
$CaCl_2 \cdot 2H_2O$	10 g×l^{-1}	1 ml	0.01 g×l^{-1}
Thiamine-HCl	1 g×l^{-1}	1 ml	1 mg×l^{-1}
K_2HPO_4	95 g×l^{-1}	20 ml	1.9 g×l^{-1}
KH_2PO_4	104 g×l^{-1}	10 ml	1.04 g×l^{-1}
$ZnSO_4 \cdot 7H_2O$	4.39 g×l^{-1}	1 ml	4.39 mg×l^{-1}
$CuSO_4 \cdot 5H_2O$	79 mg×l^{-1}	1 ml	79 µg×l^{-1}
$Na_2MoO_4 \cdot 2H_2O$	53 mg×l^{-1}	1 ml	53 µg×l^{-1}
$MnCl_2 \cdot 2\ H_2O$	70 mg×l^{-1}	1 ml	70 µg×l^{-1}
Insoluble components:			
ß-sitosterol	20 g×l^{-1} in dichloromethane	2.5 ml	0.05 g×l^{-1}
$CaCO_3$	-	2.5 g per flask	Not soluble

3.2. Microsome preparation

After 3 weeks of growth, the mycelium was poured onto a flat surface and washed with cold tap water in order to eliminate the agar and the calcium carbonate. The mycelium was then collected on a 300 µm nylon mesh to continue the washing with tap water and finally distilled water. The washed mycelium was either frozen and stored at –20°C or processed immediately.

To prepare microsomal fractions, the mycelium was homogenized in a Waring blender with 5 volumes of Microsome Buffer (Recipe 3). The homogenate was checked by light microscopy to ensure at least 80% disruption of the mycelium.

The homogenate was filtered through a 300 µm nylon mesh and centrifuged at 3,500×*g* for 20 min (4,500 rpm in a GS-3 rotor at 4°C). The supernatant was collected and centrifuged at 40,000×*g* for 45 min (20,000 rpm in a SS-34 rotor at 4°C). The supernatant was discarded and the pellets collected, frozen in liquid nitrogen and stored at -80°C until used. No loss of activity was detected after even 6 months of storage at –80°C.

Before any further step, membrane pellets were washed by resuspension in buffer A (Recipe 4) and centrifuged at 40,000×*g* for 45 min at 4°C.

Recipe 3: Microsome Buffer

	Amount for 1 liter
50 mM Tris-HCl, pH 7.5	6 g
1 M Sucrose	342.3 g
10 mM EGTA	3.8 g
10 mM NaF	0.42 g
5 mM $MgCl_2$	1 g
Filtrate through 0.22 µm membrane	

Recipe 4: Buffer A

	Amount for 1 liter
50 mM Tris-HCl, pH 7.5	6 g
1 M Sucrose	342.3 g
10 mM NaF	0.42 g
Filtrate through 0.22 µm membrane	

3.3. Glucan synthase assay

Glucan synthase activity was assayed as previously described by Cabib and Kang (1987), with minor modifications. The incorporation of radioactivity from UDP-[^{14}C]glucose into acid-insoluble product was measured.

A final volume of 40 µl was used. Usually 36 µl of the mixture to be tested was complemented with 4 µl of GS master mix (Recipe 5), leading to a final concentration of 5 mg/ml BSA and 5 mM UDP-[^{14}C]glucose (7.4 MBq/mol).

The reaction was carried out for 1 h at RT, terminated by the addition of 0.5 ml of cold 20% (w/v) trichloroacetic acid and filtrated through 2.5 cm Whatman GF/B filters. The filter with the insoluble product was washed once with 10 ml water and transferred to a scintillation vial. The β emission was measured in presence of 4 ml of scintillation cocktail per vial.

Recipe 5: GS master mix

Equal volumes of:
100 mg/ml BSA
0.1 M UDP-[^{14}C]glucose (7.4 MBq/mmol, see Recipe 6)

Recipe 6: 7.4 MBq/mmol UDP-[^{14}C]glucose

1 vol. 0.5 M UDP-glucose
4 vol. 925 kBq/ml UDP-[^{14}C]glucose (stock)

3.4. Solubilization of glucan synthase activity

Membrane pellets were washed by resuspension with a tissue homogenizer in buffer A (Recipe 4) and centrifuged at 40,000×g for 45 min at 4°C. The pellets were resuspended in the same buffer and the amount of protein was determined. The suspension was then adjusted to a final concentration of 3 mg/ml CHAPS and 2 mg/ml protein. After 1 h of gentle stirring at 4°C, followed by 1 h of slow shaking on an ice tray, the suspension was centrifuged at 180,000×*g* for 50 min at 4°C (40,000 rpm in a Beckman 45Ti rotor). The supernatant was collected and the pellet re-extracted under the same conditions in a third of the initial volume with buffer A containing 3 mg/ml CHAPS. The latter solubilized material was called second extraction quality and was used for analytical purposes.

For solubilization tests, the reaction with different detergents and concentrations was performed with 0.5 to 1 ml final volume, in 2 ml polypropylene tubes, with 1 h slow shaking on ice, followed by 30 min ultracentrifugation at 80,000 rpm in a TLA 100.3 rotor (Beckman) at 4°C.

The solubilized material was filtered through a 0.45 µm pore membrane, frozen in liquid nitrogen and stored at –80°C until used. No loss of activity was detected after 6 months of storage.

3.5. Product analysis

3.5.1. Preparation of radioactively labeled product

A detergent-solubilized protein fraction (0.1 mg protein) was incubated for 2 h with 5 mM UDP-[^{14}C]glucose (7.4 MBq/mmol) as done for a glucan synthase assay (section 3.3), in a total reaction volume of 250 µl. The reaction was terminated by heating at 100°C for 10 min. The sample was then centrifuged at 15,000×*g* for 20 min at room temperature. The supernatant was discarded and the pellet (white and gelatinous) was washed 3 times by resuspension in 1 ml of distilled water and centrifugation at 15,000×*g* for 10 min. The final pellet was resuspended in 250 µl of water.

3.5.2. Preparation of non-radioactive product

A detergent-solubilized protein fraction was incubated for 2 h with 10 mM UDP-glucose. Glucans were collected by centrifugation at 15,000×*g* for 20 min at RT. The glucan pellet was washed extensively (3 or 4 times) with 2 M NaCl followed by 10 washing steps with distilled water and was then freeze dried.

3.5.3. Quantification of carbohydrates

A modified anthrone reaction as described by Dische (1962) was used for measuring the amount of carbohydrates. The sample (0.2 ml final volume) was mixed with 1 ml of anthrone reagent (Recipe 7), incubated for 15 min at 100°C, and after cooling down the sample to RT, absorption was measured at 620 nm. As the anthrone method is incompatible with the presence of DMSO, water-insoluble glucans were completely dissolved in 0.36 % NaOH.

Recipe 7: Anthrone reagent

63 mg anthrone
1.25 g thiourea
90 ml H_2SO_4
30 ml H_2O
The components were mixed slowly, keeping the flask on ice, to minimize heat generation.

The standard curve was determined with glucose dissolved in 0.36% NaOH (w/v) (Figure 4). Five microliters of the serial dilutions were used.

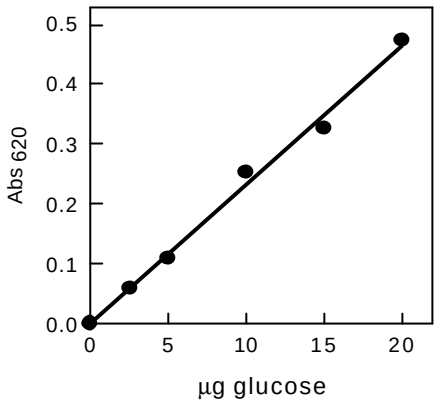


Figure 4: Glucose determination using the anthrone assay. Data are means of two independent experiments. Abs=0,0232×µg glucose

3.5.4. Enzymatic digestion of products

Aliquots of 50 µl each of radioactively labeled glucan (section 3.5.1) were incubated with additional 50 µl consisting of either:

- 2 mg/ml laminarinase solution in 10 mM sodium acetate buffer, pH 5.0, or
- 10 mg/ml α-amylase solution in 25 mM Tris-HCl pH, 7.5, or
- only buffer for controls.

After incubation at 37°C for 2 h, the mixtures were centrifuged at 15,000×*g* for 10 min at RT and 10 µl aliquots of the supernatants were spotted onto HPTLC plates (section 3.5.5). Additional 5 µl aliquots were used for measuring radioactivity by scintillation counting.

3.5.5. TLC analysis of carbohydrates

Carbohydrate standards and digestion products of the enzymatically-formed glucan were loaded onto 20×20 cm Merck silica gel 60 aluminum-backed HPTLC plates. Separation was carried out in butanol/acetone/water (4:5:2 (v/v/v)) (Monschau et al. 1997).

The non-radioactive standards of glucose, laminaribiose and gentiobiose were visualized by spraying the plate with 5% H_2SO_4 in methanol and incubating at 180°C until the charred

compounds became visible. For radioactive samples, the plates were air dried and scanned using a Fujifilm BAS-1500 phosphorescence image analyzer.

3.5.6. NMR analysis of the product of the glucan synthase

NMR analysis was carried out by Dr. Norbert Hertkorn at the Institut für Ökologische Chemie, GSF- Forschungzentrum für Umwelt und Gesundheit (Munich, Germany).

NMR spectra were recorded with a Bruker DMX 500 spectrometer (proton frequency: 500.13 MHz) using an inverse geometry 5 mm probehead (90 °: 10.8 µs ^{1}H; 10.0 µs ^{13}C) in DMSO-d_6 at 30°C ($^{1}H/^{13}C$: 2.49/39.50 ppm). The ^{13}C-NMR spectrum was recorded with broad band decoupling and an acquisition time of 825 ms (relaxation delay d1: 1 s). Gradient enhanced absolute value HH-COSY and phase sensitive (TPPI) HMQC spectra were acquired using Bruker standard software (gradient pulse: 1 ms, gradient recovery: 450 µs HMQC: aq: 102 ms, sw: 5040 Hz, d1: 2 s, ^{1}J /CH): 145 Hz, ^{13}C GARP decoupling: 70 µs, number of increments in F1: 257; HH-COSY: aq: 117 ms, sw: 4370 Hz; 512 increments in F1). The FID of the HMQC spectrum was multiplied by a $\pi/2$ shifted sine bell in F2 and by an unshifted sine bell in F1 dimension; the HH-COSY spectrum was processed with unshifted [square (F2)] sine bells; both spectra were zero filled providing a 1k × 1k data matrix.

3.6. Protein purification

3.6.1. Anion exchange chromatography

3.6.1.1. DEAE analytical scale

Analytical chromatography was carried out on 1 ml of Fractogel EMD-DEAE equilibrated at 4°C with MBC buffer (Recipe 8). Three milliliters of solubilized protein (second extraction quality, see section 3.4) were loaded. The column was washed with 2 ml of MBC buffer. Elution was done in two steps: a first step with 2 ml of MBC buffer containing 0.25 NaCl, and a second one with 2 ml of MBC buffer with 0.5 M NaCl.

Recipe 8: MBC buffer

	Amount for 1 liter
50 mM Tris-HCl, pH 7.5	6 g
1 M sucrose	342.3 g
10 mM EGTA	3.8 g
10 mM NaF	0.42 g
5 mM $MgCl_2$	1 g
3 mg/ml CHAPS	3 g
Filtrate through 0.22 µm membrane	

3.6.1.2. DEAE preparative scale

Chromatography in preparative scale was carried out on a prepacked 10×150 mm Fractogel EMD-DEAE Superformance cartridge (Merck). A detergent-solubilized sample (20-30 mg protein) was diluted 1:1 with distilled water and loaded onto a column equilibrated with buffer C (Recipe 9). The column was then washed with 30 ml buffer C containing 0.2 M NaCl. Elution of the active fraction was carried out using a 50 ml gradient from 0.2 M to 0.6 M NaCl in buffer C. The flow rate was 1 ml/min. UV absorbance at 280 nm was monitored on line. One milliliter fractions were collected and protein content and glucan synthase activity were estimated as described in sections 3.7.1 and 3.3, respectively. The fractions containing the enzyme activity were pooled for further purification.

Recipe 9: Buffer C

	Amount for 1 liter
25 mM Tris-HCl, pH 7.5	3 g
0.5 M sucrose	171 g
10 mM NaF	0.42 g
2 mg/ml CHAPS	2 g
Filtrate through 0.22 µm membrane	

3.6.1.3. Resource-Q chromatography

The column (1 ml Resource-Q, Pharmacia) was equilibrated in buffer C (Recipe 9) supplemented with 0.2 M NaCl at 0.5 ml/min. A 5 ml portion of the pool generated in section 3.6.1.2 was diluted 1:1 with buffer C and loaded. The column was washed with 5 ml of buffer C containing 0.25 M NaCl. Enzyme activity was eluted by using a 10 ml gradient from 0.25 to 0.6 M NaCl in buffer C at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and protein content and glucan synthase activity were estimated as described in sections 3.7.1 and 3.3, respectively.

3.6.1.4. Mono-Q chromatography

The column (1 ml Mono-Q, Pharmacia) was equilibrated as described in section 3.6.1.3 and run under the same conditions.

3.6.2. Size exclusion chromatography

A Superose 12 (Pharmacia) column was equilibrated with 0.1 M NaCl in buffer C (Recipe 9) at a flow rate of 0.4 ml/min. When Superose 6 was used, the matrix was equilibrated in the same buffer but at a lower flow rate (0.2 ml/min). As standards for calibration myoglobin (17.8 kDa), ovalbumin (45 kDa), BSA (66 kDa), aldolase (161 kDa), amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) (each 500 µg in 100 µl) were used. The calibration curves derived from the elution of the molecular mass markers are shown in Figure 5. The linear regressions were calculated by using SigmaPlot 5.0.

The sample to be tested was prepared by concentrating the pool generated in section 3.6.1.2 by ultrafiltration in Centricon-30. A portion of 100 µl was loaded onto the column. UV absorbance at 280 nm was monitored on line. Fractions of 0.4 ml were collected and protein content and glucan synthase activity were estimated as described in sections 3.7.1 and 3.3, respectively.

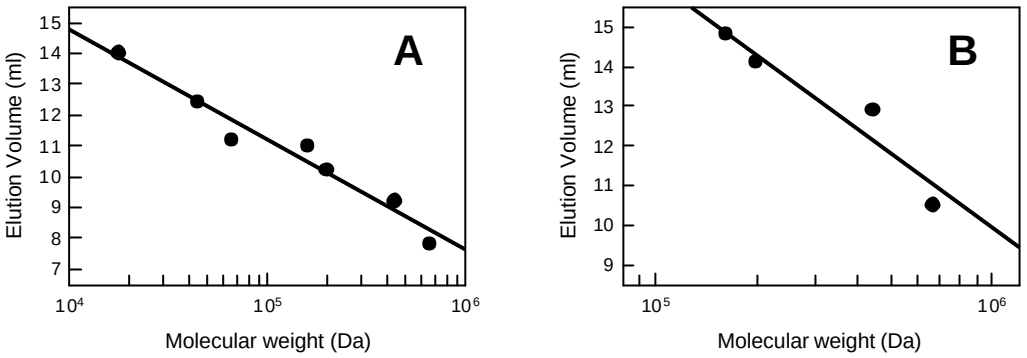


Figure 5: Calibration curves with molecular mass markers on a Superose 12 (panel A) and a Superose 6 (panel B) columns.

3.6.3. Sucrose density-gradient centrifugation

3.6.3.1. SW28 rotor

For large-scale preparations, linear 17-40% (w/v) sucrose gradients (29 ml) were poured above 4 ml cushions of 60% (w/v) sucrose in centrifuge tubes. All sucrose solutions were prepared with 50 mM Tris-HCl, pH 7.5, 25 mM NaF, 2 g/l CHAPS. The solubilized fraction was diluted and adjusted to a content of 15% sucrose. Approximately 5 ml of the diluted sample was layered on top of the gradient. After centrifugation (27,000 rpm, 41 h, 4°C; SW28 rotor), 2 ml aliquots were collected from the bottom of the gradient using a glass capillary pipette carefully placed at the bottom and sucking using a peristaltic pump. Fractions were assayed for protein content and glucan synthase activity as described in sections 3.7.1. and 3.3, respectively.

3.6.3.2. SW40 rotor

For small-scale preparations, linear 17-40% (w/v) sucrose gradients (11 ml) were poured above 1 ml cushions of 50% (w/v) sucrose in centrifuge tubes. The solutions and the sample were prepared as described in section 3.6.3.1. Approximately 1.5 ml of the sample was layered on top of the gradient. After centrifugation (36,000 rpm, 40 h, 4°C; SW40 rotor), 1-ml aliquots were collected as described in section 3.6.3.1. Protein content and glucan synthase activity were assayed as described in sections 3.7.1 and 3.3, respectively.

3.6.4. PEG precipitation

Solubilized protein fractions (0.4 ml) were mixed with different amounts of 50% PEG 6000 or PEG 4000 in 25 mM Tris-HCl, pH 7.5, yielding final concentrations from 5 to 30%, and adjusted to 1 ml with 25 mM Tris-HCl, pH 7.5. The samples were placed on ice for 1 h and then centrifuged for 30 min at 60,000 rpm in a TLA 100.3 rotor. The pellets were resuspended in 0.3 ml of MBC buffer (Recipe 8).

3.6.5. HiTrap Blue chromatography

A 5 ml HiTrap Blue (Pharmacia) column was equilibrated with buffer C at a flow rate of 1 $ml \times min^{-1}$. Solubilized protein fraction (2 ml) was loaded onto the column. The column was washed and eluted using the following program:

- 10 ml buffer C
- 1 ml gradient from 0 to 0.25 M NaCl in buffer C
- 10 ml 0.25 M NaCl in buffer C
- 1 ml gradient from 0.25 to 0.5 M NaCl in buffer C
- 10 ml 0.5 M NaCl in buffer C
- 1 ml gradient from 0.5 M to 1 M NaCl in buffer C
- 10 ml 1 M NaCl in buffer C

The elution was monitored by UV absorbance at 280 nm. Protein peaks (3 to 9 ml, 20 to 25 ml, 36 to 39 ml and 51 to 53 ml) were pooled and assayed for glucan synthase activity and protein content as described in sections 3.3 and 3.7.1, respectively.

3.6.6. HiTrap heparin chromatography

A 5 ml HiTrap heparin column (Pharmacia) was equilibrated at a flow rate of 1 ml/min with buffer C (Recipe 9). Two milliliters of solubilized fraction were loaded. The column was then washed with 10 ml of buffer C and eluted in 4 steps, using solutions of 0.15, 0.3, 0.5 and 1 M NaCl in buffer C. The elution was monitored by UV at 280 nm. The protein peaks (3 to 11 ml, 17 to 24 ml, 27 to 33 ml, 36 to 44 ml and 47 to 51 ml) were pooled and assayed for glucan synthase activity and protein content as described in sections 3.3 and 3.7.1, respectively.

3.6.7. Concanavalin A chromatography

Two milliliters of Concanavalin A-Sepharose 4B (Sigma) were equilibrated with buffer C containing, additionally, 1 mM $MgSO_4$ and 0.1 mM $MnSO_4$. Two milliliters of the solubilized fraction containing 1 mM $MgSO_4$ and 0.1 mM $MnSO_4$ were loaded. The column was washed with 4 ml of buffer C containing 1 mM $MgSO_4$ and 0.1 mM $MnSO_4$ and eluted with 4 ml of the washing solution supplemented with 0.5 M methyl α-D-mannopyranoside. The chromatography was done by gravity flow.

3.6.8. Product entrapment

Product entrapment was carried out as described by Inoue et al. (1995) and Turner et al. (1998) with minor modifications. The crude solubilized protein fraction or other pre-purified pools (e.g. the DEAE purified pool, section 3.6.1.2) was incubated with 10 mM UDP-glucose for 1 h at 25°C when used for analytical purposes, or 2 to 3 h when used for preparative purposes. The synthesized glucan, containing entrapped protein, was collected by centrifugation at 4,000×*g* for 10 min at RT and the pellet was washed twice by resuspension in buffer C containing 5 mM UDP-glucose and centrifugation as described before. The pellet was resuspended in buffer C (without UDP-glucose) and centrifuged at 200,000×*g* for 10 min. The pellet was then resuspended again in buffer C and used directly for glucan synthase assay. For SDS-PAGE, the pellet was homogenized in 1×SDS loading buffer (Recipe 15) using a Potter-Elvehjem homogenizer with a Teflon pestle, incubated at 100°C for 5 min, and centrifuged at 200,000×*g* for 10 min at 15°C. The supernatant was loaded onto a 8% or 7% polyacrylamide gel for analytical or preparative SDS-PAGE (see section 3.7.2), respectively. The remaining pellet could also be loaded onto SDS-PAGE gels with the help of a small and thin spatula.

3.6.9. Native electrophoresis and in-gel assay

Native electrophoresis was carried out as described by Kudlitka and Brown (1997) with some modifications. The gels were cast in a Biorad minigel apparatus. The separating gel (6% polyacrylamide) and the stacking gel (3%) are described in Recipe 10. No pH discontinuity was used. The chambers were filled with running buffer adapted to native electrophoresis (Recipe 11). A solubilized protein fraction with few crystals of bromophenol blue was used

for native electrophoresis separation. As a control, a solubilized protein fraction incubated at 100°C for 30 min was used.

Recipe 10: Gels for native electrophoresis

Stacking gel
4.03 ml H_2O
0.33 ml 1.5 mM Tris-HCl, pH 8,8
0.5 ml 30 % acrylamide/bisacrylamide (29:0.8)
0.1 ml 30 mg/ml CHAPS
10 µl TEMED
50 µl 10% APS
Separating gel
7.1 ml H_2O
0.66 ml 1.5 mM Tris-HCl, pH 8,8
2 ml 30 % acrylamide/bisacrylamide (29:0.8)
0.2 ml 30 mg/ml CHAPS
10 µl TEMED
50 µl 10% APS

Recipe 11: Running buffer for native electrophoresis

	Amount for 1 liter
192 mM glycine	14.4 g
0.6 g/l CHAPS	0.6 g
25 mM Tris-HCl, pH 8.3	3 g

The electrophoresis was performed at 4°C and at constant 20 mA per gel (voltage was starting at 80 and finishing at 200 V) for 2.5 h. Thereafter, the gel was incubated in 10 ml of reaction buffer (Recipe 12) for 3 h with gentle agitation at 26°C. The gel was then quickly rinsed with water and incubated for 20 min in dye buffer (Recipe 13). Subsequently the gel was washed several times with water until the background disappeared. The glucan was visualized with UV light (365 nm).

Recipe 12: Reaction buffer for in-gel assay

	Amount for 1 100 ml
1 M sucrose	34,2 g
25 mM NaF	105 mg
50 mM Tris-HCl, pH 7.5	5 ml 1 M Tris-HCl, pH 7.5
0.6 g/l CHAPS	60 mg
5 mM UDP-glucose	305 mg

Recipe 13: Dye buffer for in-gel assay

	Amount for 1 100 ml
50 mM K_2HPO_4, pH 8.2	871 mg
0.01% Tinopal	10 mg

3.7. Basic methods for protein biochemistry

3.7.1. Protein estimation

Protein concentration was determined using the method described by Bradford (1976). Routinely, BioRad protein assay reagent was used. The sample was diluted to 800 µl with water and after adding 200 µl of reagent and mixing, absorbance was measured at 595 nm. Bovine serum albumin was used as standard.

3.7.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

For protein separation, the method of Laemmli (1970) was used. The compositions of the stacking and separating gels are described in Recipe 14. Gels were usually poured to a size of 7×11 cm and 0.75 mm thickness for analytical and 1.5 mm thickness for preparative purpose, using a BioRad minigel apparatus. Liquid samples to be analyzed by SDS-PAGE were mixed with 1/5 volume of 6×SDS loading buffer (Recipe 15). Pellets of product entrapment or acetone precipitation were resuspended or re-dissolved in 1×SDS loading buffer. Samples precipitated with TCA (final concentration 10% w/v, 10 min on ice and centrifuged at

14,000×*g* for 10 min) were resuspended in the same buffer, containing, in addition, Na_2CO_3 (Recipe 16). Once the samples were dissolved in the desired buffer system, they were incubated for 2 min at 100°C, spun for 5 min and loaded into the gel slots with the help of a Hamilton syringe. Unless further specified, electrophoresis was performed at room temperature, after filling the chambers with SDS-PAGE running buffer (Recipe 17), at 100 V until the samples reach the separating gel, and then continued at 200 V. Commercially available mixtures of protein molecular markers were used as reference.

Recipe 14: Gels for SDS-PAGE

Stacking gel	Amount for 10 ml
4% acrylamide	1.25 ml 30 % acrylamide/bisacrylamide (29:0.8)
125 mM Tris-HCl, pH 6.8	1.25 ml 1M Tris-HCl, pH 6.8
0.1% SDS	100 µl 10% SDS
0.1% (v/v) TEMED	10 µl
0.04% APS	40 µl 10% stock solution
Separating gel	**Amount for 20 ml**
7 or 8 or 10 % acrylamide	4.67, 5.33 or 6.67 ml 30 % acrylamide/bisacrylamide (29:0.8)
375 mM Tris HCl, pH 8.8	5 ml 1.5 M Tris-HCl, pH 8.8
0.1% SDS	200 µl 10% SDS
0.05 % TEMED	10 µl
0.05% APS	50 µl 10% stock solution

Recipe 15: 6×loading buffer for SDS-PAGE

	Amount for 10 ml
60 mM Tris-HCl, pH 6.8	0.1 ml 1 M Tris-HCl, pH 6.8
12% SDS	1.2 g SDS
30% glycerol	3 ml 100% glycerol
0.2 M DTT	0.3 g
0.001% bromophenol blue	0.1 mg (few crystals)

Recipe 16: 1×loading buffer for TCA proteins after TCA precipitation

	Amount for 10 ml
0.1 M Na_2CO_3	106 mg
2% SDS	2 ml 10% SDS
0.2 M DTT	0.3 g
5% glycerol	0.5 ml 100% glycerol
0.001% bromophenol blue	0.1 mg (few crystals)

Recipe 17: 10×running buffer for SDS-PAGE

	Amount for 1 liter
192 mM glycine	144 g glycine
25 mM Tris-HCl, pH 8.3	30 g Tris
0.1% SDS	100 ml 10% SDS

3.7.3. Silver staining of SDS-PAGE gels

Visualization of proteins in SDS-PAGE was done according to the method of Wray et al. (1981). The gel was first soaked in 50% reagent grade methanol for at least 1 h or overnight for thicker gels. Then the gel was incubated for 15 min in staining solution (Recipe 18) with gentle agitation followed by washing in distilled water for 5 min. The gel was then soaked in developing solution (Recipe 19) until bands appeared (usually less than 10 min). The reaction was immediately stopped by washing the gel with 45% methanol/10% acetic acid solution (v/v). After equilibration in 10% (v/v) glycerol in water, the gels were dried in between cellulose-acetate sheets and stored at RT.

Eventually, the gels were totally destained by soaking the gels in a solution of few grains of commercial film cleaning solution (Tetenal) in water or until the background was minimized.

Recipe 18: Staining solution

Solution 1: 0.8 g silver nitrate dissolved in 4 ml distilled water
Solution 2: 21 ml of 0.36% NaOH mixed with 1.4 ml of 14.8 M NH_4OH
Solution 1 was added dropwise into solution 2 with constant stirring, and then volume was increased to 100 ml with water. To be used immediately.

Recipe 19: Developing solution

1 ml 1% citric acid
100 µl 38% formaldehyde
water was added to a final volume of 200 ml

3.7.4. Coomassie staining of SDS-PAGE gels

After electrophoresis, the gels were soaked in Coomassie solution (0.1% Coomassie brilliant blue in water/methanol/acetic acid (40:50:10 (v/v/v))) for 30 min at RT. The gels were then destained with destaining solution (water/methanol/acetic acid (40:50:10 (v/v/v))) until the background disappeared. The gels were equilibrated in 10% glycerol (v/v) and dried.

Gels used for protein sequencing were stained with 0.3% Brilliant blue R in water/methanol/acetic acid (40:50:10, (v/v/v)) and destained with 10% acetic acid.

3.7.5. 2D-electrophoresis

2D-electrophoresis was performed by Dr. B. Müller at the Botanisches Institut der LMU (Munich).

For the IEF separation, precast strips of pH 3 to 10 were used. After the first dimension was performed, the strip was placed over a 8% polyacrylamide SDS-PAGE gel and the second dimension separation was done. Three hundred micrograms of protein were used per sample.

3.7.6. Protein extraction from yeast

Fifteen milliliters yeast culture grown for 48 h (see section 3.9.14) were centrifuged at 2,000×*g* for 5 min at 4°C. The supernatant was discarded and the cell pellet resuspended in

2 ml of disruption buffer (Recipe 20). The cells were transferred into a 15 ml polypropylene tube and an equal volume of glass beads (200 µm) was added. Then the cells were strongly shaken for 30 sec followed by 30 sec on ice, repeating the cycle 10 times. The disruption of the cells was controlled by light microscopy.

The glass beads were allowed to settle at the bottom of the tube and the supernatant was collected. The glass beads were washed five times with 1 ml of disruption buffer and the supernatant of each wash was collected and combined.

The supernatant was centrifuged at 1,500×*g* for 5 min at 4°C in order to remove unbroken cells and cell debris. The supernatant was collected and centrifuged at 100,000×*g* for 30 min at 4°C. The supernatant was collected and the microsome pellet was resuspended in 1 ml of disruption buffer. Protein content and glucan synthase activity were estimated in the supernatant and in the microsomal fraction.

For 2D-electrophoresis, the microsomal fraction was used directly after centrifugation for 20 min at 20,000×*g*. The soluble protein was precipitated by adding 4 volumes of acetone and incubation for 30 min at -20°C. The precipitate was collected by centrifugation for 15 min at 15,000×*g* at 4°C, and dried for 2 min in a vacuum dryer.

Recipe 20: Disruption buffer

	Amount for 100 ml
50 mM Tris-HCl, pH 7.5	5 ml 1 M Tris-HCl, pH 7.5
5% glycerol	5 ml 100% glycerol
1 mM EDTA	0.2 ml 0.5 M EDTA, pH 8

3.8. Protein sequencing

Protein sequencing was performed by Dr. F. Lottspeich (Max Planck Institut für Biochemie, Martinsried). Proteins with an apparent molecular mass of 108 kDa and 50 kDa were cut from the gels after preparative SDS-PAGE (section 3.7.2), and cleaved into peptides by treatment with LysC. The peptides were separated by HPLC and sequenced.

3.9. Methods in molecular biology

3.9.1. Electrophoresis of DNA

The DNA sample to be analyzed (in water or any buffer) was mixed with one tenth volume of DNA loading buffer (Recipe 21). The samples were separated in submerged agarose gels in 1×TAE buffer containing ethidium bromide. The concentration of agarose varied depending on the samples to be run: 0.6% for digested genomic DNA, 0.8 to 1.4% for average size fragments, 2% for small fragments (250 bp or less). Electrophoresis was started at 30 mA until the samples had moved into the gel, and then the separation was carried out at 50 mA.

Recipe 21: DNA loading buffer

600 µl 50% glycerin
20 µl 50×TAE
380 µl water
a few crystals of Orange G

Recipe 22: 50×TAE

	Amount for 1 liter
2 M Tris	242 g
1 mM acetic acid	57.1 ml acetic acid
50 mM EDTA	100 ml 0.5 M EDTA, pH 8.0

3.9.2. Electrophoresis of RNA

In a sterile 1.5 ml microcentrifuge tube, RNA (2-3 µg total RNA for staining, 20 µg total RNA for hybridization) was mixed with one fifth of the volume of 6×RNA loading buffer (Recipe 24, according to Pellé and Murphy (1993)). The mixture was incubated at 75°C for 5 min, immediately placed on ice, and then loaded onto the agarose gel. Routinely 1.2% agarose gels were used (Recipe 23). RNA was electrophorezed using 10 mM sodium phosphate buffer, pH 6.8. Constant re-circulation of the buffer was maintained using a peristaltic pump to prevent the formation of an undesirable pH gradient which can lead to

degradation of the RNA during electrophoresis. Ethidium bromide was usually not included, neither in the buffer nor the gel. Electrophoresis was performed at not more than 50 mA.

Recipe 23: Gels for RNA electrophoresis

	Amount for 100 ml
1.2% agarose (RNase free)	1.2 g
10 mM sodium phosphate, pH 6.8	1 M sodium phosphate, pH 6.8
autoclave	

Recipe 24: 6×RNA loading buffer

	Amount for 10 ml
30% glycerol	3 ml 100% glycerol
1.2% SDS	1.2 ml 10% SDS
60 mM sodium phosphate, pH 6.8	0.6 ml 1 M sodium phosphate, pH 6.8
	a few crystals of bromophenol blue
filter sterilized	

3.9.3. DNA preparation from *P. sojae*

DNA was prepared according to the method of Sacks et al. (1995). Frozen two week old *P. sojae* mycelium from liquid cultures was ground to a fine powder with quartz sand in liquid nitrogen using a mortar. TES buffer (Recipe 25) was added to the powder to a final concentration of 2 mg/ml. Proteinase K was added to a final concentration of 200 µg/ml and the mixture incubated for 45 min at 56°C. The mixture was extracted once with phenol, three times with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and another time with chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated by adding 0.04 volume of 5 M NaCl and 0.8 volume of 2-propanol. After 10 min of centrifugation at 5,000 rpm in a SS-34 rotor, the pellet was dissolved overnight in TE buffer (Recipe 26).

Recipe 25: TES buffer

	Amount for 1 liter
100 mM Tris-HCl, pH 8.0	12.1 g
10 mM EDTA	25 ml 0.5 M EDTA, pH 8.0
2% SDS	200 ml 10% SDS

Recipe 26: TE buffer

	Amount for 1 liter
10 mM Tris-HCl, pH 8.0	1.21 g
1 mM EDTA	0.2 ml 0.5 M EDTA, pH 8.0
autoclaved	

3.9.4. RNA preparation from *P. sojae*

RNA was isolated according to the method of Chang et al. (1993), with minor modifications. RNA extraction buffer (12 ml) (Recipe 27) was warmed up to 65°C in a 50 ml polypropylene tube, and then 2 to 3 g of ground tissue were added and mixed completely by inverting the tube. The mixture was then extracted two times with 12 ml of chloroform:isoamyl alcohol (24:1 v/v) (carefully releasing the excess of pressure by opening briefly the tube). Separation of the phases was achieved by centrifugation in a 30 ml Corex tube at 10,000 rpm in a SS34 rotor at room temperature.

After collecting the aqueous phase, the RNA was precipitated by adding one third volume of 8 M LiCl and incubating overnight at 4°C. RNA was collected by centrifugation at 10,000 rpm in a SS-34 rotor for 20 min at 4°C. The pellet was dissolved in 500 µl of SSTE (Recipe 28) and extracted one more time with chloroform:isoamyl alcohol (24:1 v/v). RNA was precipitated by adding two volumes of ethanol and incubating for at least 30 min at -80°C. The precipitate was collected by 20 min of centrifugation in a microcentrifuge. The pellet was dried and resuspended in autoclaved DEPC-treated water (Recipe 29).

Recipe 27: RNA extraction buffer

	Amount for 100 ml
2% CTAB	2 g
2% PVP	2 g
100 mM Tris-HCl, pH 8.0	1.21 g
2.0 M NaCl	11.9 g
25 mM EDTA	5 ml 0.5 M EDTA, pH 8.0
0.25 g/L spermidine	25 mg
Mixed and autoclaved	
2% ß-mercaptoethanol (add immediately prior to use)	

Recipe 28: SSTE

	Amount for 100 ml
1.0 M NaCl	5.8 g
0.5% SDS	5 ml 10% SDS
10 mM Tris-HCl, pH 8.0	1 ml 1 M Tris-HCl, pH 8.0
1 mM EDTA	0.2 ml 0.5 M EDTA, pH 8.0
autoclaved	

Recipe 29: DEPC-treated water

0.1% DEPC in distilled water
stirred overnight in a fume-hood
autoclaved

3.9.5. DNA purification from agarose gels

DNA purification from agarose gels was performed using the Clean-A-Gene DNA purification kit.

After visualizing the DNA in an agarose gel, the band to be purified was excised from the gel and transferred into a 1.5 ml microcentrifuge tube. The weight was estimated and three volumes of binding buffer (including chaotropic agents) were added. Then, the agarose was

melted at 55°C for 5 min with occasional mixing. The mixture was allowed to reach room temperature, and 5 µl of binding matrix were added. The mixture was incubated at RT for 5 min or longer with occasional mixing. The matrix-DNA complex was pelleted for 10 sec in a microcentrifuge. The supernatant was removed, 1 ml of washing buffer was added and the matrix resuspended by whirling the tube, followed by centrifugation for 10 sec. The washing step was repeated two more times. Fifteen microlitres of sterile water, TE buffer or 10 mM Tris-HCl, pH 7.5, were added to the matrix to elute of the DNA. The matrix was resuspended in the elution volume, incubated for 3 min at 48°C and centrifuged for 30 sec. The elution step was repeated once more and the two elution supernatants were combined.

3.9.6. Small scale plasmid preparation

3.9.6.1. Alkaline lysis

The method described by Birnboim and Doly (1979) was followed. A 1.5 ml aliquot of an overnight culture of *E. coli* was centrifuged in 1.5 ml sterile microcentrifuge tubes for 30 sec. The supernatant was discarded and the pellet centrifuged again in order to remove any trace of medium.

The bacterial pellet was resuspended in 50 µl solution I (Recipe 30) by whirling and incubated 5 min at RT. Then, 100 µl of solution II (Recipe 31) were added to the cells and mixed carefully. The lysate was chilled 5 min on ice. Then, 75 µl of solution III (Recipe 32) were added, mixed immediately and incubated on ice for 5 min.

The mixture was centrifuged for 10 min at 4°C at maximum speed of a tabletop centrifuge. The supernatant (200 µl approximately) was transferred into a new microcentrifuge tube and extracted with 200 µl phenol/chloroform/isoamylalcohol (25:24:1, v/v/v). After 5 min centrifugation at RT, the upper phase was transferred to a new microcentrifuge tube, mixed with 500 µl ethanol, incubated 10 min at RT and centrifuged for 10 min. The pellet was then washed with 200 µl 70% ethanol, dried and dissolved in 25 - 50 µl of water or TE buffer (Recipe 28)

Recipe 30: Solution I for plasmid preparation

	Amount for 1 liter
50 mM D-glucose	9 g
25 mM Tris-HCl, pH 8	3 g
10 mM EDTA	20 ml 0.5 M EDTA, pH 8.0
autoclaved	

Recipe 31: Solution II for plasmid preparation

	Amount for 1 liter
1% SDS	10 g
0.2 N NaOH	8 g

Recipe 32: Solution III for plasmid preparation

	Amount for 1 liter
3 M potassium acetate, pH 4.8	294.45 g
filter sterilized	

3.9.6.2. Small and midi-scale plasmid preparation

When good quality of plasmid preparation was required (e.g. for sequencing), the Jetstar kit from Genomed was used, following the indications of the provider.

3.9.7. PCR

The Polymerase Chain Reaction (PCR) was used in order to amplify DNA from different sources. Plasmid DNA, genomic DNA, cDNA or a bacterial colony picked directly from an agar plate could be used as templates for the reaction. To avoid contamination, all solutions used for PCR were exclusively pipetted using tips containing cotton stoppers.

The oligonucleotides used for PCR were custom-made (usually by Metabion) and provided lyophilized. The oligonucleotides were dissolved in water to 1 nmol/µl and from this stock solution a 50 pmol/µl working solution was prepared. A typical 15 µl PCR reaction was performed in a 200 µl tube adding the following components (Recipe 33).

Recipe 33: Standard PCR

For a 15 µl reaction
Template
1.5 µl 10×Taq Buffer (Appligene) (100 mM Tris HCl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 1% TritonX-100 and 2 mg/ml of BSA)
0.3 µl 50 mM $MgCl_2$
0.3 µl 10 mM dNTPs
0.3 - 0.5 µl Primer 1
0.3 - 0.5 µl Primer 2
0.375 units Taq DNA Polymerase (Appligene)

Reactions were performed at 1.5 or 1 µM primer concentration when degenerated oligonucleotides or not-degenerated oligonucleotides were used, respectively. The reaction was carried out in a thermocycler with heating lid set to 110°C. The reaction mixtures were not sealed with oil. The programs used had a 3-min incubation step at 94°C before starting the cycle. Annealing was performed at 5°C below the melting point of the primers for 45 sec and, if degenerated oligonucleotides were used, annealing temperature was increased in every round. The next step in the cycle was an incubation at 72°C for 1 min (1.5 min if the fragment to be amplified was longer than 1 kbp), and the last step was for 1 min at 94°C. The cycle was repeated 35 times and the program finished with 10 min incubation at 72°C. The samples could be used directly for electrophoresis.

3.9.7.1. Colony PCR

Colony PCR was used to detect the presence of a vector with the desired insert after ligation and transformation. Each colony from a plate was slightly touched with a sterile pipette tip and the bacterial cells on the tip were introduced into a 200 µl PCR tube by touching the bottom. The same tip could be used to inoculate a preculture with the appropriate medium and antibiotic for selection. Fifteen microlitres of the PCR reaction mixture were added to the tube, and the PCR reaction was performed as described before but with 4 min at 94°C prior to the cycle and only 30 cycles. The samples could be analyzed directly by electrophoresis.

3.9.7.2. PCR of *P. sojae* actin cDNA

PCR was performed as described in section 3.9.7, using 0.4 µl of cDNA library (section 3.10.3, Table 4) and 0.3 µl of each "56up ac pmg" and "891 acainti pmg" primers (section 2.3.2) The PCR program with constant a 54°C annealing temperature and 35 cycles of amplification was chosen.

Preparative PCR was performed under the same conditions as described above in a total volume of 100 µl.

3.9.7.3. PCR with P50-derived primers

A 288 bp fragment was generated by PCR using the primer oligonucleotides "iqarsense" and "yenanti" (section 2.3.2, Table 5). The reaction was carried out with 35 cycles and a temperature gradient for annealing from 45 to 49.9°C, using the *P. sojae* cDNA as template.

3.9.8. Northern blotting

After electrophoresis (section 3.9.2), the agarose gel was placed on Whatman paper soaked with 20×SSC (Recipe 34) that was in contact with the same buffer in a chamber below. A nitrocellulose membrane was placed over the gel avoiding bubbles, covered by two sheets of Whatman paper, and more drying paper placed on top. The set was slightly pressed overnight, and then the transferred RNA was crosslinked to the membrane by UV light irradiation. The markers were cut out, incubated for 15 min in 5% acetic acid with gentle shaking and then incubated for 15 min in RNA staining solution (Recipe 35). The membrane was washed with water until the background disappeared.

Recipe 34: 20× SSC

	Amount for 1 liter
3 M NaCl	175.4 g
0.3 M sodium citrate, pH 7.2	88.2 g
filtrate and autoclave	

Recipe 35: RNA staining solution

	Amount for100 ml
0.5 M sodium acetate, pH 5.2	4.1 g
0.04% Methylene Blue	4 mg

3.9.9. ^{32}P labeling of DNA probes

The prime-a-Gene Labeling System (Promega) was used routinely. The DNA sample to be labeled (25 ng) was dissolved in 13 µl water, placed in a microcentrifuge tube, denatured by heating at 100°C for 2 min and immediately placed on ice. To the sample 5 µl of labeling 5×buffer (containing random primers) was added, followed by 1 µl of a mixture of deoxynucleotides excluding dCTP at 20 µM, 1 µl 400 µg/ml BSA, 2 µl of deoxycitidine 5´-[α-^{32}P]triphosphate and 0.5 µl of the Klenow fragment of the DNA polymerase I (2.5 units). The mixture was gently mixed and incubated for 1 h at room temperature. A 0.5 µl aliquot was collected for scintillation counting. The reaction mixture was then loaded onto a S-200 spin column (Pharmacia) (prepared by 1 min centrifugation at 3000 rpm in an Eppendorf microcentrifuge prior to use) and centrifuged 2 min at 3000 rpm. This eliminated non incorporated nucleotides and small DNA fragments. The eluate was collected, the volume estimated and an aliquot of 0.5 µl measured by scintillation counting. The incorporation yield of ^{32}P was usually between 30 to 50%.

The sample to be used for hybridization was heated 2 min in a boiling water bath and chilled immediately on ice. After cooling down and centrifugation, the radioactively labeled probe was added to the pre-hybridization buffer.

3.9.10. Hybridization of membrane-bound nucleic acids

For pre-hybridization, the membranes (from Northern blots or from the transfer of bacteria or phages) were incubated in a hybridization glass tube with 4 ml of hybridization buffer (Recipe 36) for 4 to 6 h at 42°C with constant rotation. After pre-hybridization, the radioactive probe was added to the buffer (final 1 to 3×10^6 cpm/ml), gently mixed and incubated overnight. The membranes were then washed two times with 2×SSC supplemented with 0.1% SDS at RT and incubated two times with 1×SSC supplemented with 0.1% SDS during 20 min each at 42°C with constant rotation. The membranes were then washed with 1×SSC until the SDS was completely eliminated, placed in a sealed plastic bag and exposed to a Fujifilm BAS-1500 phosphorescence image analyzer.

Recipe 36: Hybridization Buffer

Amount for 100 ml:
45 ml buffer N
5 ml buffer N2
45 ml deionized formamide
5 ml 2 mg/ml salmon sperm DNA (passed several times through a narrow needle and heated 5 min at 100°C).

Recipe 37: Buffer N

	Amount for 100 ml
1.8 M NaCl	10.5 g
0.1 M NaH_2PO_4	1.2 g
10 mM EDTA	2 ml 0.5 M EDTA, pH 8
autoclaved, supplemented with 0.2% SDS	

Recipe 38: Buffer N2

dissolve in 5 ml TE Buffer:
100 mg PVP-40
100 mg Ficoll-400
100 mg BSA

3.9.11. Preparation of electrocompetent *E. coli* cells

Eighty milliliters of LB medium (Recipe 39) were inoculated with 2 ml of an overnight culture of *E. coli* and incubated at 37°C with shaking until the OD_{600} reached 0.6 to 0.8. The cells were then transferred into sterile 50 ml polypropylene tubes (from then on work was carried out at 4°C) and centrifuged for 5 min at 5,000×*g* in a swing out rotor. The supernatant was discarded and the cells resuspended in 40 ml sterile distilled water. The resuspended cells were centrifuged again and the whole process repeated two times more. The volume of the cell suspension was estimated and glycerol added to a final concentration of 10%. The cells were transferred into sterile microcentrifuge tubes in 40 µl aliquots, frozen in liquid nitrogen and stored at –80°C.

Recipe 39: LB medium

	Amount for 1 liter
1% Bacto-Tryptone	10 g
0.5% Bacto-Yeast extract	5 g
0.5% NaCl	5 g
pH adjusted to 7.5; autoclaved	

3.9.12. Transformation of competent *E. coli* cells

Up to two µl of plasmid solution was used for transforming a 40 µl aliquot of *E. coli* electrocompetent cells (see section 3.9.11). The plasmid was dissolved in water or in a buffer with low ionic strength. The competent cells were thawed on ice and the plasmid solution added with gentle stiring with the pipette tip. The mixture was then transferred to a cold (below 0°C) electroporation-cuvette (0.1 to 0.2 cm width) taking care that the cells stayed on the bottom and there were no bubbles. The cuvette was then placed in the electroporator chamber and a current pulse was given (200 Ω, 25 µF, 2,5 kV). The cells were immediately resuspended in 1 ml of 42°C warmed SOC medium (Recipe 40) and shaken for 45 min at 37°C. The cells were streaked out on the appropriate selection medium.

Recipe 40: SOC medium

	Amount for 100 ml
2% Bacto-Tryptone	2 g
0.5% Bacto-yeast extract	0.5
10 mM NaCl	54 mg
2.5 mM KCl	18.6 mg
autoclaved, complemented with filter sterilized stock solutions to:	
10 mM $MgCl_2$	1 ml 1 M $MgCl_2$
10 mM $MgSO_4$	1 ml 1 M $MgSO_4$
20 mM glucose	1 ml 2 M glucose

3.9.13. Generation and transformation of competent *Saccharomyces cerevisiae* cells

One colony of the yeast (*Saccharomyces cerevisiae*) strain INVSc2 was picked from a YPD agar plate (Recipe 42) for inoculation of 10 ml of YPD medium (Recipe 41). The yeast cells were incubated at 30°C with shaking until the OD at 600 nm reached 0.5 units. The cells were then transferred into a sterile 50 ml Falcon tube and centrifuged for 10 min at 5,000×*g* at RT. The supernatant was discarded and the cells resuspended in 5 ml of sterile water. The yeast cells were then centrifuged for 5 min at 5,000×*g*, the supernatant discarded and the cells resuspended in 2.5 ml 0.1 M lithium acetate in TE buffer. The cells were then centrifuged 5 min at 3,000×*g*, the supernatant discarded and the cell pellet resuspended in 250 µl of 0.1 M lithium acetate in TE buffer. The cells were transferred into sterile 2 ml microcentrifuge tubes in 50 µl aliquots.

Recipe 41: YPD medium

	Amount for 1 liter
2% Bacto peptone	20 g
1% Yeast extract	10 g
2% glucose	20 g
autoclaved	

Recipe 42: YPD agar medium

	Amount for 1 liter
2% agar-agar	20 g
2% Bacto peptone	20 g
1% Yeast extract	10 g
2% glucose	20 g
autoclaved	

One microlitre of the plasmid DNA for transformation and 5 µl of salmon sperm DNA (2 mg/ml, denatured by incubation at 100°C for 5 min followed by chilling on ice) were added to the yeast cell suspension. The mixture was whirl-mixed and incubated for 7 min at 30°C with gentle shaking. Three hundred microliters of LiPEG (Recipe 43) were added, and the sample was mixed and incubated for 15 min at 30°C with shaking. Then, 36 µl of DMSO were added and the mixture was incubated for another 15 min at 30°C with shaking.

Recipe 43: LiPEG

	Amount for 100 ml
40% PEG 4000	40 g
0.1 M lithium acetate	1 g
10 mM Tris-HCl, pH 7.5	1 ml 1 M Tris-HCl, pH 7.5
1 mM EDTA	0.2 ml 0.5 M EDTA, pH 8.0

The mixture was placed in a water bath at 42°C for 15 min (heat shock). One milliliter of YPD medium (Recipe 41) was added and the mixture centrifuged at 1,000×*g* for 2 min at RT. The supernatant was discarded, the pellet resuspended in 1 ml of YPD medium and centrifuged again at 1000×*g* for 2 min at RT. The cells were resuspended in 200 µl sterile water and plated onto a YnbCasGlc agar plate (Recipe 44). The plates were incubated at 30°C until yeast colonies appeared, usually after three days. The plates were then stored at 4°C.

Recipe 44: YnbCasGlc agar medium

	Amount for 1 liter
0.1% casamino acids	1 g
0.67% yeast nitrogen base w/o amino acids	6.7 g
2% glucose	20 g
2% agar-agar	20 g
autoclaved and complemented with	
0.01% adenine (filter sterilized)	10 ml 1% adenine (filter sterilized)
0.005% tryptophan (filter sterilized)	10 ml 0.5% tryptophan (filter sterilized)

3.9.14. Heterologous expression in *S. cerevisiae*

One yeast colony from a YnbCasGlc agar plate was transferred to 2 ml of YnbCas glucose medium (Recipe 45) and incubated overnight at 30°C with shaking. The cells were then transferred to a 2 ml microcentrifuge tube and centrifuged for 5 min at 1,500×*g*. The supernatant was discarded and the cells were resuspended in 1 ml of sterile water and centrifuged for 5 min at 1,500×*g*. The cells were then resuspended in 0.5 ml of YnbCas galactose medium (Recipe 46) and transferred to 25 ml of YnbCas galactose medium. The OD_{600} of an aliquot was measured and an aliquot corresponding to one unit of absorbance was centrifuged, the supernatant discarded and the cells frozen at -80°C for further analysis. The culture was incubated at 30°C with shaking for 48 h. Aliquots of one unit of absorbance were taken at different times during incubation.

Recipe 45:Ynbcas glucose

	Amount for 1 liter
0.1% casamino acids	1 g
0.67% yeast nitrogen base without amino acids	6.7 g
2% glucose	20 g
autoclaved and complemented with	
0.01% adenine (filter sterilized)	10 ml 1% adenine (filter sterilized)
0.005% tryptophan (filter sterilized)	10 ml 0.5% tryptophan (filter sterilized)

Recipe 46: Expression medium (Ynbcas galactose AdeThr)

	Amount for 1 liter
0.1% casamino acids	1 g
0.67% yeast nitrogen base w/o amino acids	6.7 g
2% galactose	20 g
autoclaved and complemented with	
0.01% adenine (filter sterilized)	10 ml 1% adenine (filter sterilized)
0.005% tryptophan (filter sterilized)	10 ml 0.5% tryptophan (filter sterilized)

3.9.15. Ligation of DNA fragments

Several ligation procedures were applied during this work. Unless otherwise indicated, T4 ligase (MBI) was used. Usually 25 ng of vector DNA were ligated to 3 to 8 equimolar amount of insert. The conditions of the reaction also varied depending on the kind of DNA to be ligated: low temperatures (4°C) for blunt-ended DNA and higher temperatures (16°C to RT) for DNA with cohesive ends. The incubation was carried out overnight, however, shorter incubation times are possible for intramolecular reactions. The reaction was performed in a final volume of 5 or 10 µl and, once the reaction was concluded, the volume was taken to 50 µl with water. The DNA was then precipitated by adding 5 µl 3 M sodium acetate, pH 5.2, and 165 µl ethanol followed by 2 h incubation at -80°C. After 30 min centrifugation at 15,000×*g* at 4°C and washing the pellet with 70% ethanol, the DNA was dissolved in water or 50 mM Tris-HCl, pH 7.5.

3.9.16. DNA sequencing

DNA sequencing was done by members of Professor R. Hermann's group (Botanisches Institut der LMU, Munich) using the ABI377 system. The samples were supplied in a volume of 8 µl including 0.5 µg of DNA to be sequenced and 5 pmol of the oligonucleotide primer.

3.9.17. Filling of DNA ends by the use of Klenow enzyme

To a DNA pellet the following components were added:

19 µl H_20

3 µl Klenow buffer (MBI)

4 µl 0.1 M DTT

3 µl 10 mM dNTPs

1 µl Klenow enzyme (MBI, 1 unit$\times\mu l^{-1}$)

The mixture was incubated for 30 min at 30°C and then used for ligation procedures.

3.9.18. Generation of deletion clones

Several deletion clones of pZL-IY were generated in order to obtain overlapping partial sequences. In general, 1 µg of pZL-IY was digested with different restriction enzymes. When two enzymes were used for a double digestion and any of the DNA ends were sticky, Klenow filling to the end was done (section 3.9.17).

After ligation (section 3.9.15) the mixture was incubated for 10 min at 70°C. One microliter of the reaction mixture was used directly for electrotransformation of DH5α cells (section 3.9.12) and streaked out on LB plates complimented with ampicillin (100 µg/ml).

Single colonies were used for inoculation of liquid medium and after overnight incubation used for plasmid isolation using the Jet-star kit (section 3.9.6.2).

The following restriction enzymes or enzyme combinations were used for generating different deletion clones of pZL-IY:

- *Pst* I
- *Sma* I
- *Ecl*136 II + *Sal* I
- *Bgl* II + *Sal* I

- *Eco*52 I
- *Aat* II

3.10. Generation of a cDNA library of *P. sojae*

3.10.1. Total RNA extraction

RNA was extracted from 10 and 14 day old mycelium as indicated in section 3.9.4. The RNA extracted from the 10 days mycelium had a final concentration of 14.7 mg/ml and a 260/280 nm ratio of 1.98. The RNA from 14 days old mycelium had a final concentration of 7.98 mg/ml and a 260/280 ratio of 1.86.

3.10.2. Messenger RNA isolation

Two milligrams of total RNA derived from each 10 and 14-days-old mycelium were used for isolation of mRNA, using the PolyATract mRNA Isolation System from Promega with minor modification of the instructions of the manufacturers. One milligram of total RNA was placed per 2 ml microcentrifuge tube and diluted to a volume of 500 µl with DEPC-treated water. The tube was placed in a 65°C heating block for 10 min. Three microliters of Biotinylated-Oligo(dT) Probe and 13 µl of 20×SSC were added to the RNA. The sample was gently mixed and allowed to reach room temperature.

The Streptavidin-Paramagnetic Particles (SA-PMPs) were resuspended by gently flicking the bottom of the tube until completely dispersed and were captured by placing the tube in a magnetic stand until the SA-PMPs have collected at the side of the tube. The supernatant was removed and discarded. The SA-PMPs were washed three times with 0.5× SSC (0.3 ml per wash), each time capturing them using the magnetic stand and removing the supernatant. The SA-PMPs were resuspended in 0.1 ml 0.5× SSC and added to the RNA tube. The mixture was incubated at RT for 10 min, the SA-PMPs captured by placing the tube in the magnetic stand and the supernatant was removed. The particles were washed 4 times with 0.1× SSC (0.3 ml per wash).

The mRNA was eluted two times from the SA-PMPs by resuspending them in 0.2 ml DEPC-treated water and separating the matrix from the supernatant by the magnetic field.

An amount of 10.9 µg of mRNA was isolated from the 10 day old mycelium material and 5.4 µg from the 14 day old mycelium material. The 260/280 absorbance ratios were 1.99 and 2.10 respectively. Both mRNA samples were precipitated by adding 0.1 volumes of 3 M NaCl and one volume of isopropanol, overnight incubation at –20°C, followed by centrifugation for 30 min at 14,000×*g* at 4°C. Both pellets showed a light brownish color, probably due to the presence of material from the magnetic beads used for the isolation. Both pellets were dissolved in 0.45 ml DEPC-treated water each, combined, centrifuged for 30 min at 14,000×*g* and the supernatant was transferred to a new sterile tube. The concentration was 14.44 µg/ml with a 260/280 ratio of 2.10.

3.10.3. Generation of cDNA

Seven micrograms of mRNA (485 µl) were precipitated by adding one tenth volume of 3 M NaCl and 3 volumes ethanol, followed by incubation for 1 h at -80°C. After centrifugation for 30 min at 14,000×*g* and washing with 75% ethanol, the pellet was re-dissolved in 5 µl of DEPC-treated water. Then the instructions of the SuperScript™ Lambda System for cDNA synthesis and λ cloning from Gibco were followed with minor modifications.

Two microlitres of *Not* I primer adapter was added to the mRNA. The tube was heated to 70°C for 10 min, and shock-cooled on ice. After a brief spin the next components present in the kit for first strand synthesis were added: 4 µl "5× First Strand Buffer", 2µl 0.1 M DTT, 1 µl 10 mM dNTP mixture, 1 µl H_2O

The mixture was gently whirl-mixed, briefly centrifuged and placed for 2 min at 37 °C to equilibrate the temperature. Then 5 µl of SuperScript II reverse transcriptase was added, gently mixed and incubated at 37°C for 1 h.

For the synthesis of the second strand cDNA, the following components were added to the first strand reaction mixture:

91 µl DEPC-treated water

30 µl "5× Second Strand Buffer"

3 µl 10 mM dNTP mixture

1 µl *E. coli* DNA ligase (10 U/µl)

4 µl *E. coli* DNA polymerase I (10 U/µl)

1 µl *E. coli* RNase H (2 U/µl)

The tube was then gently mixed and incubated for 2 h at 16°C. Then 2 µl (10 units) of T4 DNA polymerase were added and the mixture was incubated at 16°C for 5 min.

The mixture was placed on ice and 10 µl of 0.5 M EDTA, pH 8.0, were added. The mixture was then extracted with 150 µl of phenol/chloroform/isoamyl alcohol (25:24:1), whirl-mixed and centrifuged at RT at 14,000×*g* for 5 min. Hundred forty microliters of the aqueous phase were removed and placed into a new 1.5 ml microcentrifuge tube. Seventy microliters of 7.5 M ammonium acetate followed by 0.5 ml ethanol (-20°C) were added. The tube was mixed thoroughly, and immediately centrifuged at 14,000×*g* for 20 min at RT. The supernatant was discarded and the pellet washed with 0.5 ml 70% ethanol and centrifuged for 2 min. The pellet was dried at 37°C.

For the *Sal* I adapter addition the following components were added to the ice-cooled cDNA:

25 µl DEPC-treated water

10 µl "5× T4 Ligase Buffer"

10 µl *Sal* I adapters

5 µl T4 DNA ligase

The reaction was mixed gently and incubated overnight at 16°C.

The reaction was extracted with 50 µl phenol/chloroform/isoamyl alcohol (25:24:1) and 45 µl of the upper phase was transferred to a fresh 1.5 ml microcentrifuge tube. The cDNA was precipitated by adding 25 µl of 7.5 M ammonium acetate and 150 µl ethanol (-20°C), centrifuged at 14,000×*g* for 20 min at RT and the pellet was washed with 70% ethanol and dried.

The pellet was then digested with *Not* I by adding the following components:

41 µl DEPC-treated water

5 µl REact 3 Buffer

4 µl *Not* I

The mixture was incubated for 2 h at 37°C and then extracted with 50 µl phenol/chloroform/isoamyl alcohol (25:24:1). A 45 µl portion of the upper phase was transferred to a new 1.5 ml tube. The cDNA was precipitated by adding 25 µl of 7.5 M ammonium acetate followed by 150 µl ethanol (-20°C), and centrifuged at 14,000×*g* for 20 min at RT. The pellet was washed with 70% ethanol and dried.

For size fractionation by size exclusion chromatography, the cDNA was dissolved in 100 µl TEN buffer (Recipe 47). The column was first equilibrated by washing 4 times with 0.8 ml TEN buffer. The cDNA was loaded onto the column and the effluent collected into the first tube. Hundred microliters of TEN buffer was added to the column and collected in tube 2. Then, 100 µl aliquots of TEN buffer were loaded onto the column and single-drop fractions

were collected in different tubes. The volume of every fraction was estimated. Samples 10 to 23 (217 µl total volume) were combined.

One-fifth (44 µl) of the cDNA was precipitated by adding 22 µl 7.5 M ammonium acetate and 132 µl ethanol at –20°C overnight. The sample was centrifuged and the pellet washed with 70% ethanol and dried.

Recipe 47: TEN buffer

	Amount for 100 ml
10 mM Tris-HCl, pH 7.5	1 ml 1 M Tris-HCl, pH 7.5
0.1 mM EDTA	20 µl 0.5 M EDTA, pH 8.0
25 mM NaCl	146 mg
autoclaved	

To ligate the cDNA to the vector, the following components were added to the dried pellet:

1 µl 5× λ ligation buffer

2 µl λZipLox *Not* I/*Sal* I arms

1 µl DEPC-treated water

1 µl T4 ligase

The sample was mixed gently by pipetting, and the mixture was incubated at RT for 3 h.

3.10.4. *In vitro* packaging of ligated cDNA

Packagene Lambda DNA Packaging System from Promega was used. The packagene extract was thawed on ice. Then 5 µl of the ligation reaction were added to 25 µl of the packaging extract (half of what was provided in one tube) and was mixed gently by tapping the bottom of the tube. The mixture was incubated for 3 h at RT.

220 µl of phage buffer (Recipe 48) and 25 µl of chloroform were added to the packaging mixture. The sample was mixed by inversion and the chloroform was allowed to settle to the bottom of the tube.

Recipe 48: Phage buffer

	Amount for 100 ml
20 mM Tris-HCl, pH 7.5	2 ml Tris-HCl, pH 7.5
100 mM NaCl	0.58 g
10 mM $MgSO_4$	120 mg
0.05% gelatin	50 mg

3.10.5. Titration of the cDNA library

One day before titer determination, a single colony of *E. coli* Y1090(ZL) from a freshly streaked LB-agar plate (Recipe 49) was used to inoculate 3 ml of LB medium (Recipe 39) supplemented with 0.2% maltose and 10 mM $MgSO_4$. The inoculated medium was incubated at 37°C with agitation until the OD_{600} reached 0.6 - 0.8. The cells could be stored at 4°C for up to 24 h.

Serial dilutions (1:100; 1:1000; 1:10,000) of the packaging reaction were made with phage buffer (Recipe 48). One hundred microliters of each dilution were added an equal volume of *E. coli* suspension in a 10 ml tube and the phages were allowed to absorb for 30 min at 37°C.

Five milliliters of molten (45°C) top agar (Recipe 50) were added to the tube and the mixture was poured immediately onto fresh LB plates pre-warmed to 37°C. After hardening of the top agar, the plates were incubated at 37°C overnight. The cDNA library had 600 pfu/µl and 120,000 pfu in total.

Recipe 49: LB-agar

	Amount for 1 liter
1% Bacto-Tryptone	10 g
0.5% Bacto-Yeast extract	5 g
0.5% NaCl	5 g
1.5% agar	15 g
pH adjusted to 7.5 and autoclaved	

Recipe 50: Top Agar

	Amount for 1 liter
10% Bacto-Tryptone	10 g
0.5% NaCl	5 g
0.8% agar	8 g
autoclaved	
after cooling to 60°C the agar was supplemented with $MgSO_4$ to 10 mM	5 ml 2 M $MgSO_4$ (sterile filtrated)

3.10.6. Screening of the cDNA library

The phages, prepared as described in section 3.10.5, were transferred after cooling to nitrocellulose membranes for screening. This was done by soaking the 90-mm diameter nitrocellulose membranes first in sterile 1 M NaCl and then air drying them over a Whatman paper. The dry membranes were placed on top of the agar for 3 min and then placed over another sheet of Whatman paper soaked in 1.5 M NaCl, 0.5 M NaOH. After 5 min the membranes were placed over a Whatman paper soaked in 1.5 M NaCl and 1 M Tris-HCl, pH 8.0, for 5 min. After a short wash step in 2×SSC, the membranes were incubated for 2 hours at 80°C. The membranes were then ready for hybridization as described in the section 3.9.10.

3.10.7. *In vivo* excision of phages

Plaques for *in vivo* excision were picked up from the agar plate using a sterile Pasteur pipette and transferred to 250 µl of phage buffer (Recipe 48). The tube was whirl-mixed for 10 sec and incubated for 5 min at room temperature. Twenty five microliters of phage suspension were then mixed with 100 µl of *E. coli* DH10B cells grown the day before in LB + ampicillin liquid medium.

After 10 min at room temperature, the cells were streaked out onto plates with LB agar complimented with ampicillin (100 $\mu g \times ml^{-1}$), X-gal (80 $\mu g \times ml^{-1}$)and IPTG (0.5 mM). The plates were incubated overnight at 37°C.

4. Results

4.1. Optimization of the *P. sojae* culture

As the quantity of starting material (i.e. the mycelium of *P. sojae*) is one of the main considerations in any protein purification trial, several parameters were optimized in order to maximize the amount, quality and growth rate of the mycelium.

The presence of $CaCO_3$ was fundamental for the growth. The use of high quality (p.a.) $CaCO_3$, instead of the 95% purity used earlier, reduced the incubation time for one or two days, and harvesting was easier and faster. Washing out completely the $CaCO_3$ turned out to be useful for a good microsomal preparation and for the solubilization of the glucan synthase activity.

The amount of ß-sitosterol was doubled and the ratio of phosphates exchanged (previously half of K_2HPO_4 and double of KH_2PO_4 was used). This increased the growth rate and the quality of the mycelium. The use of incubation temperatures of 25-26°C instead of 22°C also increased mycelial growth rate.

With these modifications, the growth on liquid medium was shortened to 3 weeks or less, instead of the 4 to 6 weeks required in first trials.

4.2. Preparation of the microsomal fraction

The microsomal fraction, collected as a fatty brownish pellet after centrifugation (see section 3.2), contained 1 to 5 mg of membrane protein per flask of culture. Variations were probably due more to external factors during the manipulation of big volumes than to changes in mycelium composition.

Poor glucan synthase activity was obtained when frozen mycelium was used. Therefore, whenever possible, the microsomal fraction was prepared from freshly harvested mycelium. After storage for 2 days at 4°C, the microsomal preparation still showed 75% of the initial activity.

4.3. Effect on glucan synthase activity

Several substances were tested for their effects (activating or inhibiting) on glucan synthase activity in microsomal fractions of *P. sojae*. Some of these are summarized in Table 6.

The substances were tested in 50 mM Tris-HCl, pH 7.5, 25 mM NaF, 1 M sucrose, 5 mg/ml BSA (control condition).

Table 6: Effect on glucan synthase activity

Substance	Concentration		Activity (%)
Control			100
Ca^{2+}	10.0	mM	95
Mg^{2+}	10.0	mM	93
Mn^{2+}	10.0	mM	31
Fe^{2+}	10.0	mM	37
Zn^{2+}	1.0	mM	17
	10.0	mM	0
Cu^{2+}	1.0	mM	23
	10.0	mM	0
EGTA	10.0	mM	100
EDTA	10.0	mM	90
DTT	1.25	mM	100
GTP	4.0	mM	100
GTPγS	0.05	mM	100
PPi	5.0	mM	38
UDP	10.0	mM	22
Cellobiose	10.0	mM	108
Laminarin	5.0	mg/ml	68
Glucan DP 5	5.0	mg/ml	100
Glucan DP 7	5.0	mg/ml	100
Glucan DP 13	5.0	mg/ml	93
Glucan HMW	5.0	mg/ml	74
α-Amylase	50.0	units/ml	100
Laminarinase	0.1	units/ml	50
Control –NaF	0		30
Control –Sucrose	0		50
Control –BSA	0		70

The divalent cations Ca^{2+} and Mg^{2+}, had no effect on glucan synthesis at concentrations tested, while Fe^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} had strong inhibiting effect. No effect was found when cellobiose, GTP or its analogue GTPγS was included in the mixture. UDP and PPi were found to have inhibitory effects on glucan synthase activity.

No glucan synthase activity was found in the cytosolic fraction (supernatant of microsomal preparation). Addition of cytosolic fraction to the microsomal fraction did not affect glucan synthase activity.

4.4. Solubilization of glucan synthase activity

In order to find the best detergent for solubilization of glucan synthase activity, solubilizations were done in volumes of 0.5 ml (section 3.4) at 2 mg/ml protein concentration including different detergents or detergent mixtures in a concentration of 10 mg/ml. The detergents tested were CHAPS, octyl glucoside, Zwittergent 3-12, SDS and CHAPS in combination with octyl glucoside. The results are summarized in Table 7.

Table 7: Effects of different detergents on solubilization of glucan synthase activity and total protein

Detergent	% protein solubilized	% activity solubilized	% activity in pellet
CHAPS	15	31	40
Octyl glucoside	48	0	0
CHAPS + Octyl glucoside	26	0	9
Zwittergent 3-12	59	0	0
SDS	Not measurable[1]	0	0

[1] SDS interferes with the protein assay

From the data shown in Table 7 it was evident that only CHAPS was able to solubilize and preserve glucan synthase activity from the *P. sojae* microsomal fraction.

Further experiments were carried out at different concentrations of protein and detergent in order to find out the best conditions for solubilization. The first set of experiments was performed using constant protein concentration of 1 and 2 mg/ml and several different concentrations of CHAPS. The results are summarized in Figure 6. Samples with higher concentration of detergent were diluted for the activity assay, with no change in specific enzyme activity (results not shown).

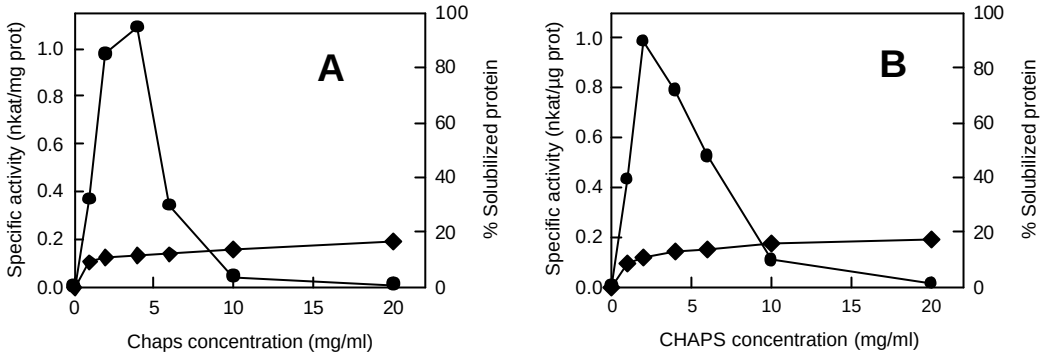


Figure 6: Solubilization of glucan synthase activity from a *P. sojae* membrane fraction using CHAPS**.** Panel A shows the experiments at 1 mg/ml protein concentration and panel B at 2 mg/ml protein concentration. Specific activity of the solubilized fraction (●); total solubilized protein (◆).

A protein concentration of 2 mg/ml was established to be the most efficient, since the total amount of solubilized protein was higher (the percentage of protein solubilizated was similar in both cases, then, the total amount of protein solubilized was double at 2 mg/ml). More accurate solubilization experiments were done to find out the best concentration of CHAPS. The results of these experiments are shown in Figure 7.

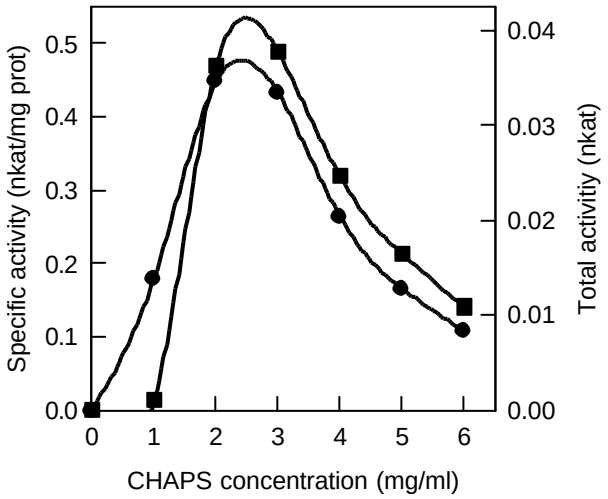


Figure 7: Solubilization of glucan synthase in the range of 0 to 6 mg/ml CHAPS at 2 mg/ml protein concentration. Specific enzyme activity (■); total solubilized activity (●).

Suitable conditions for solubilization at preparative scale were, thus, established at 2 mg/ml protein concentration and 3 mg/ml CHAPS as a compromise between a high total activity and high specific activity.

The membrane pellet remaining after solubilization was re-extracted with CHAPS as described in section 2.4. Solubilized material obtained at the second extraction had a specific activity of one forth to one third of the first one, and was only used for test experiments and never mixed with material of a first extraction.

4.5. Dependence of enzyme activity on time and protein concentration

The dependence of glucose incorporation on time and protein concentration in the activity assay was studied in order to determine the ranges in which the glucan synthase assay leads to reproducible and reliable data. The results are shown in Figure 8 to 9.

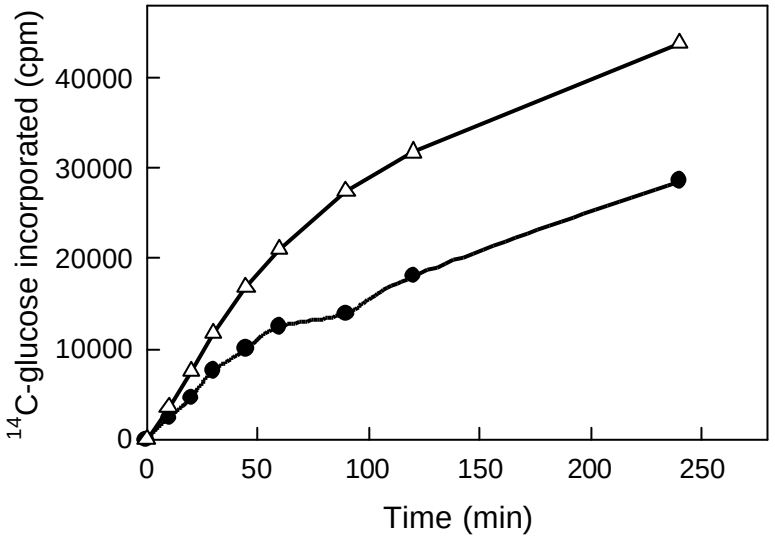


Figure 8: Time dependency for glucan synthase activity of *P. sojae*.
Microsomal fraction (●); CHAPS solubilized fraction (?).

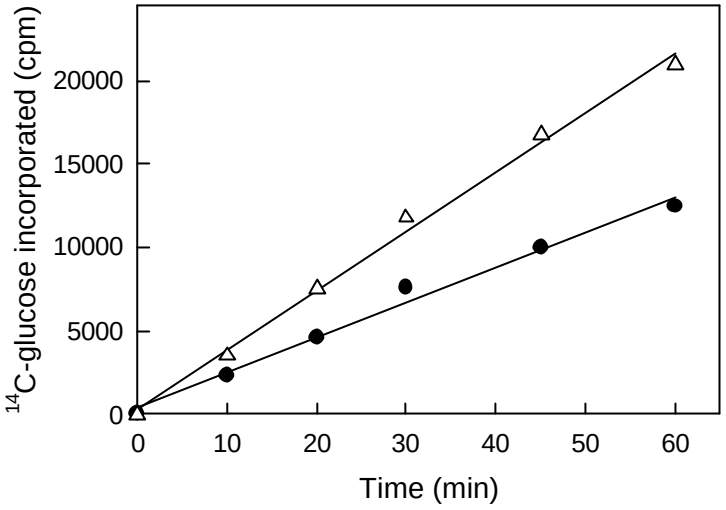


Figure 9: Linear regressions of the time dependence for the glucan synthase activity of *P. sojae* during the first hour. Microsomal fraction (●); CHAPS-solubilized fraction (?).

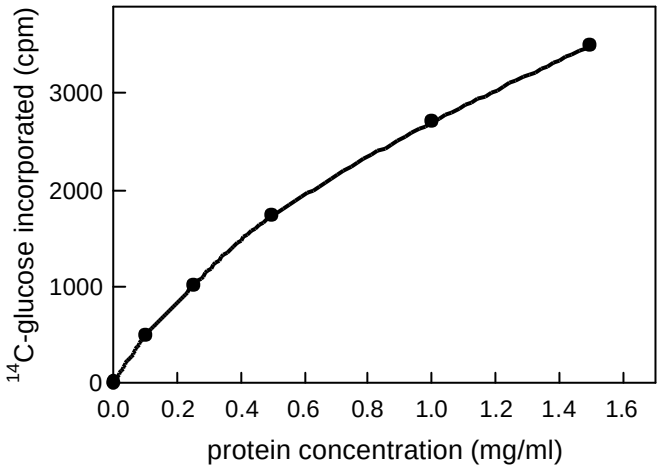


Figure 10: Dependence of glucan synthase activity on protein concentration, with microsomal fraction as protein source.

When plotting time versus product formation (Figure 8), one could observe that the velocity of the reaction decayed in the 4 h range, but remained constant during the first hour (Figure 9). The correlation coefficients (r^2) were 0.989 for microsomal fraction and 0.995 for solubilized fraction. Based on this observation, the duration of the glucan synthase assay was then set to one hour.

Figure 10 shows that the relationship of product formation to protein concentration deviated from linearity when protein concentration increased up to (1.5 mg/ml). The protein concentration in assays was therefore routinely kept below this value for both microsomal and solubilized fractions.

4.6. Dependence of product formation on substrate concentration

Substrate saturation was examined in the presence of microsomal fraction and CHAPS-solubilized protein (Figure 11) in order to determine the catalytic constants K_m and V_{max}. These were calculated from the saturation curve by non-linear regression using the Sigma Plot program. Lineweaver-Burk plots (inserts in Figure 11) were performed to verify the non-linear regressions. The saturation curves shown in Figure 11 were representative for two independent experiments. No significant differences were found in the catalytic parameters among these experiments. Catalytic constants are shown in Table 8.

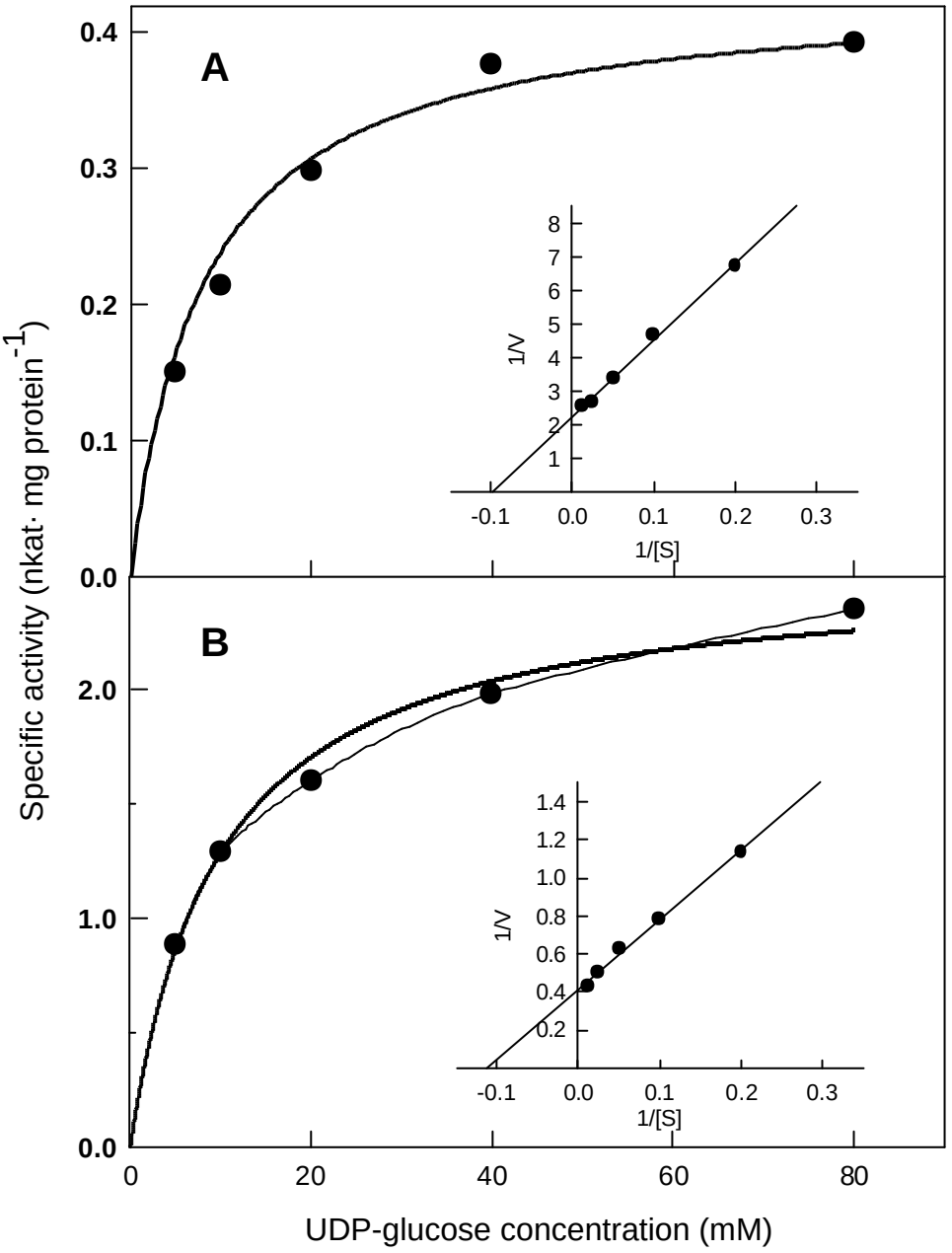


Figure 11: Dependence of β-glucan formation on UDP-glucose concentration in the presence of glucan synthase from *P. sojae*. Panel A shows product formation in the presence of the microsomal fraction and panel B in the presence of CHAPS-solubilized protein. The inserts represent the Lineweaver-Burk plots of the data. The data points in the curves represent means of two independent experiments.

Table 8: Catalytic constants for UDP-glucose of the reaction catalyzed by glucan synthase

	K_m (mM)	V_{max} (nkat·mg protein^{-1})
Microsomal fraction	10.6	0.4
CHAPS-solubilized protein	10.7	2.5

4.7. Product characterization

4.7.1. Estimation of glucose content in the reaction product of glucan synthase

The amount of sugar in the product of the catalysis was estimated by the anthrone method as described in section 3.5.3.

When 1.1 mg of enzymatically formed unlabeled glucan (section 2.5.2) was dissolved in 0.2 ml 0.36% NaOH (w/v), and 5 µl of this solution was used in the anthrone assay, an absorption of 0.727 was obtained. This corresponds to 0.172 µmol of glucose. Considering that in the linear glucan one residue of glucose has a molecular weight of 162, the glucan sample analyzed corresponded to 0.170 µmol. It was concluded that the product of the glucan synthase reaction consisted of 100% carbohydrate.

4.7.2. Enzymatic degradation

^{14}C-labelled glucan produced by the *P. sojae* glucan synthase was degraded using two enzymes and the radioactivity in the supernatant of the reaction mixtures was measured (section 3.5.4). Degradation studies with different glucanases gave information about the characteristics of the enzymatically formed product.

Neither the supernatant after α-amylase treatment nor the buffer control contained radioactivity. In contrast, the supernatant of the reaction with laminarinase contained 50% of the initial radioactivity of the pellet. Thus the product can be degraded at least partially by laminarinase but not by α-amylase.

The supernatants of the reactions were analyzed by HPTLC as described in section 2.5.5. In the case of the laminarinase treatment, the radioactive spot had the same R_f as glucose, thereby excluding laminaribiose and gentiobiose.

4.7.3. NMR analysis

NMR spectroscopy is a powerful technique used for the elucidation of chemical structures.

NMR spectra of the unlabelled glucan, dissolved in DMSO-d_6, showed resonances typical for carbohydrates (Figures 12 to 15). The ^{13}C-NMR spectrum (Figure 13) displayed only six resonances, while the ^{1}H-NMR spectrum (Figure 12) showed broadened signals indicating a short transverse relaxation time corresponding to a high molecular weight polymer. Two of the low field proton resonances did not produce cross peaks in the HMQC-NMR spectrum (Figure 14), therefore representing non (or slowly) exchanging hydroxyl groups. These could be assigned by their vicinal couplings in an HH-COSY spectrum (Figure 15). The assignment was confirmed by HH-COSY and ^{1}H, ^{13}C-HQMC-NMR spectra which gave chemical shift values (Table 9) closely resembling literature values for (1→3)-ß-D-glucans (Yu et al., 1993; Müller et al., 1996; Gutierrez et al., 1996; Kottutz and Rapp, 1990).

Table 9: Chemical shifts (δ, ppm) of NMR spectra obtained from the glucan product.

Position	Chemical shift values (δ)		
	C from ^{13}C-NMR	OH from ^{1}H-NMR	H from ^{1}H-NMR
1	103.2	--	4.50 d $J_{12(obs)}$=6.9 Hz
2	72.82	5.15 s (br)	3.30
3	86.22	--	3.44
4	68.39	4.60 s (br)	3.20
5	76.32	--	3.25
6	60.85	4.58 s (br)	3.69 d $J_{6a6b(obs)}$=9.3 Hz 3.44 m

The Lorentzian tails associated with the rather high linewidth of the ^{1}H-NMR resonances (approximately 7 Hz), combined with the low dispersion of the chemical shift, precluded an accurate estimate of impurities from the proton and HH-COSY-NMR spectra alone. However, in both spectra no resonances were visible other than those from a (1→3)-ß-glucan. The purity of the sample was deduced from the signal to noise ratio in the ^{13}C-NMR spectrum and from the absence of additional cross peaks in the ^{1}H,^{13}C-HMQC-NMR spectrum. Both spectra are in accordance with those of a single uniform glucan with impurities and branching other than (1→3)-ß-D-glucan not exceeding 3 to 4%.

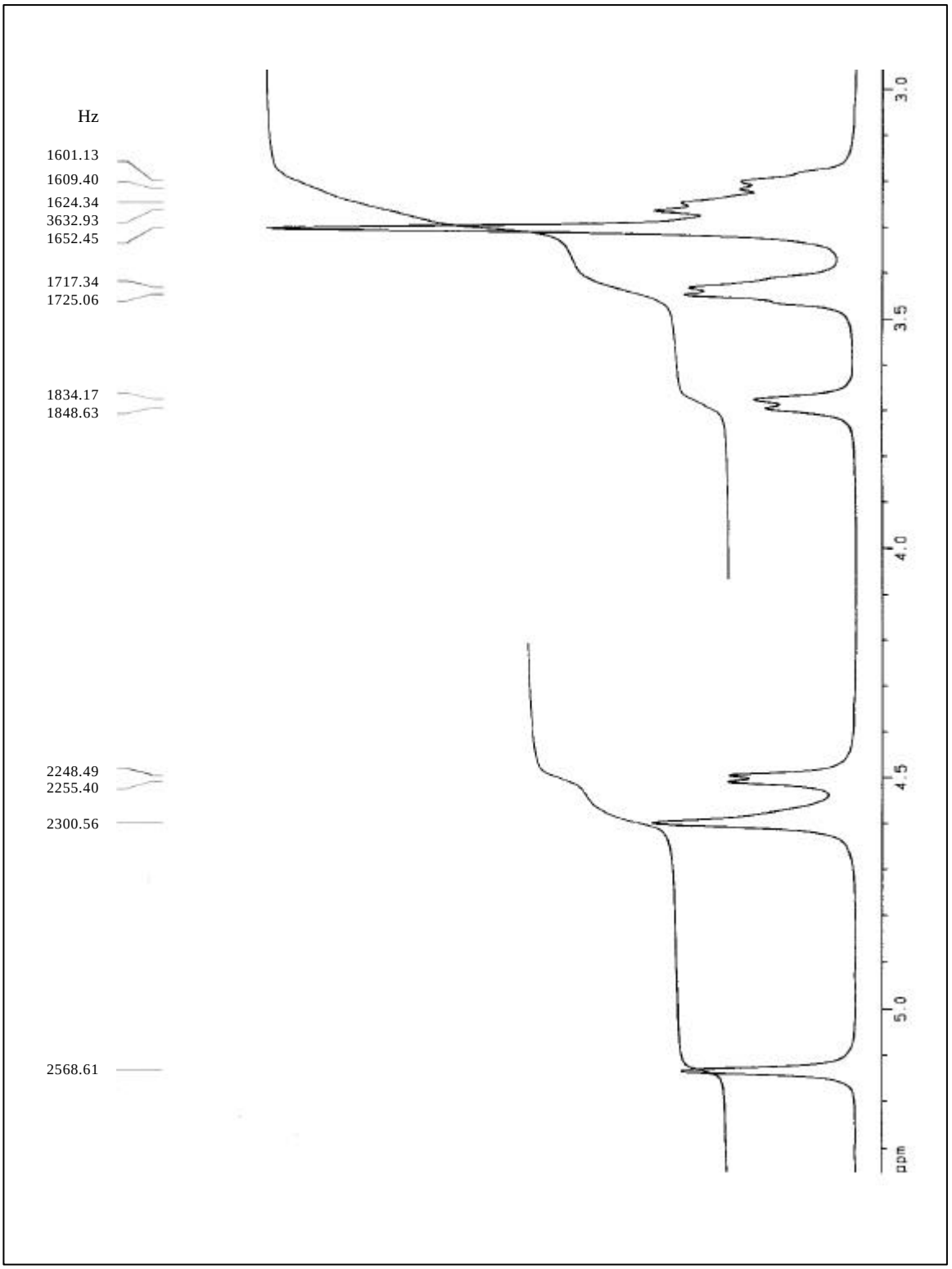


Figure 12: ^{1}H-NMR spectrum of the product of the CHAPS-solubilized glucan synthase.

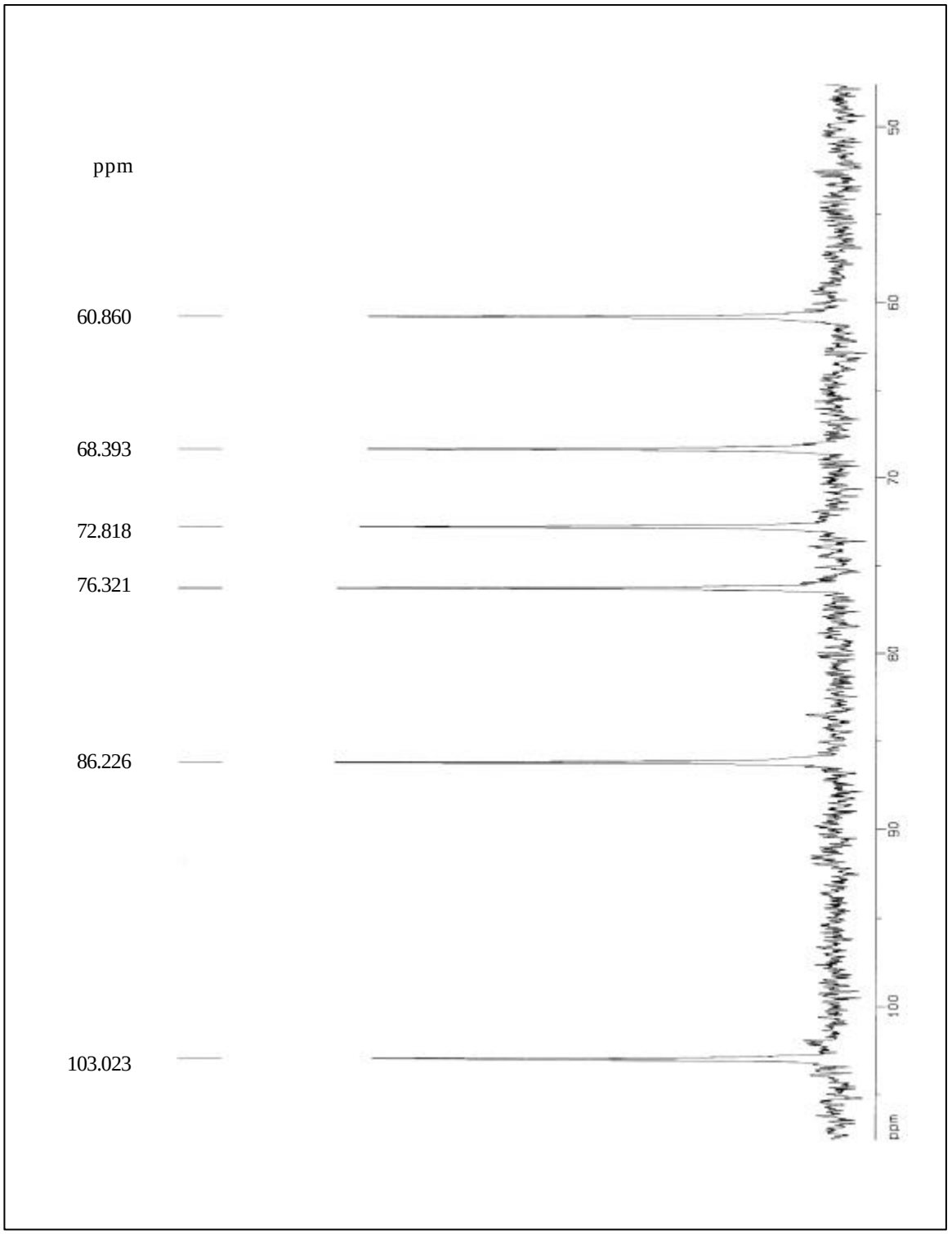


Figure 13: ^{13}C-NMR spectrum of the product of the CHAPS-solubilized glucan synthase.

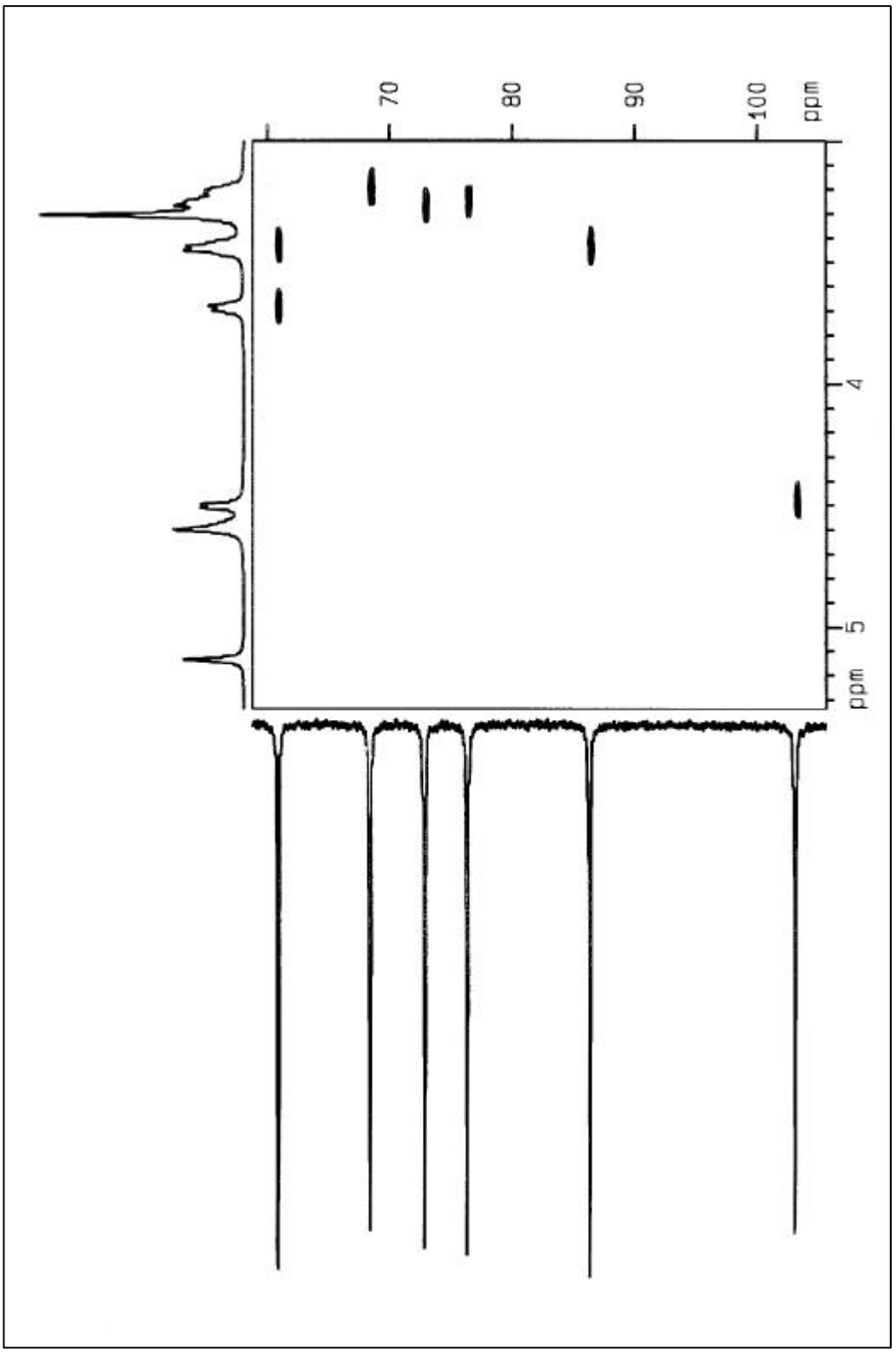


Figure 14: ^{1}H (vertical axis), ^{13}C (horizontal axis) and HMQC-NMR spectra of the product of the CHAPS-solubilized glucan synthase.

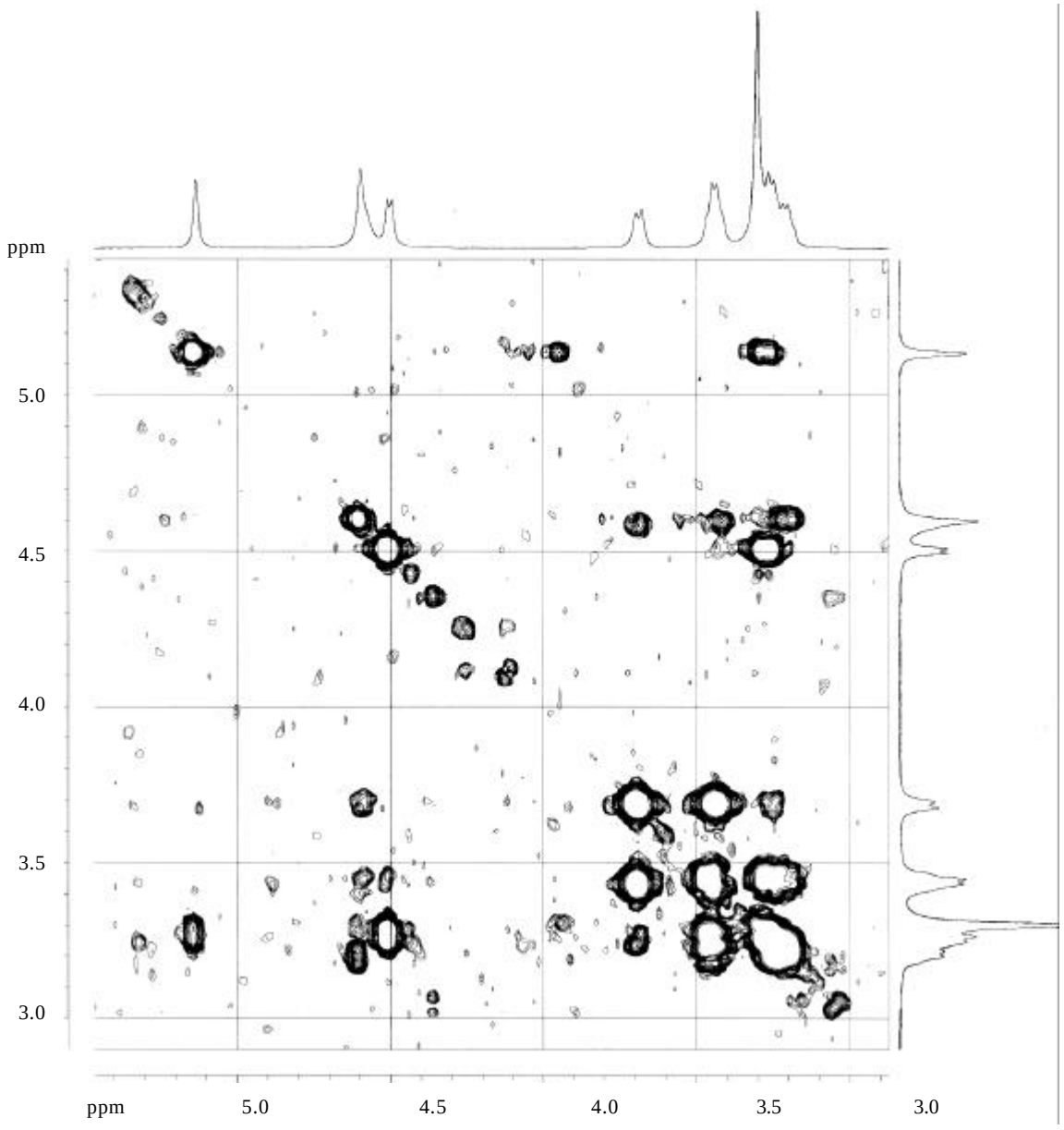


Figure 15: COSY-NMR spectrum of the product of the CHAPS-solubilized glucan synthase.

4.8. PEG precipitation of glucan synthase activity

There is a variety of neutral water-soluble polymers with high-molecular-weight that can cause aggregation of proteins without their denaturation. Among them, polyethylene glycol (PEG) is the most used because of the relative low viscosity of its solutions.

In order to evaluate PEG precipitation as a method for concentrating or partially purifying glucan synthase activity, precipitation experiments were performed as described in section 3.6.4. The results are summarized in Figure 16.

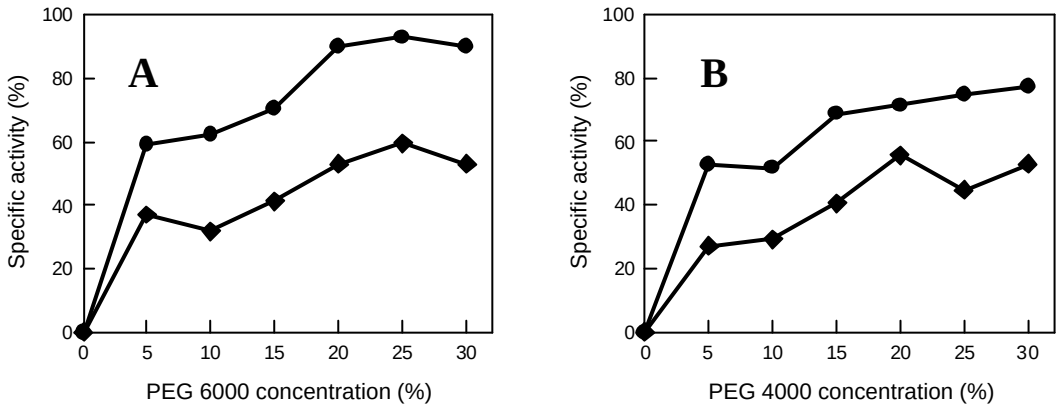


Figure 16: Precipitation of glucan synthase activity with PEG 6000 (panel A) and PEG 4000 (panel B). Specific activity (●) and recovery of total activity (◆).

None of the experiments (with either PEG 6000 or PEG 4000) resulted in an increase of specific activity. Moreover, in both cases the recovery (never higher than 50%) was too low to consider this method suitable for partial purification or concentration.

4.9. Sucrose-density gradient centrifugation

Centrifugation is an effective method for separation of macromolecules. In density gradient centrifugation the proteins pass through the gradient and are separated according to their different sedimentation coefficients.

Sucrose-gradient centrifugation with CHAPS-solubilized protein was performed as described in section 3.6.3. The results of the experiment performed in the SW40 rotor are shown in Figure 17.

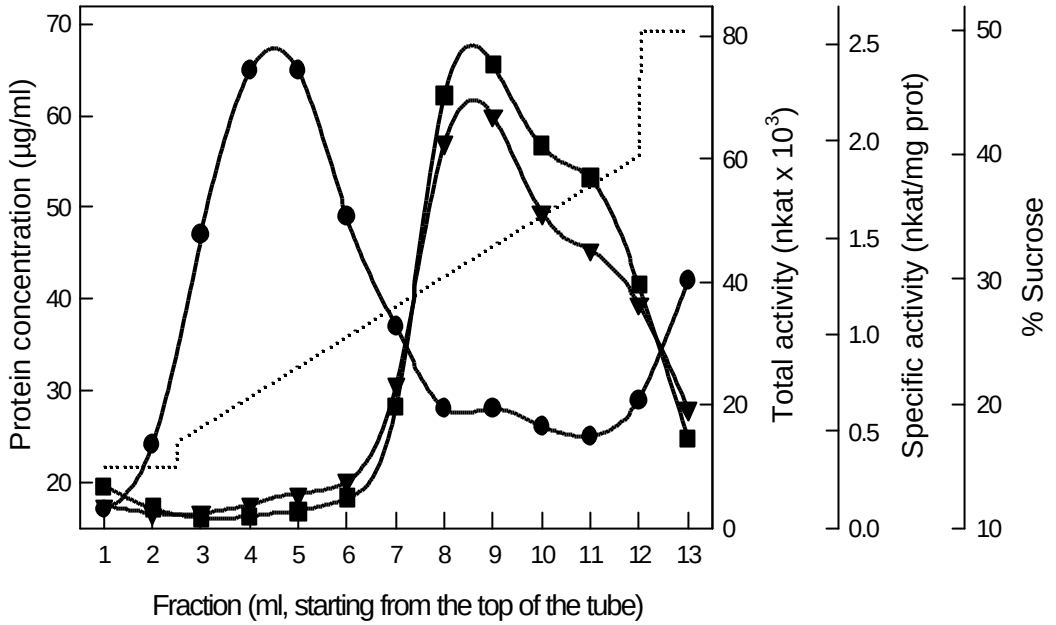


Figure 17: Sucrose-gradient centrifugation in a SW40 rotor. Protein concentration (●), specific activity (■), total activity (▼), sucrose concentration, dotted line.

Sucrose-gradient centrifugation in a SW40 rotor resulted in a good separation of the glucan synthase activity: as is shown in Figure 17, the main protein peak has its maximum at fraction 4, while the peak of glucan synthase activity has its maximum at fraction 9. Due to the small capacity of SW40 rotor tubes, further experiments were performed using a bigger SW28 rotor.

The profiles of the experiments with the SW28 rotor were basically similar, with the main peak of protein content arising in the upper third of the tube and the main peak of activity in the lower third, at a sucrose concentration slightly higher than 30%. All six tubes in both experiments showed the same profile. The specific activity of the most active fractions was increased 5-fold compared to the crude solubilized fraction, while the total recovery was 60%.

4.10. Anion exchange chromatography

Proteins bind to ion exchangers by electrostatic forces between protein surface charges (mainly) and the dense clusters of charged groups on the exchangers. Usually, elution is done by increasing the ionic strength and, thereby, weakening the electrostatic interaction between protein and adsorbent.

The analytical scale chromatography performed in a 1 ml Fractogel EMD-DEAE column (see section 3.6.1.1) resulted in a 1.7 fold increase in specific activity in the fraction eluted with 0.5 M and in a 50% recovery of total activity.

The results of the preparative scale DEAE chromatography are shown in Figure 18. At pH 7.5, the activity was retained and eluted with a NaCl gradient between 0.35 and 0.45 M, between the two main peaks of protein. A recovery of about 50% was obtained, and the increase of specific activity was about six fold. The fraction obtained from this step was one-tenth of the volume of the original crude solubilized fraction.

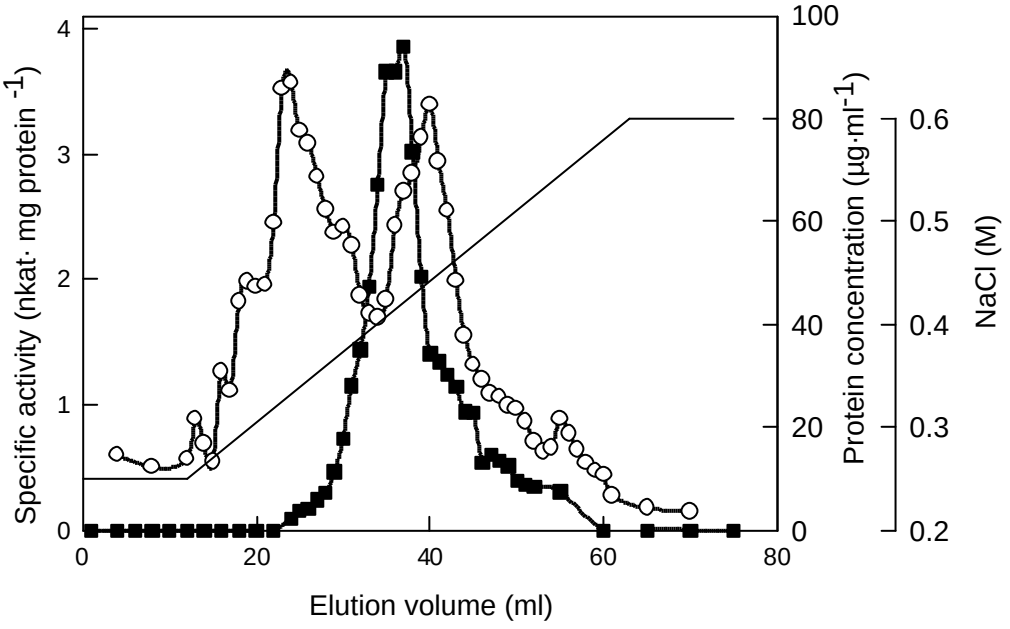


Figure 18: Anion exchange chromatography of detergent-solubilized glucan synthase, carried out on a DEAE-Superformance column (10×15 mm, Merck). Specific enzyme activity (■) and protein concentration of the fraction (○). The continuous line shows the salt gradient.

Chromatography experiments with Resource-Q and Mono-Q (sections 2.6.3 and 2.6.4) resulted in two- to three-fold increases in specific activity. The activity also eluted between 0.35 and 0.45 M NaCl. The recovery of total activity was below 50%.

4.11. Size exclusion chromatography

In a size exclusion chromatography the matrix consists of an open, cross-linked, three-dimensional molecular network. The pores within the beads are of such average sizes that are not accessible to large molecules. In contrast, smaller molecules can penetrate all pores and are therefore retained longer.

The experiments conducted with Superose 12 and Superose 6 columns are described in section 3.6.2 and the standard curves for the molecular markers are shown in Figure 5.

In the experiments performed with the Superose 12 column, the peak of glucan synthase activity was distributed in fractions eluting at 7.6 and 8.0 ml. This corresponded to an apparent molecular mass of about 1000 kDa. The peak of activity in the case of experiments with Superose 6 column, was distributed in the fractions between 7.6 to 8.4 ml elution volume, corresponding to an apparent molecular mass of about 2000 kDa. In both cases activity eluted out of calibration range and the molecular masses are extrapolations from the calibration curve presented in Figure 5. In both cases, the peak of activity overlapped with the only peak of absorbance at 280 nm.

4.12. HiTrap Blue chromatography

Some enzymes, namely nucleotide-binding enzymes, have the ability to bind some dyes in a semi-specific way. To explore this possibility, chromatography on a HiTrap Blue column was performed.

After loading the column, a first washing step was followed by three steps of elution with 0.25, 0.5 and 1.0 M NaCl, respectively (see 3.6.5). The peak of activity appeared in the washing fraction with a specific activity that was 1.5 times higher than that of the crude solubilized fraction loaded onto the column. The total recovery was 66%.

4.13. HiTrap heparin chromatography

Heparin is a sulfated polysaccharide, highly negatively charged, and it is used as pseudo-affinity ligand and ion exchanger.

After heparin chromatography (section 3.6.6), the activity peak eluted in the washing fraction (without NaCl) with a loss of specific activity of more than 50%. A small amount of activity was distributed in the fractions eluting from 0.15 to 0.5 M NaCl, having lower specific activity.

4.14. Concanavalin A chromatography

Concanavalin A, a lectin, is a mannose- and glucose-binding protein. Many glycoproteins have exposed mannose residues, which interact strongly with Concanavalin A, and elution is achieved by displacement with a buffer containing methyl α-D-mannopyranoside.

Chromatography was performed with solubilized fraction on Concanavalin A-Sepharose 4B (section 3.6.7). Enzyme activity was present only in the first two fractions (from washing the column) and not in the fraction with 0.5 M methyl α-D-mannopyranoside. There was no increase of specific activity and total recovery of activity was 66%.

4.15. Product entrapment

Product entrapment relies on the fact, that some enzymes have affinity to their own products, and when these are insoluble, enzymes can be recovered by centrifugation.

Product entrapment reactions were carried out at either preparative or analytical scale (as described in section 3.6.8). In a typical analytical experiment, up to 10 ml of crude solubilized fraction or DEAE purified fraction were used. Glucan synthase activity could not be released from the glucan pellet after product entrapment even after removing the sucrose from the washing buffer, adopting a high-salt regime, or using chaotropic agents such as lithium acetate. For this reason, proteins bound to the glucan pellet were extracted by SDS treatment and heating to 100°C. The proteins so obtained could be analyzed by SDS-PAGE as shown in Figure 19.

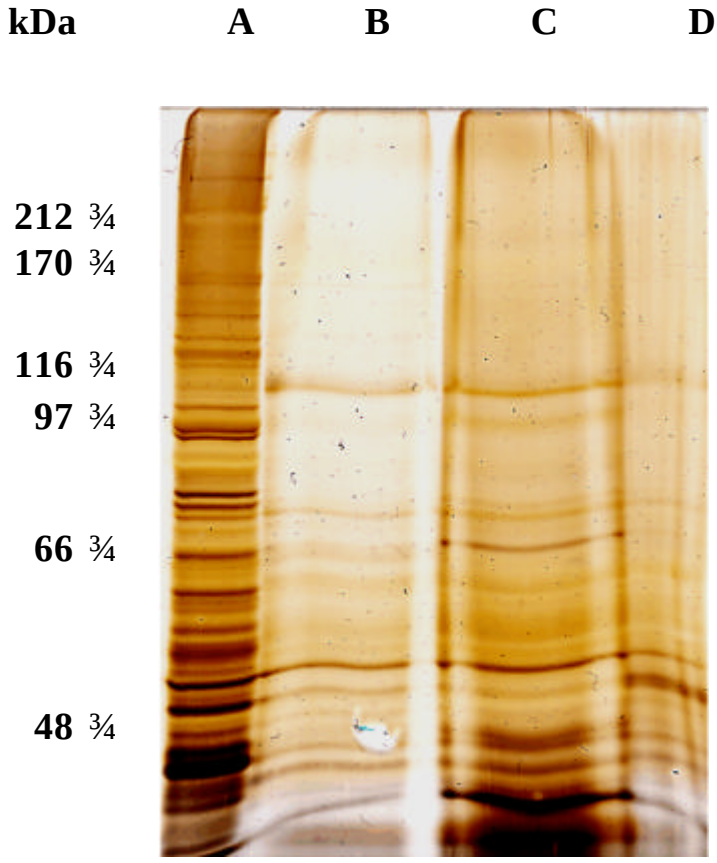


Figure 19: Silver staining of proteins after SDS polyacrylamide gel electrophoresis of crude detergent-solubilized proteins from *P. sojae* membranes and fractions after affinity purification using product entrapment. Lane A contains CHAPS-solubilized proteins used for product entrapment, lane B contains proteins extracted from the pellet after product entrapment with crude solubilizate, lane C contains proteins extracted from the product entrapment pellet that was obtained with an enzyme fraction partially purified by anion exchange chromatography, lane D contains the pellet remaining after denaturation and extraction with SDS buffer that was directly applied to the gel.

Glucan synthase activity could be measured *in situ* after product entrapment by resuspending the pellet in buffer C, adding UDP-glucose and using the standard glucan synthase assay. The glucan synthase activity recovered in the glucan pellet was only 5% of the total activity used for the product entrapment reaction.

Protein estimation in the glucan pellet was not possible because the glucan binds the dye used in the Bradford assay. Therefore, an approximate estimation of the relative amount of

protein could only be made by evaluation of the SDS-gel (Figure 19). Thus, the purification factor attributable to product entrapment could not be determined.

Two main protein bands were observed to copurify with the glucan synthase activity by product entrapment (Figure 19, lane B). One of them had an apparent molecular mass of 108 kDa, while the other was more abundant and had an apparent molecular mass of 50 kDa. Semiquantitative densitometric analysis suggested an enrichment of the 108 kDa band by at least two orders of magnitude.

Preparative product entrapment reactions (100 ml) were done in order to enrich the 50 and 108 kDa proteins. The same procedure as for the analytical scale was followed and the final sample was loaded onto a gel of 1.5 mm thickness, to run SDS-PAGE. The Coomassie-stained gel is shown in Figure 20.

The protein bands at 108 kDa and 50 kDa were excised from the gel. Protein sequencing of the two polypeptides was performed as described in section 3.8. Bands of three of such gels were provided for sequencing.

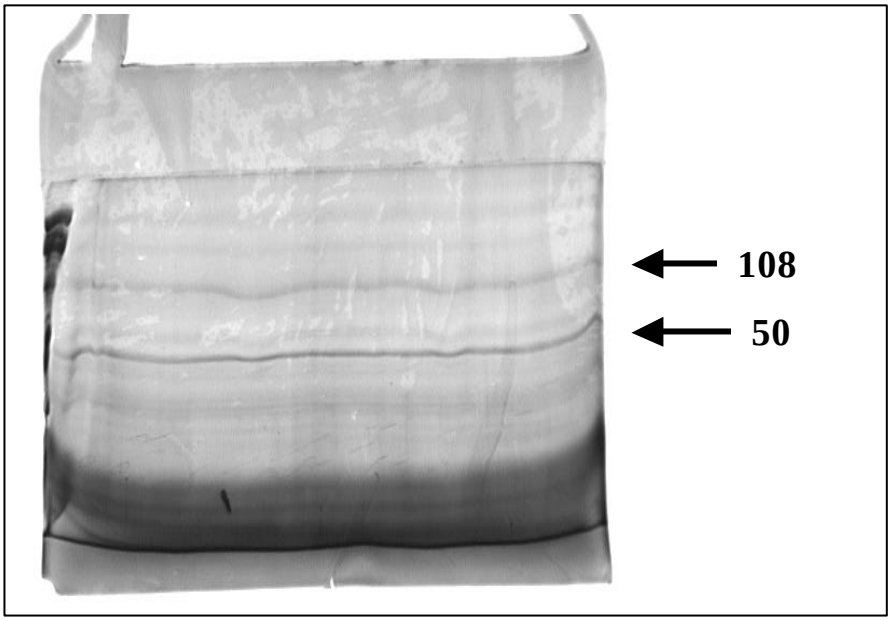


Figure 20: Analysis of protein sample by SDS-page after preparative product entrapment. The arrows indicate the bands cut for protein sequencing, with their apparent molecular masses (kDa).

4.16. Native gel electrophoresis and in-gel assay

Proteins and protein complexes can be separated as an active form by native gel electrophoresis. Enzyme activities can then be detected in-gel.

Gels were prepared and developed as described in section 3.6.9. One sample to be run was CHAPS-solubilized protein incubated for 30 min at 100°C. The other sample was not denatured. After incubation with dye for visualization of the glucan polymers, the gel was exposed to UV light and photographed (Figure 21).

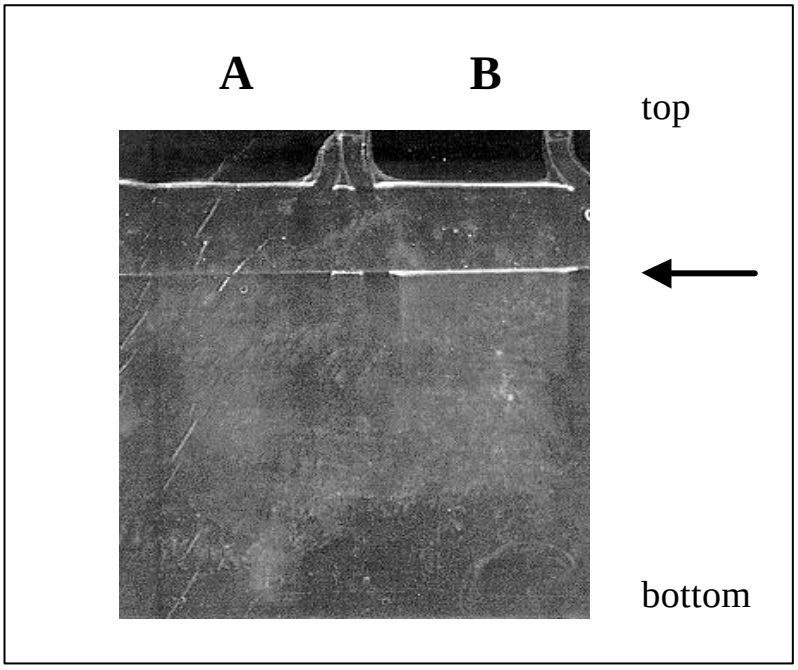


Figure 21: Native gel electrophoresis and in gel-assay. After electrophoresis, native gel was incubated with substrate (UDP-glucose) and the glucans formed visualized with tinopal as described in section 3.6.9. Lane A: CHAPS-solubilized material incubated for 30 min at 100°C; lane B: CHAPS-solubilized material without denaturation.

In Figure 21, some fluorescence can be observed at the starting zone of the gel in both lanes A and B. At the interface between the 3% and the 6% acrylamide gels (arrow), fluorescence was only observed in lane B from the sample with non-denatured enzymes. Since denatured proteins can not synthesize glucans, the fluorescence of the starting zone of the gel corresponded to unspecific interaction with tinopal, while the fluorescence band indicated by the arrow corresponded to newly formed glucans.

4.17. Peptides from protein sequencing

The proteins purified by product entrapment with an apparent molecular mass of 108 kDa (from now on called P108) and 50 kDa (called P50) were microsequenced (section 3.8). The resulting peptide sequences of P108 and P50 are shown in table 10 and table 11, respectively. Amino acids whose identity could not be totally ascertained are indicated in brackets, partly along with subscripts indicating other possible residues at that position. Degenerated oligonucleotides were designed for some of the peptides (section 2.3.2) using the coding usage for *P. sojae* as shown in the web page www.ncgr.org/pgi/psojae.html. The design of these degenerate oligonucleotides is shown in Figure 22.

Table 10: Oligopeptide sequences from P108

Peptide	name
(K)(T_{AN})YTND(V_{YG})(G_{ES})(Q_{NT})(I_P)(Q_Y)(G_{FV})(A_{GK})QV(X_P)L	tnd
M(L_T)(M_E)(A_W)(L_Q)(E_G)(Y_L)TLYLITP	lit
(K)AAVIGDTDGD(H)	vig
(K)VAASLERT(E)(A)(L)(K)	vaas
(K)VLETRRAIPLDVNEGV	rai
(K)IVRDAFDQA(K)	daf
(K)XVEEQATAV	veeq
(V_F)R(V)ERAVK	erav
(Q)EL(AS)TQ(QA)(LG)E(NS)QS(S)(A)(E)(G)	elast

Table 11: Oligopeptide sequences from P50

Peptide	name
(K)YYENHNPLEYGDFVAMD	yen
(K)VIQARVQGDAPV	iqar
(K)ARNTG(V)GIEVEQ	eve
(K)(S)(S)ATSNPGAISGVVQGSSSNNNNNNS	sat

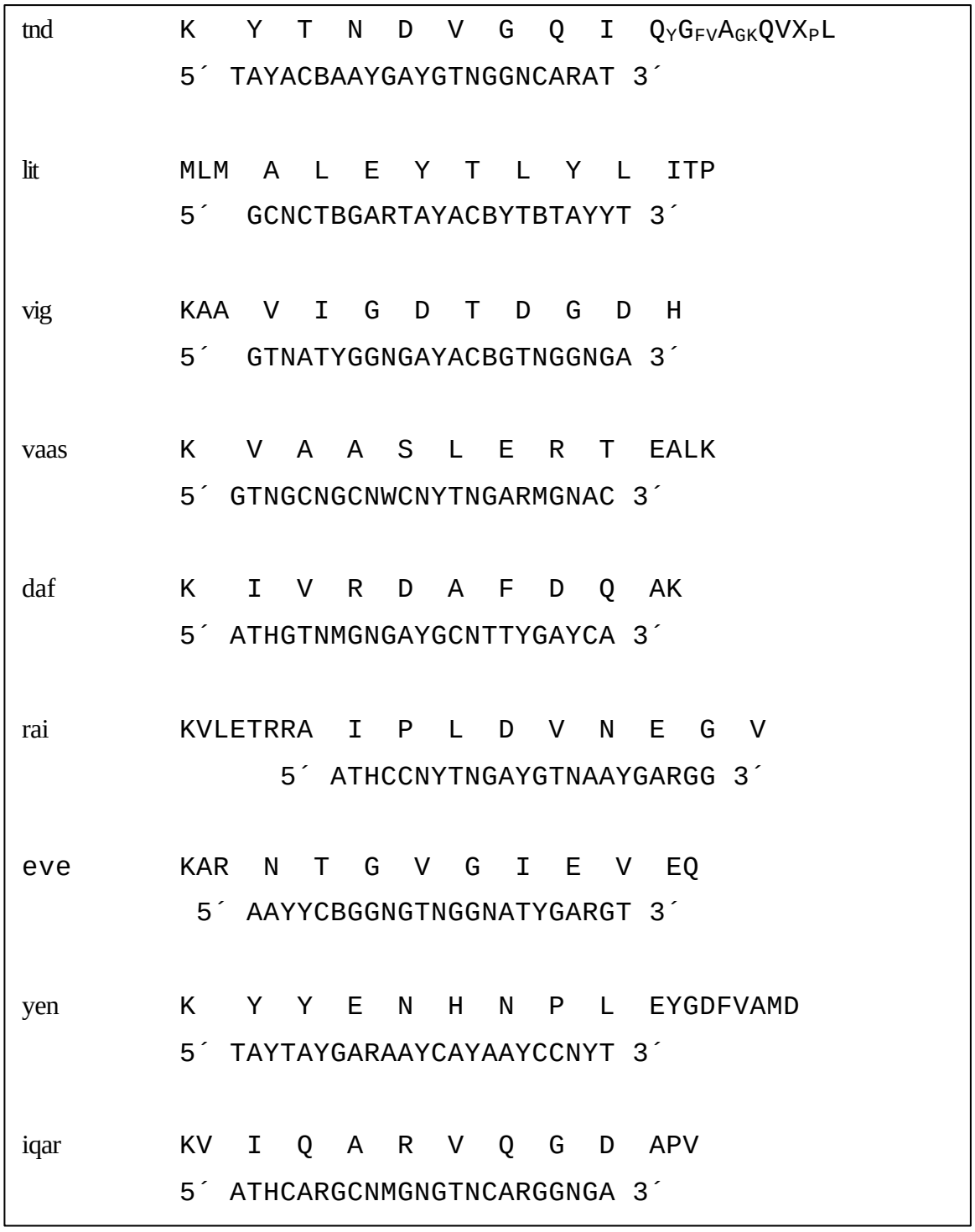


Figure 22: Design of oligonucleotide primers for the peptides as determined by microsequencing. The oligonucleotide sequences shown are in sense orientation. Sense and antisense oligonucleotides are listed in Table 5, section 2.3.2.

4.18. Cloning of P. sojae actin cDNA

In order to determine the quality of the cDNA library, the PCR and the cloning techniques, the actin gene (Dudler, 1990) was cloned. PCR was performed as described in section 3.9.7.2, using the cDNA library as template (section 3.10.3) and the oligonucleotides "56up_ac_pmg" and "891_acainti_pmg" (section 2.3.2, Table 4). When the PCR product was analyzed by electrophoresis, a single band slightly below 1000 bp was detected.

Preparative PCR was performed, the PCR product was purified (see section 3.9.5) and an aliquot was run in a 1.4% agarose gel in order to check amount and quality (Figure 23).

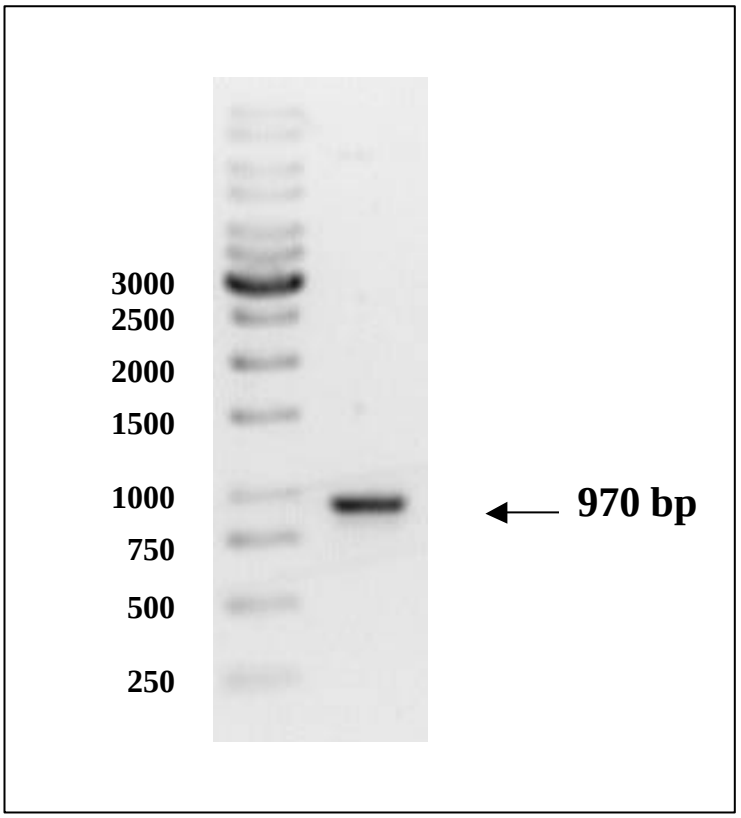


Figure 23: DNA aliquot of the PCR reaction with actin primers. After preparative PCR the sample was eluted from the gel and an aliquot run in an agarose gel. Left lane molecular weight markers (bp). The arrow indicates the expected band and the size of the actin-PCR fragment**.**

The size of the PCR product (970 bp) correlates exactly with the expected size for actin cDNA fragment between the primers used for PCR. Purified PCR product was labeled with ^{32}P (see section 3.9.9) and used for hybridization of a Northern membrane with total RNA from *P. sojae* (see sections 3.9.8 and 3.9.10). The result of this hybridization is shown in

Figure 24. A single hybridization band was detected with a size of 1.3 kb, which correlates well with the expected size of a full length mRNA of the actin gene.

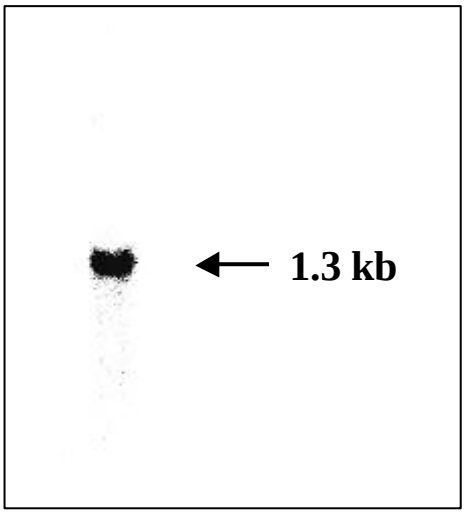


Figure 24: Northern analysis using as probe the PCR-amplified fragment of actin cDNA. The arrow indicates the apparent size of the detected band.

4.19. Cloning of the cDNA for P108

Several attempts were made to clone the cDNA of P108 that copurified with the glucan synthase activity during product entrapment. PCR reactions were carried out with combinations of all six degenerated oligonucleotides derived from the sequenced peptides (section 2.3.2). All of the six oligonucleotides in the sense orientation were combined with all others in the antisense orientation in 15 µl individual reactions using several ranges of annealing temperatures (primers are listed in Table 5). The following primer combinations resulted in the amplification of DNA fragments of the *P. sojae* cDNA library (the fragment size in brackets): "tndsense" + "litanti" (720 bp), "tndsense" + "viganti" (350 bp), "vigsense" + "litanti" (525 bp), "vaassense" + "dafanti" (600 bp), "raisense" + "litanti" (400 bp).

All PCR-amplified fragments were purified from preparative reactions, ^{32}P-labeled for Northern analysis and cloned into the vector pGEM for sequencing.

None of the Northern blot experiments showed a band with a size of about 3000 bp as expected for the cDNA of an 108 kDa protein. Sequences of PCR-amplified fragments lacked the expected known amino acids flanking the regions used for primer design (see Figure 22). Thus none of the PCR-amplified DNA fragments generated were related to the P108.

4.20. Cloning of the cDNA for P50

A 288 bp fragment was generated by PCR (see 3.9.7.3) using the primers "iqarsense" and "yenanti" (Table 5). In the following this fragment is called "iqaryen". The PCR reaction was analyzed in a 2% agarose gel and compared with control reactions lacking one of the primers or the template, as shown in Figure 25. No PCR fragment was generated when the primers "yensense" and "iqaranti" were used (data not shown).

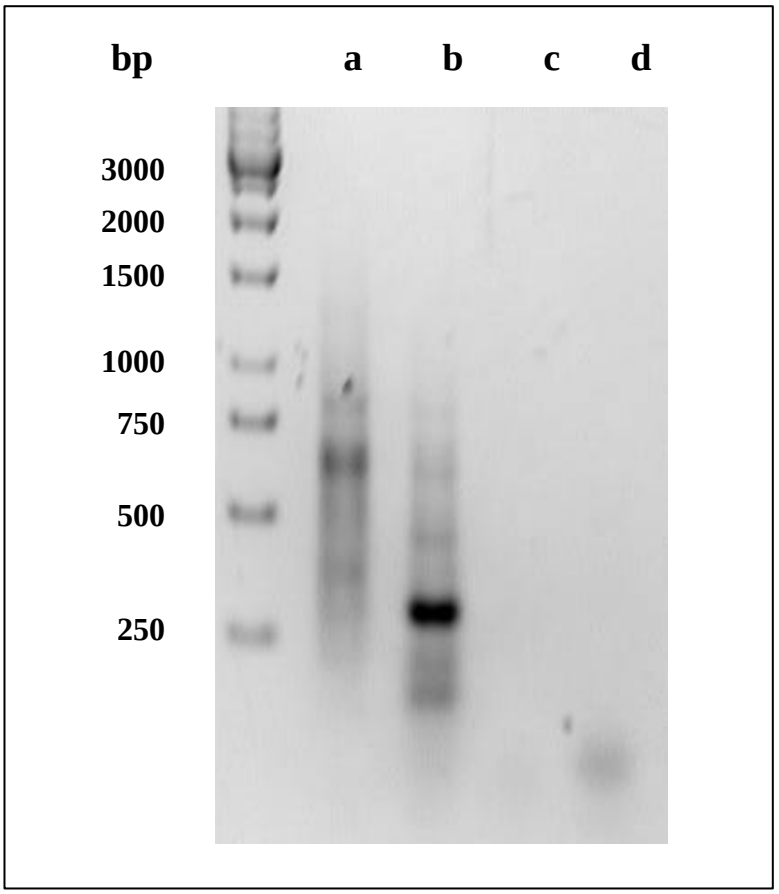


Figure 25: Analysis of PCR reactions with oligonucleotides derived from P50. Lane a: reaction lacking the oligonucleotide yenanti; lane b: full reaction with 0.3 µl cDNA as template, and both iquarsense and yenanti oligonucleotides, lane c: reaction lacking iqarsense oligonucleotide; lane d: reaction lacking the cDNA.

The fragment "iqaryen", shown in Figure 25, lane b, was purified, inserted into the vector pGEM (see section 3.9.15) and used for transformation of *E. coli* JM109 cells. Transformants were checked for incorporation of the plasmid by colony PCR using again the oligonucleotides "iqarsense" and "yenanti" as primers. From one of the colonies containing the expected PCR product (288 bp) the pGEM-iqaryen plasmid was isolated and used for

sequencing the insert. The sequence of the PCR product is shown in Figure 26. Amino acids initially found by microsequencing of "iqar" and "yen" peptides were present in the deduced amino acid sequence of "iqaryen", including some not used for primer construction.

```
  1 ATTCAAGCACGTGTACAGGGAGACGCCCCCGTGTGGCACCCGGAGGTGGGCCAGTGGCTG
     I  Q  A  R  V  Q  G  D  A  P  V  W  H  P  E  V  G  Q  W  L

 61 TCCAAGTACGGCTCCAACACGGAGCAGCAGTACATGAACAACCTGGACACCGTCAACACG
     S  K  Y  G  S  N  T  E  Q  Q  Y  M  N  N  L  D  T  V  N  T

121 GCGTCCGTGGAGGGCGCGCTCATGTACGTGCAGGCCGAGGGCATCAACGTGAACGAGCAG
     A  S  V  E  G  A  L  M  Y  V  Q  A  E  G  I  N  V  N  E  Q

181 TCGGTCAAGTGCCACCGCAAGAACGACATGCAGTACGTCGTGTTTTACGAGATGACCATC
     S  V  K  C  H  R  K  N  D  M  Q  Y  V  V  F  Y  E  M  T  I

241 GTGCAGCCCACGGCTTCGGTCAAGTACTATGAAAACCACAACCCTCTA
     V  Q  P  T  A  S  V  K  Y  Y  E  N  H  N  P  L
```

Figure 26: Deduced amino acid sequence of the "iqaryen" PCR product. Underlined are the amino acids used for the primer generation. The amino acids in boxes were not used for the primer generation but were present in the sequenced peptides.

The "iqaryen" fragment was excised by digesting pGEM-iqaryen plasmid with the restriction enzymes *Sph* I and *Spe* I. The insert was separated by gel electrophoresis, and then labeled with ^{32}P-dCTP. This radioactive probe was used for hybridizing a Northern blot with total RNA of *P. sojae* as shown in Figure 27.

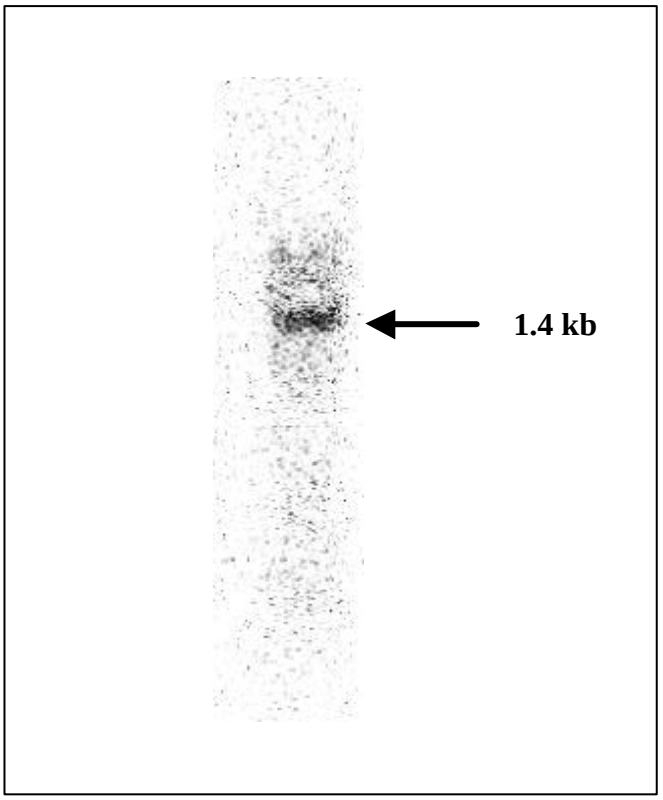


Figure 27: Northern hybridization using the ^{32}P-labeled "iqaryen" fragment as radioactive probe. Twenty micrograms total RNA of *P. sojae* mycelium were used. The arrow indicates the apparent molecular size of the detected band.

4.20.1. Screening of the P. sojae cDNA library with the "iqaryen" PCR fragment

Of the packed λ cDNA library (section 3.10), 6600 pfu (11 µl) were cultivated on agar medium as described in section 3.18.5, transferred to nitrocellulose membranes and hybridized with the ^{32}P-labelled "iqaryen" fragment (see above).

One of the membranes showed a hybridization signal of a plaque (Figure 28), which was localized and isolated. *E. coli* Y1090 cells were infected in dilutions of 1:1, 1:10, 1:100 and 1:1000. The plate infected with the 1:10 dilution resulted in around 50 plaque per plate, which were transferred to a nitrocellulose membrane. The membrane was hybridized with ^{32}P-labelled "iqaryen" fragment for a second screening.

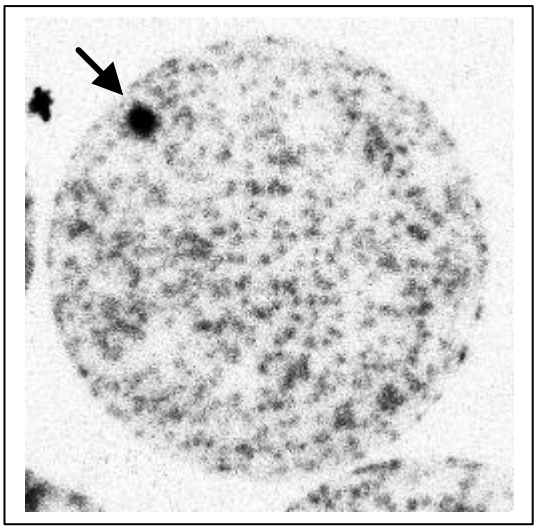
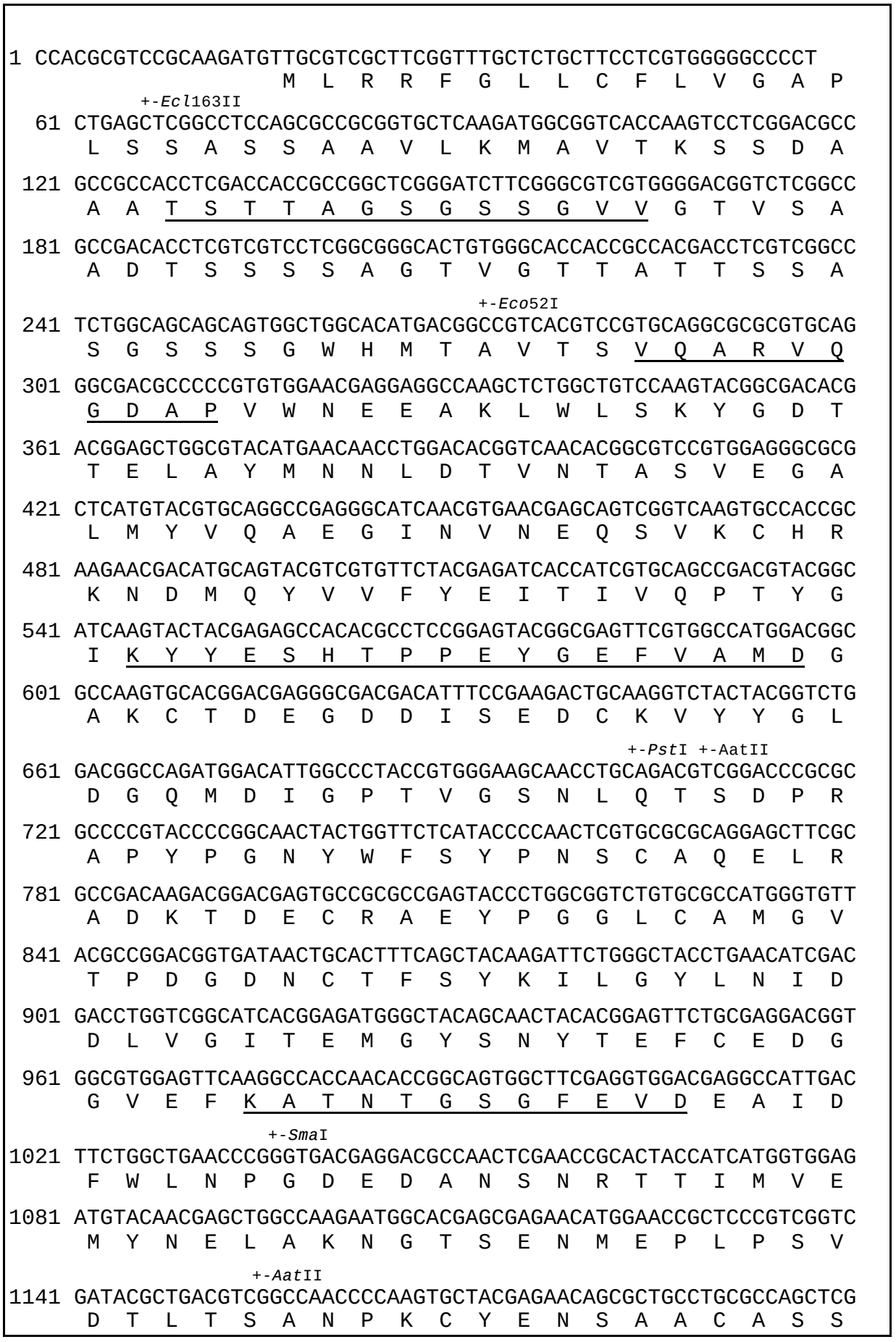


Figure 28: Screening of *P. sojae* cDNA library on nitrocellulose membranes. Membranes were hybridized with "iqaryen" PCR generated fragment. The arrow indicates the positive hybridization signal.

After the second screening, around half of the plaques gave positive hybridization signals. Four positives were picked and *in vivo* excision of the pZL-P50 plasmid was performed as described in section 3.18.7. A plasmid was isolated from one of the colonies, tested to be positive by PCR with the oligonucleotides "iqarsense" and "yenanti". This plasmid will be called from now on pZL-Ps-P50 (Ps stands for *P. sojae*). *E. coli* DH5α cells were transformed by electroporation (this *E. coli* strain gives plasmids in a supercoiled state more suitable for sequencing).

The pZL-Ps-P50 plasmid was re-isolated from DH5α cells, and a preliminary set of sequencing was done using as primers the T7 and SP6 promoter sequences (present at opposite ends of the multiple cloning site of pZL1). In order to obtain overlapping partial sequences, a variety of deletion clones were generated using the restriction enzymes *Pst* I, *Sma* I, *Ecl*136 II + *Sal* I, *Bgl* II +*Sal* I, *Eco*52 I, and *Aat* II (see section 3.9.18). These plasmids were sequenced, as well, from T7 and SP6. Based on these sequences, specific oligonucleotides were designed (section 2.3.2, table 2) for the total sequencing of both directions of the Ps-P50 clone. The sequence of Ps-P50 cDNA and its deduced amino acid sequence is presented in Figure 29.


1 CCACGCGTCCGCAAGATGTTGCGTCGCTTCGGTTTGCTCTGCTTCCTCGTGGGGGCCCCT
M L R R F G L L C F L V G A P
+-Ecl163II
61 CTGAGCTCGGCCTCCAGCGCCGCGGTGCTCAAGATGGCGGTCACCAAGTCCTCGGACGCC
L S S A S S A A V L K M A V T K S S D A
121 GCCGCCACCTCGACCACCGCCGGCTCGGGATCTTCGGGCGTCGTGGGGACGGTCTCGGCC
A A T S T T A G S G S S G V V G T V S A
181 GCCGACACCTCGTCGTCCTCGGCGGGCACTGTGGGCACCACCGCCACGACCTCGTCGGCC
A D T S S S S A G T V G T T A T T S S A
+-Eco52I
241 TCTGGCAGCAGCAGTGGCTGGCACATGACGGCCGTCACGTCCGTGCAGGCGCGCGTGCAG
S G S S S G W H M T A V T S V Q A R V Q
301 GGCGACGCCCCCGTGTGGAACGAGGAGGCCAAGCTCTGGCTGTCCAAGTACGGCGACACG
G D A P V W N E E A K L W L S K Y G D T
361 ACGGAGCTGGCGTACATGAACAACCTGGACACGGTCAACACGGCGTCCGTGGAGGGCGCG
T E L A Y M N N L D T V N T A S V E G A
421 CTCATGTACGTGCAGGCCGAGGGCATCAACGTGAACGAGCAGTCGGTCAAGTGCCACCGC
L M Y V Q A E G I N V N E Q S V K C H R
481 AAGAACGACATGCAGTACGTCGTGTTCTACGAGATCACCATCGTGCAGCCGACGTACGGC
K N D M Q Y V V F Y E I T I V Q P T Y G
541 ATCAAGTACTACGAGAGCCACACGCCTCCGGAGTACGGCGAGTTCGTGGCCATGGACGGC
I K Y Y E S H T P P E Y G E F V A M D G
601 GCCAAGTGCACGGACGAGGGCGACGACATTTCCGAAGACTGCAAGGTCTACTACGGTCTG
A K C T D E G D D I S E D C K V Y Y G L
+-PstI +-AatII
661 GACGGCCAGATGGACATTGGCCCTACCGTGGGAAGCAACCTGCAGACGTCGGACCCGCGC
D G Q M D I G P T V G S N L Q T S D P R
721 GCCCCGTACCCCGGCAACTACTGGTTCTCATACCCCAACTCGTGCGCGCAGGAGCTTCGC
A P Y P G N Y W F S Y P N S C A Q E L R
781 GCCGACAAGACGGACGAGTGCCGCGCCGAGTACCCTGGCGGTCTGTGCGCCATGGGTGTT
A D K T D E C R A E Y P G G L C A M G V
841 ACGCCGGACGGTGATAACTGCACTTTCAGCTACAAGATTCTGGGCTACCTGAACATCGAC
T P D G D N C T F S Y K I L G Y L N I D
901 GACCTGGTCGGCATCACGGAGATGGGCTACAGCAACTACACGGAGTTCTGCGAGGACGGT
D L V G I T E M G Y S N Y T E F C E D G
961 GGCGTGGAGTTCAAGGCCACCAACACCGGCAGTGGCTTCGAGGTGGACGAGGCCATTGAC
G V E F K A T N T G S G F E V D E A I D
+-SmaI
1021 TTCTGGCTGAACCCGGGTGACGAGGACGCCAACTCGAACCGCACTACCATCATGGTGGAG
F W L N P G D E D A N S N R T T I M V E
1081 ATGTACAACGAGCTGGCCAAGAATGGCACGAGCGAGAACATGGAACCGCTCCCGTCGGTC
M Y N E L A K N G T S E N M E P L P S V
+-AatII
1141 GATACGCTGACGTCGGCCAACCCCAAGTGCTACGAGAACAGCGCTGCCTGCGCCAGCTCG
D T L T S A N P K C Y E N S A A C A S S

```
                                                  +-BglII
1201 CAGTACGGCTGCAACCGCACGCTGTACTCGCAGATCTGCGCCGTGTGCTCGTCGGATGCC
      Q  Y  G  C  N  R  T  L  Y  S  Q  I  C  A  V  C  S  S  D  A
1261 GACGGTTGCGAGGCCGCTCCCTCGTCCTTCTCGTTCCCGGAGCTGACGCTGCCTTCTAAC
      D  G  C  E  A  A  P  S  S  F  S  F  P  E  L  T  L  P  S  N

1321 TCTTCGTCGGACGGCTCGTCGGACTCGACCAAGACCGGCTCCTCGGGCAGCCCGATGACT
      S  S  S  D  G  S  S  D  S  T  K  T  G  S  S  G  S  P  M  T

                     +-AatII
1381 GCGACGATGACGTCTGCCGCTGTGGCCCTCGTGGCCATGGTCGCCAGCAGCCTGCTGTAA
      A  T  M  T  S  A  A  V  A  L  V  A  M  V  A  S  S  L  L  -

1441 GCGTGTGTATTTGTACATGACATTTGATGTTCATGGTACTGCAAACGCCGCCGCTTCGGA

1501 GTGGGGAACGGAGCACCCTCGGACTGCTCGGGCCTAGCCACGTTGGCAGCGATTCGTAGG

1561 GCTTGCTACTGTGAATTCAAAGTAAACTCAAGTTCCGGTATCGAAAAAAAAAAAAAAAAA

1621 AAAAA
```

Figure 29: DNA sequence of the Ps-P50 cDNA and its deduced amino acid sequence. The underlined sections represent the amino acid sequences with high similarity to the sequenced peptides (Table 11).

The sequence shown in Figure 29 contains four partial amino acid sequences with similarity to the peptides sequenced from the protein P50 (see Table 11, underlined in the sequence shown in Figure 29). Identities and similarities of sequenced peptides to the deduced amino acid sequence of P50 are summarized in Table 12 and the sequence alignment is shown in section 8.3.

The deduced amino acid sequence between the second and the third underlined regions corresponds to the PCR fragment “iqaryen”. The similarity of these sequences are shown in Figure 30 and Figure 31.

Table 12: Percentage of similarities of sequenced peptides relative to the deduced amino acid sequence of Ps-P50. Sequence alignments are shown in section 8.3

Sequence	Identities (%)	Positives (%)
iqar	90	99
yen	77	88
eve	61	76
sat	50	64

```
Ps-P50:289 caggcgcgcgtgcagggcgacgcccccgtgtggaacgaggaggccaagctctggctgtcc 348
           || || || || ||||| ||||||||||||||| ||  |||||     |  |||||||||
PCR  : 4   caagcacgtgtacagggagacgcccccgtgtggcacccggaggtgggccagtggctgtcc 63

Ps-P50:349 aagtacggcgacacgacggagctggcgtacatgaacaacctggacacggtcaacacggcg 408
           |||||||||  ||  ||||||| |  |||||||||||||||||||| ||||||||||||
PCR  : 64  aagtacggctccaacacggagcagcagtacatgaacaacctggacaccgtcaacacggcg 123

Ps-P50:409 tccgtggagggcgcgctcatgtacgtgcaggccgagggcatcaacgtgaacgagcagtcg 468
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR  : 124 tccgtggagggcgcgctcatgtacgtgcaggccgagggcatcaacgtgaacgagcagtcg 183

Ps-P50:469 gtcaagtgccaccgcaagaacgacatgcagtacgtcgtgttctacgagatcaccatcgtg 528
           |||||||||||||||||||||||||||||||||||||| |||||||| |||||||||
PCR  : 184 gtcaagtgccaccgcaagaacgacatgcagtacgtcgtgtttacgagatgaccatcgtg 243

Ps-P50:529 cagccgacgtacggcatcaagtactacgagagccacacgcctc 571
           ||||| |||       |||||||||| || | |||||  ||||
PCR  : 244 cagcccacggcttcggtcaagtactatgaaaaccacaaccctc 286
```

Figure 30: Alignment of the nucleotide sequences of Ps-P50 and the PCR fragment "iqaryen". Sequences presented have 86.9% identity.

Score = 250 bits (581), Expect = 3e-65

Identities = 79/95 (83%), Positives = 84/95 (88%)

```
Ps-P50:95  VQARVQGDAPVWNEEAKLWLSKYGDTTELAYMNNLDTVNTASVEGALMYVQAEGINVNEQ 154
           +QARVQGDAPVW+ E   WLSKYG  TE  YMNNLDTVNTASVEGALMYVQAEGINVNEQ
PCR  : 1   IQARVQGDAPVWHPEVGQWLSKYGSNTEQQYMNNLDTVNTASVEGALMYVQAEGINVNEQ 60

Ps-P50:155 SVKCHRKNDMQYVVFYEITIVQPTYGIKYYESHTP 189
           SVKCHRKNDMQYVVFYE+TIVQPT  +KYYE+H P
PCR  : 61  SVKCHRKNDMQYVVFYEMTIVQPTASVKYYENHNP 95
```

Figure 31: Alignment of the deduced amino acid sequences from Ps-P50 and homology between Ps-P50 clone and the PCR fragment "iqaryen".

4.20.2. Comparison of derived amino acid sequence of Ps-P50 within the sequence data bases

When the databases were searched for similarities to the deduced amino acid sequence of Ps-P50 only one known protein showed some similarity to some extent. The result of such a homology search is shown in Figure 32.

```
>sp|P78591|FET3_CANAL IRON TRANSPORT MULTICOPPER OXIDASE PRECURSOR
 emb|CAA70509.1| (Y09329) multicopper oxidase [Candida albicans]
          Length = 624

 Score = 37.1 bits (84), Expect = 0.38
 Identities = 41/170 (24%), Positives = 64/170 (37%), Gaps = 22/170 (12%)

Query: 116 KYGDTTELAYMNNLDTVNTASVEGALMYVQAEGINVNEQSVKCHRKNDMQYVVFYEITIV 175
           K GD  +L  +N  D +NT      L    A  ++  E   +C       Y+  + +T
Sbjct: 60  KKGDRVQLYLINGFDNLNTTLHFHGLFVRGANQMDGPEMVTQCPIPPGETYLYNFTVTDQ 119

Query: 176 QPTYGIKYYESHTPPEYGEFVAMDGAKCTDEGD-----------DISEDCKVYYGLDGQM 224
             TY   +Y SHT  +YG+   M G    ++ D            +S+    Y G
Sbjct: 120 VGTY---WYHSHTGGQYGD--GMRGVFIIEDDDFPYHYDEEVVLTLSDHYHKYSG----- 169

Query: 225 DIGPTVGSNLQTSDPRAPYPGNYWFSYPNSCAQELRADKTDECRAEYPGG 274
           DIGP   +    P P N+ F+   +   ++   KT   R    GG
Sbjct: 170 DIGPAFLTRFNPTGAE-PIPQNFLFNETRNATWKVEPGKTYFVRILNVGG 218
```

Figure 32: Homology search by Blast. Query is the Ps-P50. Sbjct is a multicopper oxidase of *Candida albicans***.**

4.20.3. Comparison among P. sojae ESTs.

A database of expressed sequence tags (ESTs) generated from *P. sojae*, available at www.ncgr.org/pgi/, was searched for homologies to the Ps-P50 nucleotide sequence. The search facility, provided in the web page, allows only searches that give 100% similarity (identity). To make the comparison search, random 20-base long sequences from Ps-P50 were introduced to the program.

Eight ESTs were found to have some similarity to Ps-P50. The distribution of the homologous ESTs is represented in Figure 33. The degrees of similarity of Ps-P50 to the ESTs are summarized in Table 13. In the first view it could not get clear that certain ESTs with overlapping similarities to Ps-P50 do not show significant similarities among each other. This is due to the fact that similarity rates given in Table 13 were calculated by the BLAST program over the entire sequence, and not only in the areas with similarity to Ps-P50.

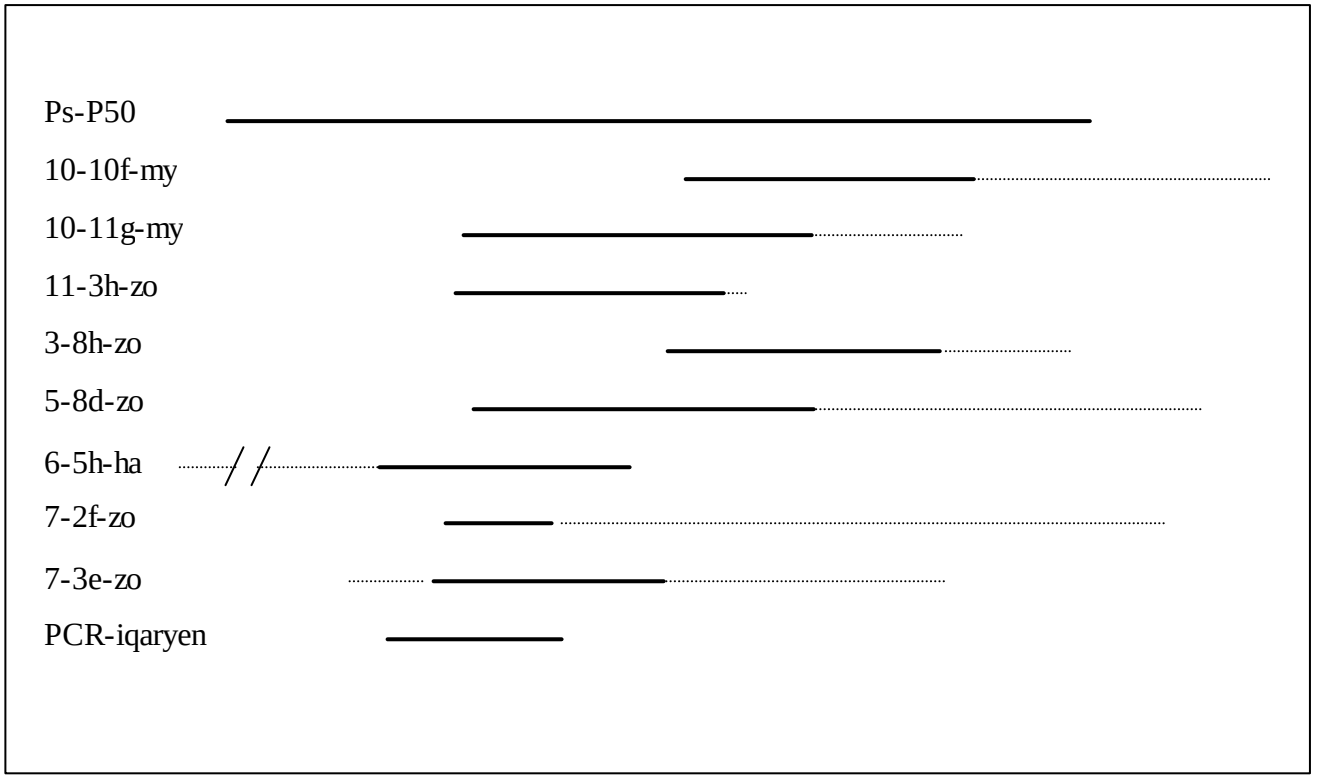


Figure 33: Diagrammatic representation of alignments between Ps-P50, the PCR fragment "iqaryen" and *P. sojae* ESTs. Solid lines indicate the area where the similarity to Ps-P50 was calculated.

Table 13: Similarity matrix of the relationships (in %) between Ps-P50, the PCR fragment "iqaryen" and the ESTs nucleic acid sequences.

10-10f-my	--									
10-11g-my	97	--								
11-3h-zo	85	80	--							
3-8h-zo	82	48	86	--						
5-8d-zo	79	79	80	100	--					
6-5h-ha	0	98	90	0	82	--				
7-2f-zo	n.s.	n.s.	77	n.s.	n.s.	77	--			
7-3e-zo	0	n.s.	77	n.s.	n.s.	77	100	--		
Ps-P50	97	97	83	74	79	84	77	65	--	
PCR-iqaryen	0	87	88	0	97	86	95	75	86	--
	10-10f-my	10-11g-my	11-3h-zo	3-8h-zo	5-8d-zo	6-5h-ha	7-2f-zo	7-3e-zo	Ps-P50	PCR-iqaryen

n.s.: not significant

4.21. Analysis of the deduced amino acid sequence of Ps-P50

All predictions were done using the PCgene software.

4.21.1. Amino acid composition and isoelectric point

The predicted protein sequence of Ps-P50 has 474 amino acid residues and a molecular weight of 49,991 Da. The amino acid composition is shown in the Table 14. The isoelectric point was predicted to be 3.86.

Table 14: Amino acid composition of the deduced Ps-P50 amino acid sequence.

Amino acid	number of residues	%
Ser	60	12.6
Ala	47	9.9
Thr	42	8.8
Gly	41	8.6
Val	32	6.7
Asp	30	6.3
Glu	29	6.1
Leu	28	5.9
Asn	24	5.0
Tyr	23	4.8
Pro	20	4.2
Met	16	3.3
Lys	15	3.1
Cys	15	3.1
Ile	12	2.5
Phe	12	2.5
Gln	11	2.3
Arg	9	1.8
Trp	5	1.0
His	3	0.6

4.21.2. Transmembrane helices

Two putative transmembrane helices were detected in the deduced amino acid sequence of Ps-P50 by using the method of Argos and Rao (1986). One of these helices is located at the amino terminus (amino acids 2 to 30) and the other resides at the carboxyl terminus (residues 453 to 474). The plot of the smoothed buried helix profile for Ps-P50 is shown in Figure 34.

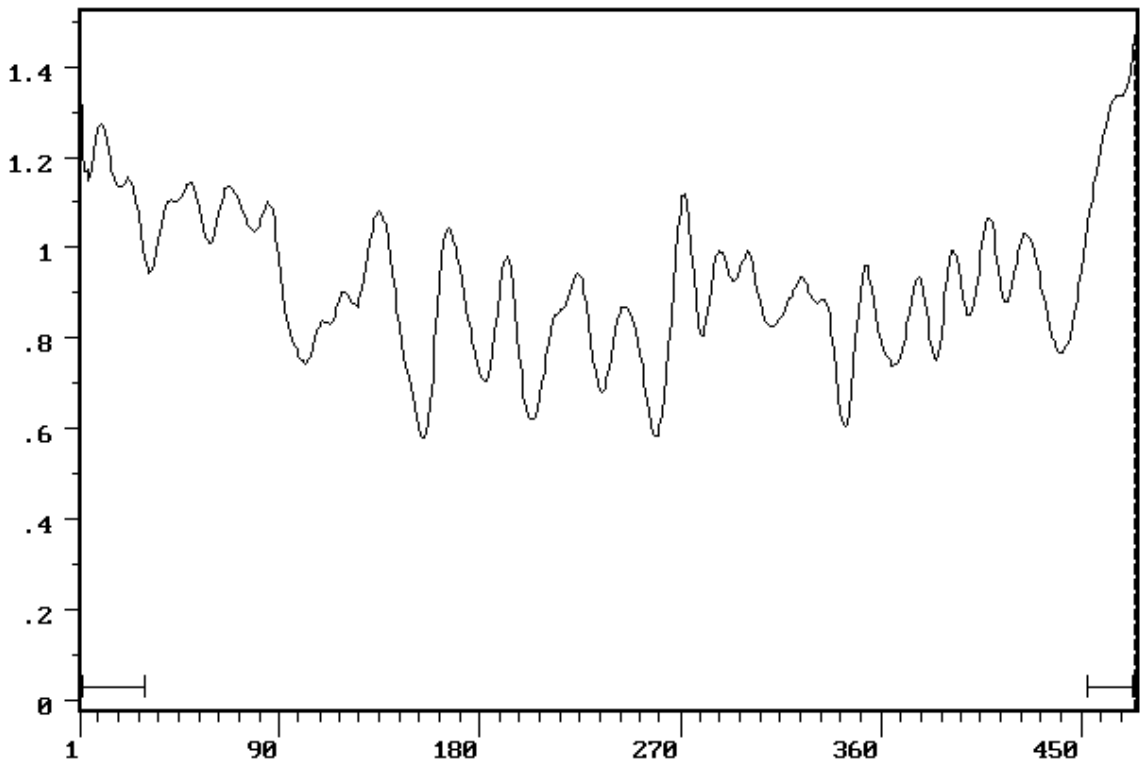


Figure 34: Smoothed curve of the derived amino acid sequence from Ps-P50 from residue 1 to 474. The y axis represents the buried helix parameter. The x axis represents the amino acid position. Areas marked by represent the putative transmembrane domains.

4.21.3. Detection of sites and signatures

The following putative modification sites were found in the deduced amino acid sequence of Ps-P50: 6 potential N-glycosylation sites were found in positions 281, 307, 348, 363, 400 and 435; one potential tyrosine sulfatation site was found at 212 position; protein kinase C phosphorylation sites, four potential sites were found at positions 150, 285, 347 and 444; casein kinase II phosphorylation sites, 6 potential sites were found at positions 54, 183, 326, 426 and 436, N-myristoylation sites, 14 potential sites were found at positions 13, 43, 48, 51, 64, 67, 77, 143, 195, 268, 364, 398, 440 and 451.

Neither mitochondrial, chloroplast nor microbody transit peptides were predicted. No part of the deduced amino acid sequence was predicted to be a DNA-binding regulatory protein.

4.22. Heterologous expression of Ps-P50 cDNA

4.22.1. Heterologous expression in E. coli

Twelve different strategies were adopted for cloning the Ps-P50 cDNA into the expression vector pQE, using one or two sticky DNA ends. In all cases, transformation was either unsuccessful or resulted in false positives (checked by colony PCR).

However, when both DNA ends were blunt, transformation was achieved, but all analyzed plasmids from 20 colonies checked contained the Ps-P50 insert in the reverse orientation.

4.22.2. Hetereologous expression in yeast

Both pYes2 and pZL-Ps-P50 plasmids were digested with *Not* I and *Ecl*136 II. These two restriction enzymes are cutting pYES2 at the multiple cloning site. pZL-Ps-P50 was linearized 5´ to the second methionine (*Ecl*136 II cuts at position 65 in the nucleotide sequence of Ps-P50) and the insert is released with *Not* I, located 3´ to the cDNA.

After ligation, the construct (pYES-Ps-P50) was transformed into *E. coli* DH5α cells (see sections 3.9.15 and 3.9.11). Around 800 colonies grew on LB medium supplemented with ampicillin. Forty-two of these colonies were checked by colony PCR (section 3.9.7.1) and half of them (21) were found to contain the inserted Ps-P50. The plasmid pYES-Ps-P50 was isolated and verified, with positive outcome, by restriction analysis.

S. cerevisiae INVSc2 was transformed (section 3.9.13) with either pYES2 (empty vector) or pYES-Ps-P50. Heterologous expression was carried out with yeast transformants as described in section 3.9.14. Aliquots at times 0, 8, 16, 24 and 48 h after starting the culture were taken and the OD_{600} measured. Fifteen milliliters of 48 h culture (stationary phase) were used for preparation of membrane and soluble fractions as described in section 3.7.6.

From each fraction, 300 µg of protein (membrane and soluble fractions of expression of pYES2 and pYES-Ps-P50) were separated by two dimensional gel electrophoresis (section 3.7.5). After silver staining of the 2D-gels, no differences were observed between the microsomal fractions of the pYES2 and pYES-Ps-P50 expressing cells (see appendix 7.3 for figures of the gels).

However, differences could be observed between the soluble fractions of pYES2 and pYES-Ps-P50. These differences appeared in a section of the 2D-gel corresponding to low pH and low molecular weight. The areas of interest are shown in Figure 35. The entire gels from this experiment are documented in section 8.2.

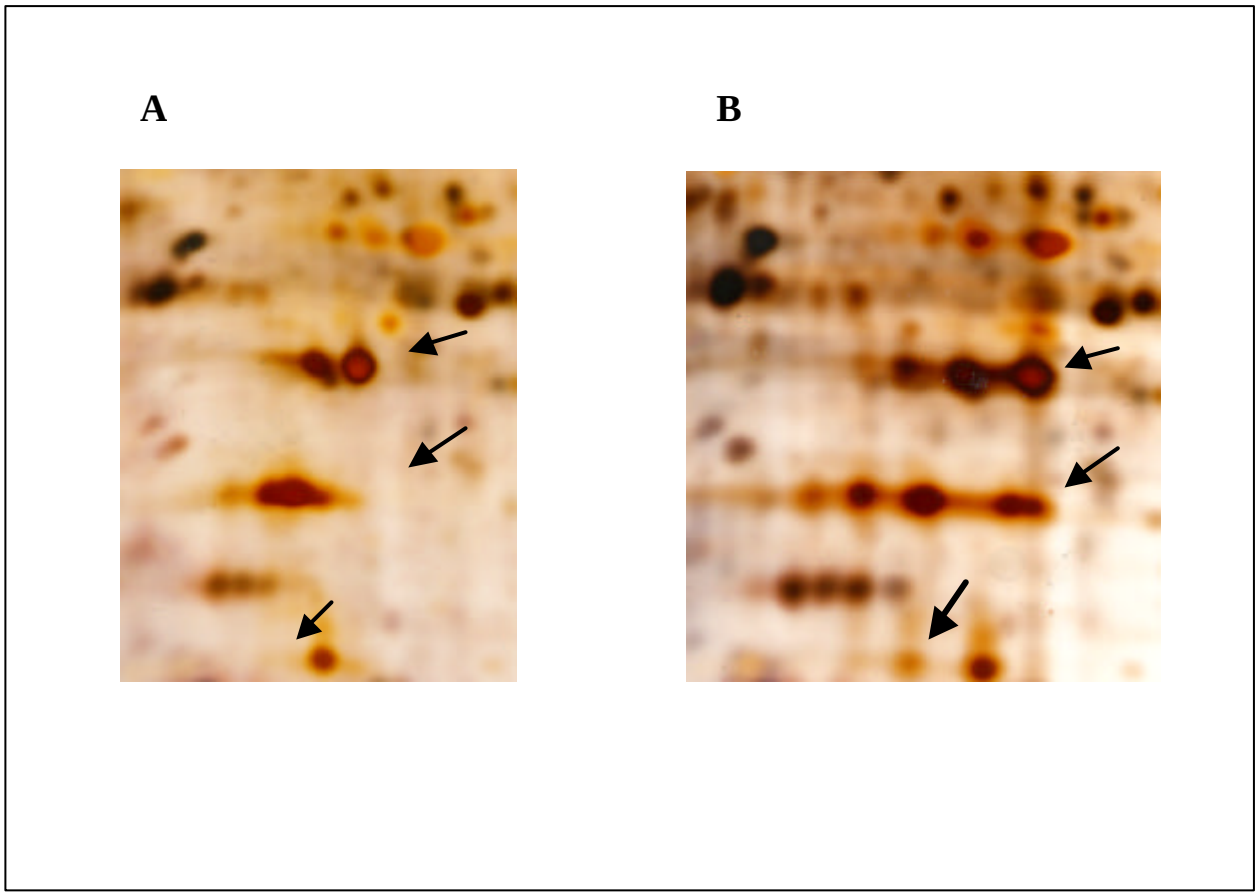


Figure 35: Selected areas of the two dimensional electrophoresis gels of soluble protein fractions of recombinant yeast. Panel A: protein extracts from the expression of pYES2 (empty vector); and panel B: protein extracts from the expression of pYES-Ps-P50. The arrows depict peptides in panel B that are not present in panel A. Complete gels are shown in the section 8.2.

5. Discussion

5.1. Optimization of growth of P. sojae mycelium

The amount of starting material is one of the main problems in any protein purification trial. In the case of the mycelium of *Phytophthora sojae* this problem turned out to be critical. It is not possible to grow the mycelium in any kind of fermentation device (Ebel, J. personal communication) so the growth should be achieved in Fernbach flasks. Only few grams (usually not more than 5 g) of wet material can be harvested per flask at the end of the growth period, and from this material not more than 1-5 mg are membrane proteins. Furthermore, not more than 20% of these membrane proteins can be solubilized efficiently for glucan synthase activity.

The method optimization reported in section 4.1 led to a reduced growing time (3 weeks instead of 4 to 6) and allowed the handling of more than 100 flasks per harvest.

5.2. Glucan synthase of P. sojae

Glucan synthase activity was easily detectable and quantifiable in microsomal preparations from actively growing *P. sojae* cultures. The method used in this work to determine the glucan synthase activity is based on the measurement of radioactivity contained in the TCA precipitated product and varies very little of what was described by Cabib and Kang (1987). The use of large volumes (500 µl instead of 40 µl) of 20% TCA for precipitating the glucans allows faster and easier manipulations and minimizes unspecific losses. Other glucan synthase assays rely on the recovery of the glucans as ethanol-precipitated material (Kottutz and Rapp, 1990), but this method requires an overnight step, while with the TCA method each sample is processed in less than one minute.

The glucan synthase activity of *P. sojae* appeared to be dependent on a proteinaceous factor: there was no activity measurable if the microsomal fraction was incubated 10 min at 100°C prior to the assay, when the assay was stopped at time point zero, or when the microsomal fraction was incubated with protein denaturing agents like SDS.

The glucan synthase activity of *P. sojae* was localized in the microsomal fraction. The cytosolic fraction did not include any glucan synthase activity and did not influence the glucan synthase activity when added to the microsomal fraction, thus there was neither a positive nor a negative regulator for glucan synthase activity *in vitro* present in the cytosolic fraction. No subcellular localization experiments were carried out in this work because it is

widely accepted that (1→3)-β-glucan synthase activity is associated with the plasma membrane in all kingdoms where this activity is present, including stramenopiles (Girard and Fèvre, 1984), and such activity is even used broadly as a marker for plasma membrane.

After some experiments to characterize the chemical conditions for maximal activity of the glucan synthase of *P. sojae*, it was possible to specifically modify the extraction buffer for microsome preparation. Sucrose and NaF were included in the buffer used to generate the microsomal fraction because they increased glucan synthase activity. EGTA was included in order to reduce the excess of Ca^{2+} from the culture medium, because Ca^{2+} affects solubilization. Apart from these additions, the method for extracting the microsomal fraction (differential centrifugation) did not differ from methods for purifying other glucan synthases (see Table 15 and Table 16 for references) or other membrane proteins (Cosio et al., 1996).

The kinetics of the *in vitro* reaction at 25°C showed linear glucose incorporation into insoluble glucan for up to 1 h followed by non-linear but significant on-going product formation for at least 4 h after addition of substrate (Figure 7). The activity in microsomal preparations was fairly stable with not more than a 25% loss being observed after 2 d at 4°C.

Several substances were tested for their effect on glucan synthesis *in vitro* (Table 1). From the compounds tested, only NaF, sucrose and BSA turned out to enhance glucan synthase activity of *P. sojae* to some extent. These substances are well known as activators or stabilizers of the fungal glucan synthases (Cabib and Kang, 1987). The role of sucrose as activator can be interpreted as that of an osmotic regulator because of the high concentration needed (1 M, lower concentrations are less effective) and because the same effect can be obtained by adding glycerol. NaF is known to be an inhibitor of phosphatases (Wang et al., 1995) but in the case of glucan synthesis the mechanism of enhancement is unclear. The activation by BSA is lower than in other reported cases (only 30% of activity is lost if it is not present) and is probably related to some stabilization of the proteins (i.e. protecting from protease activity).

Neither GTP nor GTP-γ-S stimulate the glucan synthase of *P. sojae in vitro* when used in concentrations up to 4 mM. GTP (or its non-degradable analogue GTP-γ-S) is known to be necessary for glucan synthase activity in yeast and other fungi (Cabib and Kang, 1987) because these enzymes require the presence of a GTP-binding protein identified as Rho1p (Drgonová et al., 1996; Qadota et al., 1996). In these cases, the GTPase was copurified with glucan synthase and appears to be a regulatory subunit of the enzyme complex.

In contrast, the glucan synthase of *Achlya ambisexualis*, like the one of *P. sojae*, is GTP independent (Cabib and Kang, 1987). Both species belong to the Stramenopile kingdom.

Membrane-bound enzymes of higher plants synthesize (1→3)-β-glucans (callose) in response to wounding, physiological stress, or infection (Delmer, 1987). When plant cells are physically damaged, a disruption in the normal ionic gradient across the plasma membrane results, leading to an efflux of Cl^- and K^+ ions and an influx of Ca^{2+}. This local increase in Ca^{2+} leads to immediate synthesis of callose (Bacic and Delmer, 1981). Moreover, the enzyme requires Ca^{2+} for its activity *in vitro* (Bulone et al., 1999).

In contrast, the divalent cations Ca^{2+} and Mg^{2+} have nearly no effect on the glucan synthase activity of *P. sojae*. In this aspect it differs clearly from the stress-induced callose synthase.

Callose is as well a component of specialized plant walls or wall-associated structures at a particular stage of growth and differentiation like cell plates, seeds, leaf and stem hairs, plasmodesmatal canals, sieve plates, transient walls of microsporogenic and megasporogenic tissues, and in pollen and pollen tubes (Kudlicka and Brown, 1997). Interestingly, the callose synthase of pollen tubes is Ca^{2+}-independent (Schlüpmann et al., 1993), as the *P. sojae* glucan synthase.

Other divalent cations inhibit to some extent glucan synthase of *P. sojae*, ranging from a decrease of activity of two thirds (Mn^{2+} and Fe^{2+}) up to total inhibition (Cu^{2+} and Zn^{2+}) when tested at 10 mM. This phenomenon was also observed for the glucan synthase of *Pyricularia oryzae* P_2 (Kominato et al., 1987) and for the *Lolium multiflorum* callose synthase from endosperm (Bulone et al., 1999). A hypothesis that might explain this effect is the interaction of these divalent cations with histidine residues present in the protein.

The glucan synthase of *P. sojae* is not affected by DTT when used at concentrations up to 1.25 mM. It was reported by Cabib and Kang (1987) that sulfhydryl reagents partially inhibit the glucan synthase of several fungi. The only glucan synthase that was not affected by sulfhydryl reagents was the one of *A. ambisexualis*.

Cellobiose and other β-glucosides are reported to be activators of both Ca^{2+}-dependent callose synthase (Delmer, 1999) and Ca^{2+}-insensitive pollen tube callose synthase (Schlüpmann et al., 1993). Cellobiose is also stimulating the glucan synthase of *Sclerotium glucanicum* by 20% (Kottutz and Rapp, 1990). The reason for this dependence is not clear

and, in the case of the pollen tubes of *Nicotiana alata*, this effect can be to some extent substituted by glycerol or sucrose (Schlüpmann et al., 1993). Cellobiose is usually not included in the glucan synthase assay of fungal sources. In this work, cellobiose was tested and found not to affect the glucan synthase activity of *P. sojae*.

Glucans with a low degree of polymerization, extracted from *P. sojae* mycelium (Cosio et al., 1988; Cosio et al., 1990), had no effect on glucan synthase activity. Thus, their role as primers of the enzymatic activity should be discarded. High molecular weight glucan from the same source had an inhibitory effect when used at 5 mg/ml (26% inhibition). The reason for this inhibition remains unclear, but could be due to some extent to the arrest of the glucan synthase in a similar way as it is used for product entrapment.

5.2.1. Catalytic constants of glucan synthase activity

The catalytic constants of the glucan synthases from different sources are summarized in Table 15. All data shown correspond to the glucan synthase from microsomal fractions of the named organisms.

Table 15: Catalytic constants of glucan and callose synthases.

Organism	K_m (mM)	V_{max} (nkat·mg protein^{-1})	Reference
Saccharomyces cerevisiae	3.8	0.69	Cabib and Kang (1987)
Hansenula anomala	0.67	0.033	Cabib and Kang (1987)
Neurospora crassa	2.9	0.14	Cabib and Kang (1987)
Cryptococcus laurentii	0.86	0.036	Cabib and Kang (1987)
Schizophyllum commune	0.8	0.028	Cabib and Kang (1987)
Wangiella dermatitidis	1.8	0.047	Cabib and Kang (1987)
Botritis cinerea	0.8	0.014	Monschau et al. (1997)
Pyricularia oryzae	1.2	--	Kominato et al. (1987)
Sclerotium glucanicum	0.54	--	Kottutz et al. (1990)
Entomophaga aulicae	1.7	0.11	Beauvais and Latge (1989)
Achlya ambisexualis	7.1	0.14	Cabib and Kang (1987)
Phytophthora sojae	10.6	0.4	This work

All glucan and callose synthases shown in Table 15 have a K_m in the millimolar range, but the values from *A. ambisexualis* and *P. sojae* are higher (7.1 and 10.6 mM, respectively) when compared to all other listed species.

No general conclusions could be made from the V_{max} data as they vary significantly from one organism to the other. This heterogeneity might be due not only to species variability, but also to different experimental procedures.

A. ambisexualis is the only organism shown in Table 15 belonging to the same kingdom as *P. sojae* (Stramenopiles). The glucan synthase of these two organisms have several characteristics in common that differ from all other known fungal glucan synthases:

- higher K_m value for UDP-glucose
- no stimulation by GTP,
- no inhibition by sulfhydryl agents.

With the data of only two organisms of the stramenopiles, it would not be correct to assign these characteristics to the whole kingdom. Anyway, it is notable that the glucan synthases of the stramenopiles kingdom species studied until now share these characteristics, different to the glucan synthases of the fungal kingdom species.

5.3. Solubilization of glucan synthase activity

Among the several detergents tested for the solubilization of glucan synthase activity from microsomal membranes of *P. sojae* (see section 4.4), only CHAPS was effective (to some extent). Under the experimental conditions used, with the other three tested detergents (octyl glucoside, Zwittergent 3-12 and SDS) glucan synthase activity could not be detected, neither in the solubilized fractions nor in the remaining membrane pellets (see Table 5). This strong inhibitory effect could not be reversed by decreasing the detergent concentration prior to analysis, so it turned out to be irreversible. Using a mixture of CHAPS and octyl glucoside resulted in an increase of total protein solubilization but a decrease of glucan synthase activity both in supernatant and remaining pellet.

The solubilization profile described above for the glucan synthase of *P. sojae* is similar to what is described in the literature for the glucan synthase and the callose synthase of fungi and plants, respectively, summarized in Table 16.

Table 16: Solubilization of glucan and callose synthases

Organism	Detergents	Reference
Beta vulgaris L.	0.25% CHAPS + 0.01% digitonin	Frost et al. (1990)
Lolium multiflorum	0.5% digitonin or 0.6% CHAPS	Meikle et al. (1991)
Saprolegnia monoica	0.5% CHAPS + 0.1% octyl glucoside	Girard et al. (1992)
Neurospora crassa	0.1% CHAPS + 0.5% octyl glucoside	Awald et al. (1993)
Aspergillus fumigatus	0.3% CHAPS	Beauvais et al. (1993)
Pisum sativum	0.25-0.5% digitonin	Dhugga and Ray (1994)
Saccharomyces cerevisiae	0.5% CHAPS + 0.1% cholesteryl hemisuccinate	Inoue et al. (1995)
Saccharomyces cerevisiae	0.2% CHAPS	Baginsky and Mazur (1996)
Vignia radiata	0.25% digitonin	Kudlicka and Brown (1997)
Phaseolus vulgaris	1% reduced Triton X-100	McCormack et al. (1997)
Gossypium hirsutum	0.1% and 1% digitonin	Shin and Brown (1999)

As can be seen in Table 16, most of the authors used CHAPS and/or digitonin for solubilization of different glucan synthases. The structure of these and other detergents is presented in section 8.4.

The main difference between the group of CHAPS, cholesteryl hemisuccinate, digitonin, Tergitol NP-40 and Triton X-100 on one hand, and the group of Zwittergent 3-12 and octyl glucoside on the other is, that in the first group of detergents the hydrophobic part of their structure is a non linear structure, and in the second group of detergents it is a linear alkyl chain.

The only two cases shown in Table 16 of the successful use of a detergent with an alkyl chain are reported for *Neurospora crassa* (Awald et al., 1993) and *Saprolegnia monoica* (Girard et al., 1992) glucan synthases. In both cases, a mixture of CHAPS and octyl glucoside was used. Girard et al. (1992) explained this as representing a compromise between the poor solubilization rate but good activity achieved with CHAPS and the better solubilization rate but loss of activity achieved with octyl glucoside. This correlates to some extent with experience in this work. The loss of activity caused by the presence of alkyl chains was

presumed to be due mainly to the displacement of phospholipids and other activating compounds from the enzyme complex (Girard and Fèvre, 1991).

Tergitol NP-40 was used to solubilize part of the glucan synthase complex from yeast (Mol et al., 1994), *Hansenula anomala* and *Neurospora crassa* (Kang and Cabib, 1986). In all three cases, a peripheral small GTP-binding protein, later identified as Rho1p (Mazur and Baginsky, 1996), is separated from the rest of the membrane by treatment with Tergitol NP-40. This was not affecting the final activity when both fractions (membrane and solubilized) were combined for the assay. This detergent has no alkyl chain as a hydrophobic part of the molecule, which is consistent with the characteristics of the detergents used for solubilization of glucan synthase from different sources.

As described in section 4.4, the best solubilization of the glucan synthase from *Phytophthora sojae* microsomal fraction was obtained at 3 mg/ml CHAPS and 2 mg/ml protein concentrations. It was shown in this work that this was a compromise between maximum solubilization degree (achieved with higher detergent concentrations) and maximum specific activity (achieved with lower detergent and protein concentrations).

Solubilization experiments with detergent concentrations higher than 0.3% resulted in an irreversible loss of activity. This was also reported by Li et al. (1997) for the pollen tube callose synthase of *Nicotiana alata*, and by Beauvais et al. (1993) for the glucan synthase of *Aspergillus fumigatus*. In any case, all the solubilization methods including CHAPS found in the literature and shown in Table 15, are using concentrations below the CMC (0.6%, Li et al., 1997).

The total protein solubilized from the *P. sojae* microsomal fraction, when 0.3% CHAPS was used, did not exceed 20% in most experiments. This poor solubilization extent was also reported for *Saprolegnia monoica* by Girard et al. (1992). The authors increased solubilization degrees by using octyl glucoside together with CHAPS. In the case of *P. sojae*, the concomitant use of octyl glucoside compromised the specific activity in a way, that made its use not desirable.

The CHAPS-solubilized glucan synthase from *P. sojae* has an apparent K_m for UDP-glucose similar to that found for the membrane fraction (10.6 and 10.7 mM for membrane and solubilized fraction, respectively) (Table 12). The use of CHAPS resulted in a significant increase in glucan synthase activity: the V_{max} of CHAPS-solubilized fraction is six times higher than the V_{max} of the microsomal fraction (0.4 and 2.5 nkat·mg protein^{-1} for membrane

and solubilized fraction, respectively). This effect has also been observed with the glucan synthases of *Aspergillus nidulans* (Kelly et al., 1996) and of *Saprolegnia monoica* (Girard et al., 1992) and the callose synthases of *Lolium multiflorum* (Meikle et al., 1991; Henry and Stone, 1982a), *Nicotiana alata* (Li et al., 1999; Schlüpmann et al., 1993) and *Apium graveolens* (Slay et al., 1992).

To explain this effect, Li et al. (1997) postulated that CHAPS stimulates callose synthase by permeabilizing right-side-out vesicles to the substrate UDP-glucose and through a direct interaction with otherwise inactive callose synthase. This hypothesis is summarized in Figure 36. Whether this hypothesis is valid for the activation of the *P. sojae* glucan synthase is not clear since Li et al. (1997) reported not only an increase of V_{max} but also a decrease of the K_m value. Such an effect did not occur in the case of the CHAPS solubilized glucan synthase of *P. sojae*.

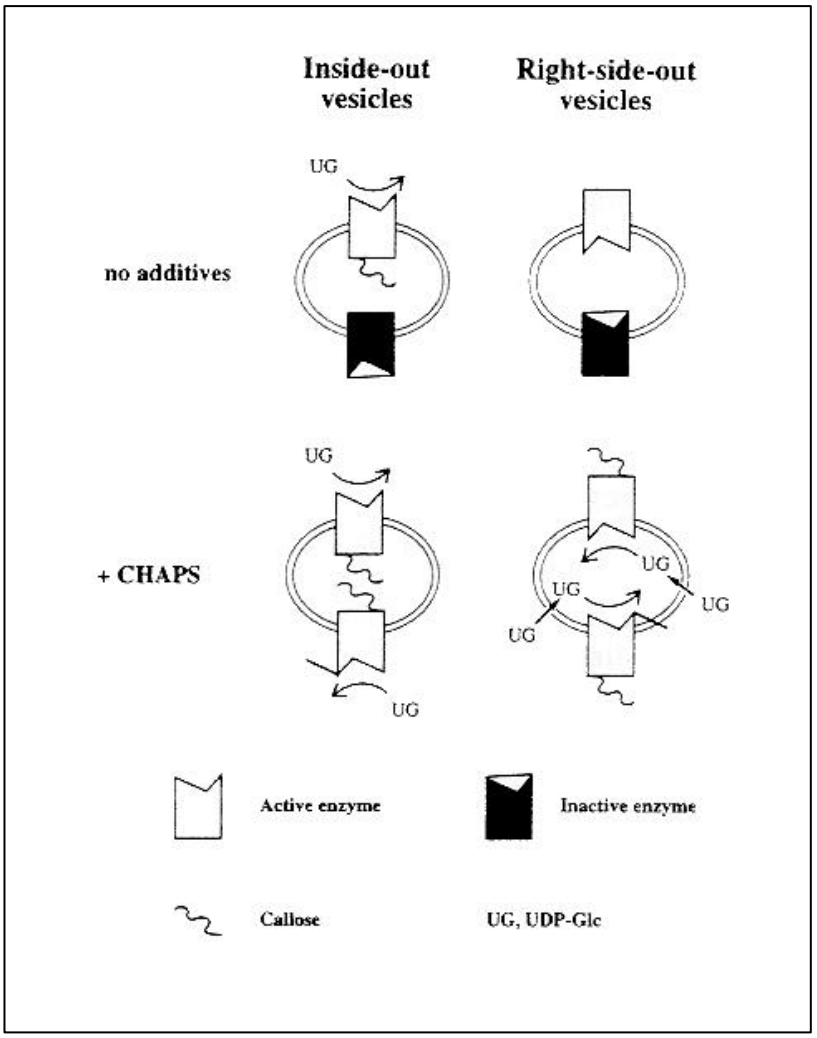


Figure 36: Diagrammatic representation of the possible mechanism of activation of pollen tube callose synthase. Adapted from Li et al. (1997).

5.4. Product analysis

Two main experimental procedures were used for the identification of the product of the solubilized glucan synthase of *P. sojae*: enzymatic degradation and NMR spectroscopy.

Laminarinase, a (1→3)-β-glucan hydrolase, can degrade the ^{14}C-labeled insoluble glucan. The sole product of this reaction was glucose as shown by thin layer chromatography. Neither (1→6)-β-linked oligosaccharides nor the disaccharide gentiobiose were detectable. The glucan was not degraded by α-amylase. This enzymatic test was used as proof for the identity of the products of several glucan synthases (Henry and Stone, 1982a,b; Wang and Bartnicki-

Garcia, 1982; Awald et al., 1993; Beauvais et al., 1993; Kudlicka and Brown, 1997; McCormack et al., 1997), but has the disadvantage that most of the commercially available laminarinases could have additional enzymatic activities like cellulase activity and others.

For a better and more conclusive elucidation of the product of the *P. sojae* glucan synthase, NMR spectroscopy was performed with glucan produced by the CHAPS-solubilized protein. The most straightforward spectrum to interpret is the ^{13}C-NMR (Figure 13): only six resonances are displayed, each corresponding to one carbon atom in the glucose monomer of the β-glucan chain. These signals (summarized in Table 9) correspond with those published for a (1→3)-β-glucan (Saito et al., 1979; Kottuz and Rapp, 1990;Yu et al., 1993; Gutierrez et al., 1996;Müller et al., 1996). Moreover, the absence of signals at 79.9 and 70.1 ppm are consistent with the lack of (1→4)-β and (1→6)-β linkages, respectively. The (1→4)-β linkages are also present in the cell wall of *Phytophthora* (Bartnicki-Garcia and Wang, 1983). The absence of any signal at 74.8 ppm indicates that all C3 carbon atoms in the glucose molecule are substituted. In summary, with the data given solely by ^{13}C-NMR it is possible to identify the product of the CHAPS-solubilized glucan synthase as a long linear (1→3)-β-glucan.

The lack of (1→6)-β linkages in the glucan produced by the CHAPS-solubilized protein is not surprising since the (1→6)-β-glucan synthase activity has not been detected *in vitro* (Roemer and Bussey, 1991). In yeast some components of the (1→6)-β-glucan synthesis have been already identified, like KRE6 and SKN1 genes, both encoding phosphorylated integral membrane glycoproteins that are most likely localized in the Golgi apparatus (Roemer et al., 1994). (1→6)-β linkages are present in the cell wall of *Phytophthora* (Bartnick-Garcia and Wang, 1983) and in the *P. sojae* hepta-β-glucoside elicitor (Ebel, 1998). In yeast, (1→6)-β-glucan interconnects several cell wall components like chitin, (1→3)-β-glucan and proteins (Kollár et al., 1997).

5.5. Purification of glucan synthase

Different strategies for protein purification were attempted for the solubilized glucan synthase of *P. sojae*. Some basic biochemical approaches like PEG precipitation, useful for other membrane proteins (Frey et al., 1993), resulted in 50% loss of total activity and no increase in specific activity and therefore had to be abandoned as a purification method. Other

methods like the sucrose-density gradient centrifugation led to some purification with good total recovery. But their high time consumption and low loading capacity made them insufficient for protein purification.

Concanavalin A chromatography is commonly used for the purification of glycoproteins (Eipper et al., 1976) and it has specificity for branched mannoses, carbohydrates with terminal mannose or glucose. The CHAPS-solubilized glucan synthase activity of *P. sojae* was not retained by the concanavalin A. This could mean that the glucan synthase lacks those glycosyl residues specifically recognized by concanavalin A. But the absence of retention of the glucan synthase activity by concanavalin A could also be due to the fact that the glycosylated domains are usually extracellular and the catalytic site of the glucan synthase is located at the cytoplasmatic side. Thus, if only the inside-out micelles are catalytically active (they expose the catalytic site to the buffer containing the substrate) no binding to concanavalin A could be achieved because the glycosylated parts are inside the micelle. Therefore, no biochemical characteristics can be deduced from the fact that the glucan synthase could not be purified by concanavalin A sepharose.

Some nucleotide-requiring enzymes can bind to Blue Sepharose matrices. This possibility was explored for glucan synthase considering its dependency on UDP-glucose. Some enrichment was achieved (1.5 fold specific activity) but not enough for purification purposes. This relatively low affinity for the matrix could be due to the high K_m value for UDP-glucose displayed by the glucan synthase of *P. sojae*.

Immobilized heparin has two main modes of interaction with proteins. It can operate as an affinity ligand, as for example with coagulation factors. Heparin also acts as a high capacity cation exchanger, due to its anionic sulfate groups. The CHAPS-solubilized glucan synthase eluted mainly with the washing fractions and only a small amount was retained by the heparin matrix, with no increase of specific activity. Thus, heparin chromatography was excluded as purification method.

Native gel electrophoresis was performed with CHAPS-solubilized material. As shown in Figure 21, glucan synthase activity is concentrated at the interface between stacking and separating gel. Similar results were obtained for the (1→3)-β-glucan synthase of mung bean (Kudlicka and Brown, 1997): the callose synthase barely entered the 6% separating gel while

the cellulose synthase was concentrated at the top of the loading well. In the case of the CHAPS-solubilized proteins of *P. sojae*, some fluorescence could be observed at the gel interface as well as at the top of the loading well, but this fluorescence appeared equally when heat-denatured material was used for electrophoresis. Thus, in the case of the solubilized proteins of *P. sojae*, the fluorescence seen at the top of the 3% gel should be assigned to unspecific binding of the dye, possibly to a residual fraction of the cell wall of *P. sojae* that might be present in the CHAPS-solubilized material.

Photoaffinity labeling was used by various groups as an attempt to identify glucan synthases. A 57 kDa peptide of *Beta vulgaris* was specifically labeled when 5-azido-UDP-glucose was used (Frost et al., 1990). Several peptides were labeled in *Apium graveolens* (Slay et al., 1992) or *Vignia radiata* preparations (Kudlicka and Brown, 1997), using the same photoaffinity ligand. A 55 kDa peptide that copurified with the callose synthase of pea was labeled using [α-^{32}P]UDP-glucose as photoaffinity ligand (Dhugga and Ray, 1994). By using 5-[3-(p-azidosalicylamide)]allyl-UDP-glucose labeled with ^{125}I, a 31 kDa polypeptide was identified in the immunoprecipitated glucan synthase of *Lolium multiflorum* (Meikle et al., 1991). But even after years of such reports and further development of other potential photoaffinity ligands (Ng et al., 1996), no primary sequence of glucan synthases was reported until now. This fact, and the relatively high K_m value for UDP-glucose of the glucan synthase of *P. sojae*, makes the use of photoaffinity analogues of UDP-glucose inadvisable for the identification of the glucan synthase of *P. sojae*.

Size exclusion chromatography was performed with Superose 6 and 12 columns. The glucan synthase activity eluted at volumes corresponding to apparent molecular masses between 1000 and 2000 kDa. These apparent values are at the limit of resolution of these columns and may represent, therefore, only rough estimates. Furthermore, there is always the risk of some artifacts, like protein precipitation during the preparation. This further reduces the reliability of this information. On the other hand, it is known for other (1$\rightarrow$3)-β-glucan synthases as well as for cellulose synthase that they are large multisubunit complexes (Kimura et al., 1999; Selitrennikoff, 1995), which would make this result appear realistic.

Anion exchange chromatography gave a clear increase of specific activity (about 6-fold) with a reasonable total recovery (50%), big loading capacity and short running times. Taking these results into account, anion exchange chromatography was considered as a possible

method for pre-purification. It was, nevertheless, not efficient enough by itself to achieve a purification level that allowed the identification of a discrete number of polypeptide candidates.

A method successfully used in the past for the purification of several glucosyl transferases is product entrapment. An outline of this method is shown in Figure 37. The technique relies on the fact that some enzymes have affinity to their own product, and if the product is insoluble, the enzyme can be enriched by collecting the pellet after centrifugation of the reaction mixture.

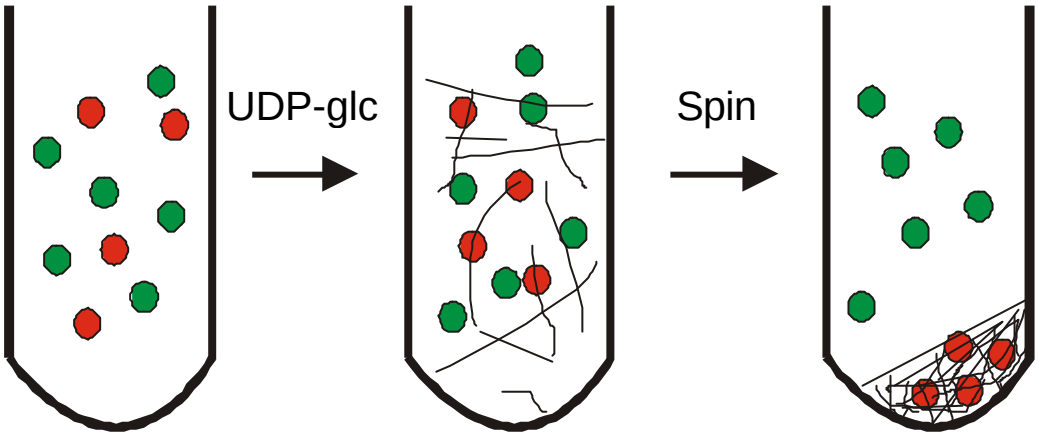


Figure 37: Schematic representation of the product entrapment procedure. Glucan synthase (red circles) can be separated from the rest of the proteins (green circles) because its affinity for the product (black lines). Since the glucan product is insoluble, the glucan synthase activity is recovered in the glucan pellet after centrifugation of the mixture.

Product entrapment was used the first time for the purification of chitin synthase (Kang et al., 1984). A 700-fold purification was reported for the (1→3)-β-glucan synthase of the ascomycete *Neurospora crassa* (Awald et al., 1993), but no specific polypeptide could be assigned as being involved in glucan synthesis.

The glucan synthase of *S. cerevisiae* was purified as well by product entrapment and the enrichment of a 200 kDa protein in SDS-PAGE was observed (Inoue et al., 1995). In this elegant work, Inoue and coworkers demonstrated biochemically and genetically the

importance of the genes GSC1 and GSC2 (encoding the 200 kDa protein) in the synthesis of (1→3)-β-glucans in yeast.

Product entrapment was used in this work for the purification of the glucan synthase of *Phytophthora sojae* (see section 4.15). After extensive washing, some of the glucan synthase activity remained in the glucan pellet. Unfortunately, the glucan synthase activity could not be removed from the glucan pellet, and consequently no protein estimation could be done. Therefore no enrichment factor could be calculated for the purification. The binding of the glucan activity was so strong that, even after SDS treatment, some protein remained bound to the glucan pellet (Figure 19, lane D).

Two main proteins were shown by SDS-PAGE to copurify with the glucan synthase when product entrapment was used for purification (Figure 19, lane B). The larger protein, with an apparent molecular mass of 108 kDa, was the only one that could be found in the high molecular mass range of the SDS-PAGE. The second protein, with an apparent molecular mass of 50 kDa, was predominant in the low molecular mass range of the SDS-PAGE.

Product entrapment performed with material pre-purified by anion exchange chromatography (Figure 19, lane C) showed nearly the same profile as the one performed directly with CHAPS-solubilized material. Therefore, anion exchange chromatography was excluded as pre-purifying method, and preparative product entrapment was carried out directly with CHAPS-solubilized protein (Figure 20). Hence, with a single purification step after CHAPS solubilization, two proteins with apparent molecular mass of 108 and 50 kDa, (called P108 and P50, respectively) were enriched in a way that they could easily be excised from SDS-PAGE gels and delivered for microsequencing.

The amount of P108 present in the preparative gels was close to the limit of detection of the sequencing methods used. This resulted in a lower reliability of the sequencing result. In any case, nine peptide sequences were obtained, with lengths from 8 to 15 amino acids (see Table 10).

On the other hand, the amount of P50 isolated after electrophoresis was clearly sufficient for microsequencing.

5.6. Cloning of P. sojae genes

5.6.1. Method validation: cloning of the actin cDNA.

The cloning of the cDNA for actin (Dudler, 1990) was performed in order to validate the quality of cDNA and PCR techniques. This turned out to be necessary since the accuracy of

protein sequencing was not high, *Phytophthora sojae* genome has a high percentage of G+C (up to 58%, Qutob et al., 2000), and the knowledge on oomycetes genetics in general is low (Judelson et al., 1992).

In *P. sojae,* the actin protein is encoded by a single gene without any introns. The protein has a length of 375 amino acids and its sequence diverges remarkably from any other actin known. It is one of the few middle size proteins with known sequence in *P. sojae* (by checking the data bases via www.ncbi.nlm.nih.org). This makes the actin gene an appropriate marker gene.

Two oligonucleotides, one starting 56 bases upstream of the starting ATG, and the other one, in antisense orientation, 891 bases after the ATG, were used for the PCR reaction. As template, the cDNA library generated as described in section 3.10 was used. As expected, a 970 bp fragment was generated. The ^{32}P-labeled fragment hybridized with RNA at the expected size for the actin mRNA (1.3 kb, Figure 24). It was concluded that the method is suitable for cloning genes out of the cDNA library constructed for this work.

5.6.2. Cloning of the cDNA encoding P108

Cloning of the cDNA encoding P108 using a PCR strategy was unsuccessful. As primers for the PCR reaction, degenerated oligonucleotides derived from the sequenced peptides were used.

A first explanation for the failure of the PCR experiments is the low accuracy of the peptide sequences and the resulting oligonucleotides. Moreover, the repeated presence of lysine and serine, amino acids encoded by six different codons, introduced high degeneration in the oligonucleotides. This may produce lower affinity of the oligonucleotide primers for the DNA template.

There is, as well, the possibility that not all the peptides sequenced are derived from the same protein: two or more polypeptides could have the same apparent molecular mass and be present in the same band after staining the SDS-PAGE gel. Thus, in the same piece of acrylamide gel cut for microsequencing, several polypeptides would be present, each giving several peptides suitable for sequencing. Therefore, two oligonucleotide primers would not bind to the same, but to distinct genes, and no amplification would be possible in the PCR experiment.

Another factor that should be considered is the use of *Not*I restriction enzyme for the generation of the cDNA that was used as template in the PCR experiments. The recognition

site for this endonuclease is GCGGCCGC which is extremely rare in most cDNAs. But the high percentage of G and C in the genome of *Phytophthora sojae* (Qutob et al., 2000) increases the probability for the occurrence of this sequence, and thus, this 8-base recognition site could be one or more times present in the cDNA encoding P108. In this case, the cDNA would be cut in pieces, and the amplification by PCR would be impossible.

5.6.3. Cloning of the cDNA encoding P50

A 288 bp DNA fragment was generated by PCR when oligonucleotides derived from the partial peptide sequences of P50 were used. The deduced amino acid sequence of the PCR fragment showed the presence of some amino acids of the sequenced peptides that were not used for the generation of the oligonucleotides. This suggests that the PCR product is closely related to the gene encoding P50.

The significance of this PCR fragment as part of the cDNA encoding P50 was further checked by Northern analysis. A 1.4 kb RNA was specifically hybridizing with the PCR fragment (Figure 27), corresponding to the expected size of a mRNA encoding a 50 kDa protein.

The DNA fragment generated by PCR was used as a probe for the screening of the cDNA library representing actively growing mycelium of *P. sojae*. After screening a relative low number of phages, one positive phage was detected and isolated. The plasmid pZL-Ps-P50 was obtained after *in vivo* excision of the positive phage. This plasmid contains a 1625 bp long insert (Ps-P50).

Ps-P50 has two ATG codons relatively close to the beginning to its sequence (nucleotides 18 and 96, respectively), both in frame, that could encode the starting methionine. The second one is situated in a Kozak translation initiation sequence, ANNATGG (Kozak, 1987, 1990, 1991), and because of that it was chosen as starting codon for the heterologous expression in yeast. No assumption was taken about which ATG codon corresponds to the starting methionine, since not much is known about the upstream and non-coding sequences in oomycetes (Judelson et al., 1992), and the difference in molecular mass of the two putative polypeptides is not significant. As well, nothing is known about the signal peptides in these organisms. P50 is likely to be a membrane-bound protein, because it has been isolated from the microsomal fraction and the deduced amino acid sequence of Ps-P50 has two putative transmembrane domains. Thus, a signal peptide should be present for the right localization of

the polypeptide within the cell. This information could be useful to assign the role as starting codon to any of them.

The deduced amino acid sequence of Ps-P50 is in agreement with the peptides that were sequenced. Four peptides can be found in the amino acid sequence encoded by Ps-P50 that are similar to the peptides sequenced from the purified P50, showing similarities from 64% to 99% (see section 8.3., figures 42 to 45). In the deduced amino acid sequence of Ps-P50, all the peptides are in frame with each other. This corroborates strongly the conclusion that the four peptides are derived from the same protein. The PCR fragment is also present in Ps-P50, with 86.5% identity at the nucleotide level, and 88% at the amino acid level. No gap is present in the homologous section of the PCR fragment when aligned to the Ps-P50 sequence and it is in frame with the rest of the peptide sequences.

The fact that the Ps-P50 is neither completely identical to the PCR product, nor to the sequenced peptides of P50 is a hint that Ps-P50 may be a member of a gene family.

Running a BLAST search, no significant homologies were found at the nucleotide level. At the amino acid level, only one hit with low significance was found: a fragment of the iron channel multicopper oxidase of *Candida albicans* shows 37% homology with one third of the putative protein encoded by Ps-P50 (Figure 32). Because of the low level of homology, the presence of several gaps, and the short length of sequence fragments paired, this similarity result may not be significant. However, the fact that the glucan synthase is inhibited by copper, and, therefore, some binding of copper ions to any of the polypeptide of the glucan synthase complex should occur, leads to the idea that some structural motifs could be shared by both proteins. Although enzymatic activities of both proteins are not related, they could have in common a copper binding motive or a transmembrane domain.

The *Phytophthora* Genome Initiative (PGI) provides a data base which presently contains the sequences of 3126 ESTs of *P. sojae*. Unfortunately, the search algorithm within this data base is designed for nucleotide patterns (e.g., microsatellites or restriction sites). This means that only 100% homologies are considered as a match. Thus, random subsequences of 20 to 25 nucleotides length of Ps-P50 cDNA were used to search the PGI data base. Eight ESTs were found to have high similarity to Ps-P50. Whether there are more ESTs with homology to Ps-P50 still has to be demonstrated by using a better algorithm.

The presence of several ESTs, some with a homology close to 100%, demonstrates that Ps-P50 is a gene expressed in *Phytophthora sojae*. Moreover, the existence of at least 5 groups of ESTs with different degrees of similarity to Ps-P50 corroborates the idea of the presence of a gene family for Ps-P50.

The putative protein encoded by Ps-P50 has a molecular mass of 49,991 Da when translated from the first ATG. This correlates well with the apparent molecular mass in SDS-PAGE of the P50 polypeptide. From the 474 amino acids, 21.4% are serine and threonine. The high presence of these amino acids is known for proteins involved in the metabolism of carbohydrates (Erbeznik et al., 1998; Kuranda and Robbins, 1991; Liebl et al., 1997).

Two putative transmembrane domains were proposed in the Ps-P50 protein sequence. This fits with the finding that the glucan synthase activity resides in the membrane fraction, not only in *P. sojae*, but in all known glucan-producing organisms.

Heterologous expression was attempted in *E. coli* and in *S. cerevisiae*. For *E. coli*, ligation of Ps-P50 to the expression vector in the right orientation was not possible and the only viable transformants had the vector containing the Ps-P50 cDNA in the opposite orientation. As both orientations had theoretically the same probability, the existence of a selection factor has to be assumed. One hypothesis is that the basal expression of the Ps-P50 gene in the transformants with the right orientation was lethal for the bacteria, and, therefore, no colony was detected.

Transformation of yeast with a plasmid construct containing the Ps-P50 gene was successful. After activation of the expression with galactose, proteins of soluble and membrane fractions, from yeast carrying the pYES-Ps-P50 or an empty vector instead, were separated by two dimensional gel electrophoresis. Differences in the protein pattern between the pYES-Ps-P50 carrying transformant and control were detected only in the soluble protein fraction, in an area of IEF-SDS-PAGE corresponding to low molecular mass (around 20 kDa) and low pI (around 4). The low pI correlates with the putative pI for the Ps-P50 encoded protein, but not the molecular mass. It was expected that the heterologously expressed polypeptide would be present in the microsomal fraction, since there are two putative transmembrane helixes. However, preliminary sequencing experiments of the proteins isolated from two dimensional gels, indicate that they are proteins not expressed in the untransformed control. The possible toxicity of the expressed Ps-P50 protein in bacteria may be correlated with the presence of these new polypeptides in the transformed yeast, since they could be a stress response in yeast.

No additional glucan synthase activity was detected in the transformed yeast, in both microsome and soluble fractions, when compared with the control. This is not very surprising if it is taken in account that glucan synthesis probably resides in a complex of several polypeptides and, so far, no functional heterologous expression was reported for any of the known glucan synthases. Moreover, it is not guaranteed that the expressed polypeptide has the right folding and post-translational modifications (like glucosylation or myristylation), even when using an eukaryotic system. And further, it has to be taken into account that P50 may not be related to glucan synthase activity at all, but it was simply copurifing.

5.7. Outlook

In this work, a cDNA coding for a 50 kDa protein was isolated. P50 was co-purified with the glucan synthase activity by product entrapment. The basis of this purification method is the affinity to β-glucans and, therefore, other proteins with similar affinity but different function could co-purify with the glucan synthase. It would be interesting to assign a physiological role to P50. This could be achieved by generation of antibodies against the polypeptide and performing experiments with the purified material, like immunoprecipitation of the glucan synthase activity and inhibition of the glucan synthase activity. Moreover, the use of specific antibodies would allow the immunolocalization of P50 within the cell.

For the generation of antibodies, it would be helpful to design and use a better heterologous system, such as the use of a fusion protein for purification of the recombinant protein, or the use of other eukariotic systems than yeast.

It would be interesting, as well, to study the expression of the P50 at different life stages of *P. sojae*, as well as during the plant infection.

A significant amount of genetic information about glucan synthesis has been generated in other organisms. Most of it is from *Saccharomyces cerevisiae*, ascomycetes or imperfect fungi. Although belonging to a different kingdom than *Phytophthora sojae*, this information could be used for the generation of specific genetic probes that might lead to the identification of relevant genes. This information could be combined with the EST sequences in order to assign candidates involved in glucan synthesis.

It was demonstrated in this work that the Ps-P50 cDNA, that encodes the 50 kDa protein, is part of a gene family. It would be of interest to identify from the cDNA library other members of the family, and look for different expression patterns and functions.

In this work, it was not possible to clone the gene that codes for the 108 kDa polypeptide. The main obstacles appeared to be quantity and purity of the sample, as well as certain properties of the protein, such as its hydrophobic nature. Another possible reason for the failure in cloning the cDNA encoding the P108 was the low reliable peptide sequences provided by Edman degradation. The use of other protein sequencing techniques like tandem-MS/MS, with better resolution (Andersen and Mann, 2000), could help to get more reliable sequences using even lower amounts of purified protein, making the reverse genetics a promising approach.

6. Summary

Glucans, with the (1→3)-β-glucosidic linkage as major feature, are present in most of the higher plants, in many lower plants, as well as in microorganisms (Stone and Clarke, 1992). The synthesis of (1→3)-β-glucan *in vivo* is catalysed by the enzyme (1→3)-β-glucan synthase (EC 2.4.1.34; UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyl transferase) using UDP-glucose as substrate. The (1→3)-β-glucan synthase was characterised in a number of fungi and plants, but not much work was done with oomycetes (Stone and Clarke, 1992), even though one of the earliest successful *in vitro* assays for glucan synthase activity was done using *Phytophthora cinnamomi* (Wang and Bartnicki-Garcia, 1976, Selitrennikoff 1995).

In this work, the glucan synthase of the oomycete *Phytophthora sojae* was characterised, solubilized, and partially purified, and the cDNA for a protein co-purifying with the glucan synthase activity was cloned.

The glucan synthase of *P. sojae* had several features that distinguish it from what is known for glucan synthases from fungi and plants (callose synthases). Its apparent K_m value for UDP-glucose was higher than reported for other glucan synthases. The activity was GTP-independent and shown not to be activated by divalent cations like Mg^{2+} or Ca^{2+}, and shown to be inhibited by some others, like Cu^{2+} or Zn^{2+}. Some of these properties are shared with the glucan synthase from *Achlya ambisexualis* (Cabib and Kang, 1987), an organism that belongs to the same kingdom as *P. sojae*: the Chromista.

It was also demonstrated by NMR analysis and enzymatic degradation that the sole product of the CHAPS-solubilized glucan synthase of *P. sojae* was composed of long linear (1→3)-β-glucan chains.

The glucan synthase was purified by product entrapment. Two proteins, with apparent molecular masses of 108 and 50 kDa, were enriched and microsequenced. With the degenerated oligonucleotides derived from the sequenced peptides, PCR experiments were performed using as a template a cDNA library of actively growing *P. sojae* mycelium. No positive result could be obtained by using the oligonucleotides derived from the 108 kDa protein. In contrast, a full length cDNA (named Ps-P50) was cloned, using the oligonucleotides derived from the 50 kDa protein (P50). The deduced amino acid sequence of Ps-P50 cDNA contains sequence motifs homologous to the peptides sequenced from P50. This cDNA encodes a protein with a molecular mass of 49.991 Da with no homology found

in the data bases. Diversity between the PCR product and the cDNA clone, and various different homologous ESTs indicates that Ps-P50 is a member of a gene family.

Zusammenfassung

Glucane mit hauptsächlich (1→3)-β-glycosidischer Bindung kommen in den meisten höheren und niederen Pflanzen vor, ebenso wie in Mikroorganismen. Die Synthese wird *in vivo* durch das Enzym (1→3)-β-Glucansynthase katalysiert (EC 2.4.1.34;UDP-Glucose:1,3-β-D-Glucan 3-β-D-Glucosyltransferase), das UDP-Glucose als Substrat verwendet. Die (1→3)-β-Glucansynthase wurde bereits in einigen Pilzen und Pflanzen charakterisiert, doch bisher wurde noch wenig mit Oomyceten gearbeitet (Stone and Clarke, 1992), obwohl einer der ersten erfolgreichen *in vitro*-Tests auf Glucansynthase-Aktivität in *Phytophthora cinnamomi* durchgeführt wurde (Wang and Bartnicki-Garcia, 1976, Selitrennikoff 1995).

In der vorliegenden Arbeit wurde die Glucansynthase des Oomyceten *Phytophthora sojae* charakterisiert, solubilisiert und teilweise aufgereinigt. Darüber hinaus wurde die cDNA für ein Protein kloniert, das zusammen mit der Glucansynthase-Aktivität aufgereinigt wird.

Die Glucansynthase von *Phytophthora sojae* unterscheidet sich in mehreren Eigenschaften von denen aus Pilzen und Pflanzen (Callose-Synthasen). Der scheinbare K_m-Wert für UDP-Glucose ist höher als bei anderen Glucansynthasen berichtet. Die Aktivität ist GTP-unabhängig und wird nicht durch zweiwertige Kationen, wie Mg^{2+} oder Ca^{2+}, stimuliert und durch einige andere, wie Cu^{2+} oder Zn^{2+}, inhibiert. Einige dieser Eigenschaften findet man auch bei der Glucansynthase von *Achlya ambisexualis* (Cabib and Kang, 1987), einem Organismus, der dem selben systematischen Reich angehört wie *Phytophthora sojae*, den *Chromista.*

Es wurde auch durch NMR-Analyse und enzymatischen Abbau gezeigt, daß die CHAPS-solubilisierte Glucansynthase von *Phytophthora sojae* als einziges Produkt lange lineare (1→3)-β-Glucan-Ketten hervorbringt.

Die Glucansynthase wurde durch „product entrapment“ aufgereinigt. Zwei Proteine mit scheinbaren relativen Molekularmassen von 108 kDa und 50 kDa wurden angereichert und der Mikrosequenzierung unterzogen.

Mit den aus der Mikrosequenzierung erhaltenen Oligopeptid-Sequenzen wurden degenerierte Oligonucleotide entworfen und in PCR-Experimenten mit einer cDNA-Bank aus wachsendem *Phytophthora sojae*-Mycel eingesetzt. Nach Einsatz der vom 108 kDa-Protein abgeleitet Oligonucleotide konnte kein positives Ergebnis erzielt werden. Oligonucleotide, die Peptide des 50 kDa-Protein (P50) repräsentierten, führten zur Isolierung einer cDNA-Vollängenklons für das Ps-P50. Die Aminosäuresequenz, die sich von Ps-P50 cDNA ableitet, weist Homologie zu den sequenzierten Peptiden aus P50 auf. Die cDNA codiert für ein

Protein mit der molekularen Masse von 49,991 Da, zu dem keine Ähnlichkeiten in den Datenbanken gefunden wurden. Abweichungen zwischen PCR-Produkt und dem cDNA-Klon, sowie Homologien zu verschiedenen ESTs, weisen darauf hin, daß Ps-P50 ein Mitglied einer Genfamilie ist.

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8. Appendix

8.1. P. sojae EST sequences

10-10f-my

AATTCGGCACGAGGTTACGCCGGACGGTGATAACTGCACTTTCAGCTACAAGATTCTGGGCTACCTGA
ACATCGACGACCTGGTCGGCATCACGGAGATGGGCTACAGCAACTACACGGAGTTCTGCGAGGACGGT
GGCGTGGAGTTCAAGGCCACCAACACCGGCAGTGGCTTCGAGGTGGACGAGGCCATTGACTTCTGGCT
GAACCCGGGTGACGAGGACGCCAACTCGAACCGCACTACCATCATGGTGGAGATGTACAACGAGCTGC
CAAGAATGGCACGAAGCGAGAACATGGAACCGCTTCCCGTCGGTCGATACCTGACGTCGGCCAACCCC
AAATGCTACGAAAACAAGCGCTTNCTTGCGCCAACTNGCAATACGGNTTGAACCCCACGCTTTACTTC
GCAAGATCTTGCGCCCGTGTGCTCGTCGGATGCCCGACGGTTGCGAGGCCGCTCCCTTCGTCCTTCTC
GTTCCCGGAAGCTGACGCTTGCCTTTTTAACTNTTTCGTCGGACGGGTTGTCGGAACTNGAACCAAAA
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GGGNCTTGNAAAAANCCCCCCCCTTTTNGNANNNGGGGGAAANGGANAAACCCTTNGNAATTGTTTGG
GGCCTTANCCCCCTTTGGNANNNNATTTNNNAANGGGNTTGGTTTNTTGGNAATTNAAAANNAAAACT
NAANNTTCCCGGTTTTGNAAAANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAAAAANNTNNNNAAANNNTTTTTTNAAAGGGGGNNNNGG
GNCCCCCNNNNNTTTTTNCCNCCCCNGGGGGGGGGNCCCCCNNNNAANNNNNCCCCNANTNNCCCCCN
TNNNNGGGGGGGNNNTTNAAAAANNAANNGGGNNNNNGNNTTTNAAAANNNNNNNNNNGGGGAAAAAA
NCNNGGNNNNCCNNNNTTAANNNNTTNNNNNAAAAANNCCNTTTTTNNNNNNNGGGNNNAAAAAAAAA
ANNNNNNNNNNNNNNNNTTTNNAAAANNNNNCNNNNNNNNNGNNNANGGNANNNNNNTTTNNNGNTNA
NTTTN

10-11g-my

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CGTCGTGTTCTACGAGATCACCATCGTGCAGCCGACGTACGGCATCAAGTACTACGAGAGCCACACGC
CTNCGGAGTACGGCGAGTTCGTGGCCATGGACGGCGCCAAGTGCACGGACGAGGGCGACGACATTTCC
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GCAGACGTCGGACCCGCGCGCCCCGTACCCCGGCAACTACTGGTTCTNATACCCCAACTCGTGCGCGC
AGGAGCTTCGNGCCGACAAGACGGACGAGTGCCGNGCCGAGTACCCTGGCGGTCTGTGCGCCATGGGT
GTTACGCCGGACGGTGATAACTGCACTTTCAGCTACAAGATTCTGGGCTACCTGAACATCGACGACCT
GGTCGGNATCACGGAGATGGGCTACAGCAACTACACGGAGTTCTGCGAGGACNGTGGCGTTGGAGTTC
AAGGCACCAACACCNGCAGTGGCTTCTANGTGGACGAGGCCATTGACTTCTGGCTGAACCCGGTGACG
AGGACNCCAACTNGACNGGACTACATTATGGTGGAGATTNAACNAGCTGGCAAAATGGACGAGGANAC
ATGAACCGTCCGTGGTCGATACCTGACTGGCAACCAATNTCAGACAGTTGGTGGCAGTNGATCGGTGA
CCNANTGATNGAATTGCCTGGTCGCGATGCACGTGAGGCGTCTNCTTGTCGATNNTGNTTATTTTGCG
TGGTAAACGTTTGACTATGAAATTNTTGCTGNTGCANCNTACGGTTCTNTNTAGTGANCTTANGAGNC
TNTTGTCTGATAGTGTATAGAA

11-3h-zo

AATTCGGCACGAGCCAAGTACGGCGATACCAAGGAACAGCAGTACGTGAACAACCTGGACACGGTCAA
CATGGCGTCCGTGGAGGGCGCGCTCATGTACGTGCAGGCCGAGGGCATCAACGTGAACGAGCAGTCGG
TCAAGTGCCACCGCAAGAACGACATGCAGTACGTCGTGTTCTACGAGATGATCATCGTGCAGCCGACG
TATGGCATTAAGCACTACGAGAGCCACACGCCTCAAGAGTACGGCGACTTCGTGGCCATGGACGGTGC
CAAGTGCACGAACGCCGGAGAGGACCTGCCCCAGAGCTGCAAGTCGTATAACGGTCTGGACGGCGAGA
AGCGCATTGGCCCCGTGGTGGGCTGCAACCCGCAGGGATCGGACCCGCGCGCGCCATACCCCGGCAAC
TTTTGGTTCTCGTACCCGAACTCGTGCGTGCAAAAGTACCGAGCGGAGAAGACCGACCAGTGCCGTGC
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AAGATCCTGGGCTTCTGAACATCGACGACCTNGTGGGCATNANCAAGATGGCTTNAACACATACCAAC
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5-8d-zo

AATTCGGCACGAGGAACGACATGCAGTACGTCGTGTTTTACGAGATGACCATCGTGCAGCCCACGGCT
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6-5h-ha

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GGACNGGGCCNAGTGCACGGACGANGGCGACGACATTTCCGAAGACTGAAGGTNTATTACGGCTGGAC
GGNGAG

3-8h-zo

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7-2f-zo

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7-3e-zo

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8.2. Two dimensional gel electrophoresis

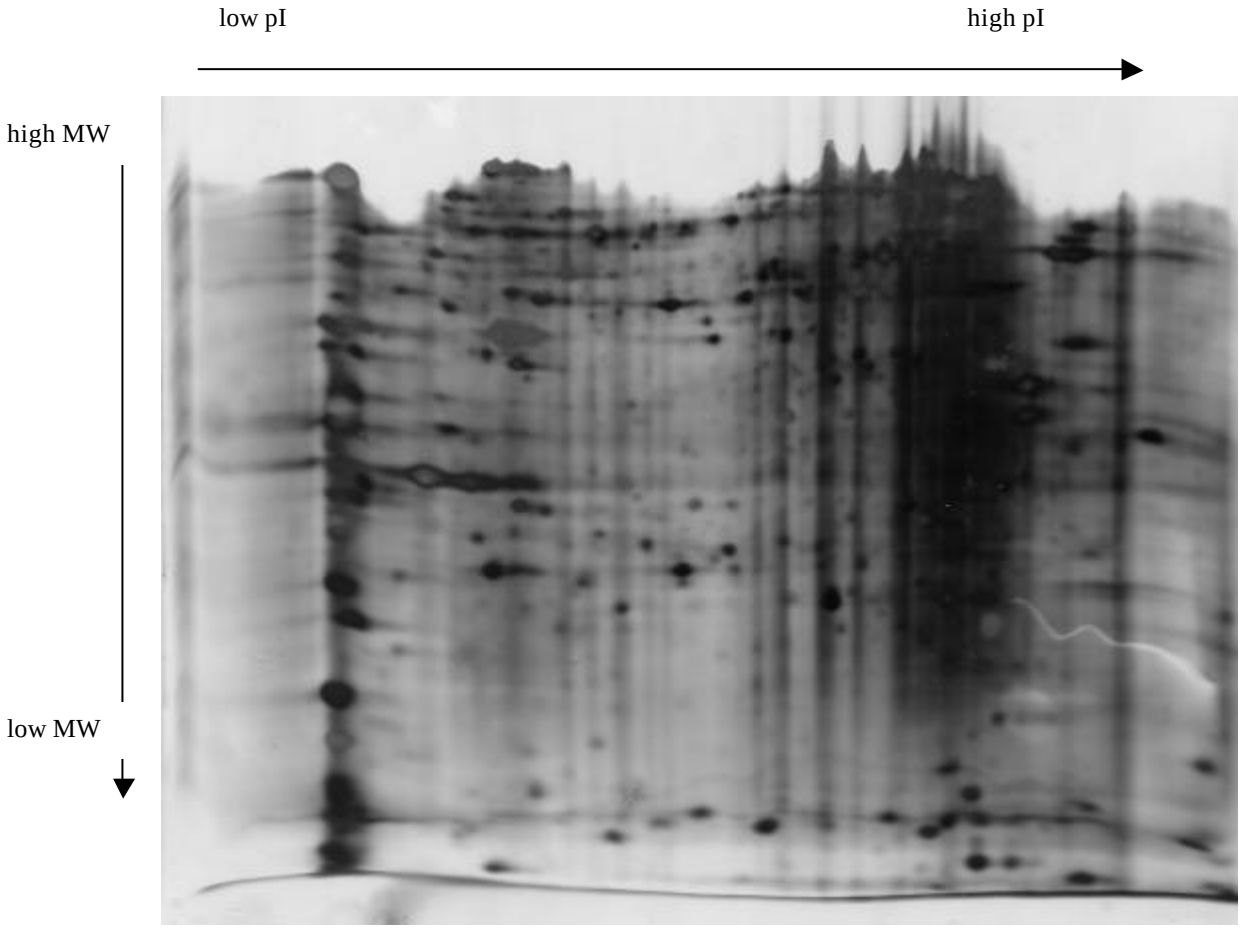

Figure 38: Two dimensional gel electrophoresis of proteins from a microsomal fraction of yeast transformed with the empty vector pYES2. IEF was performed at the horizontal axis and SDS-PAGE at the vertical axis.

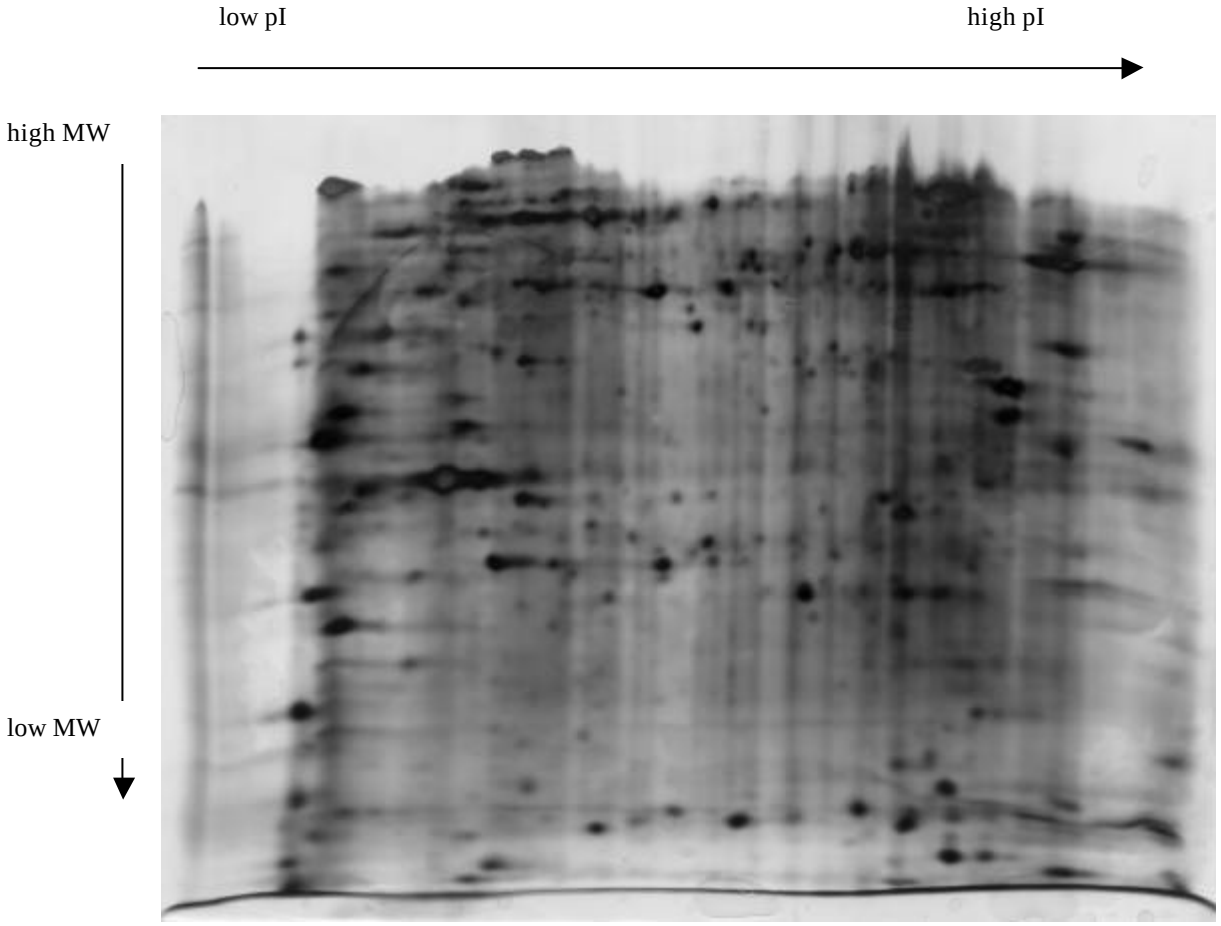

Figure 39: Two dimensional gel electrophoresis of proteins from a microsomal fraction of yeast transformed with pYES-Ps-P50. IEF was performed at the horizontal axis and SDS-PAGE at the vertical axis.

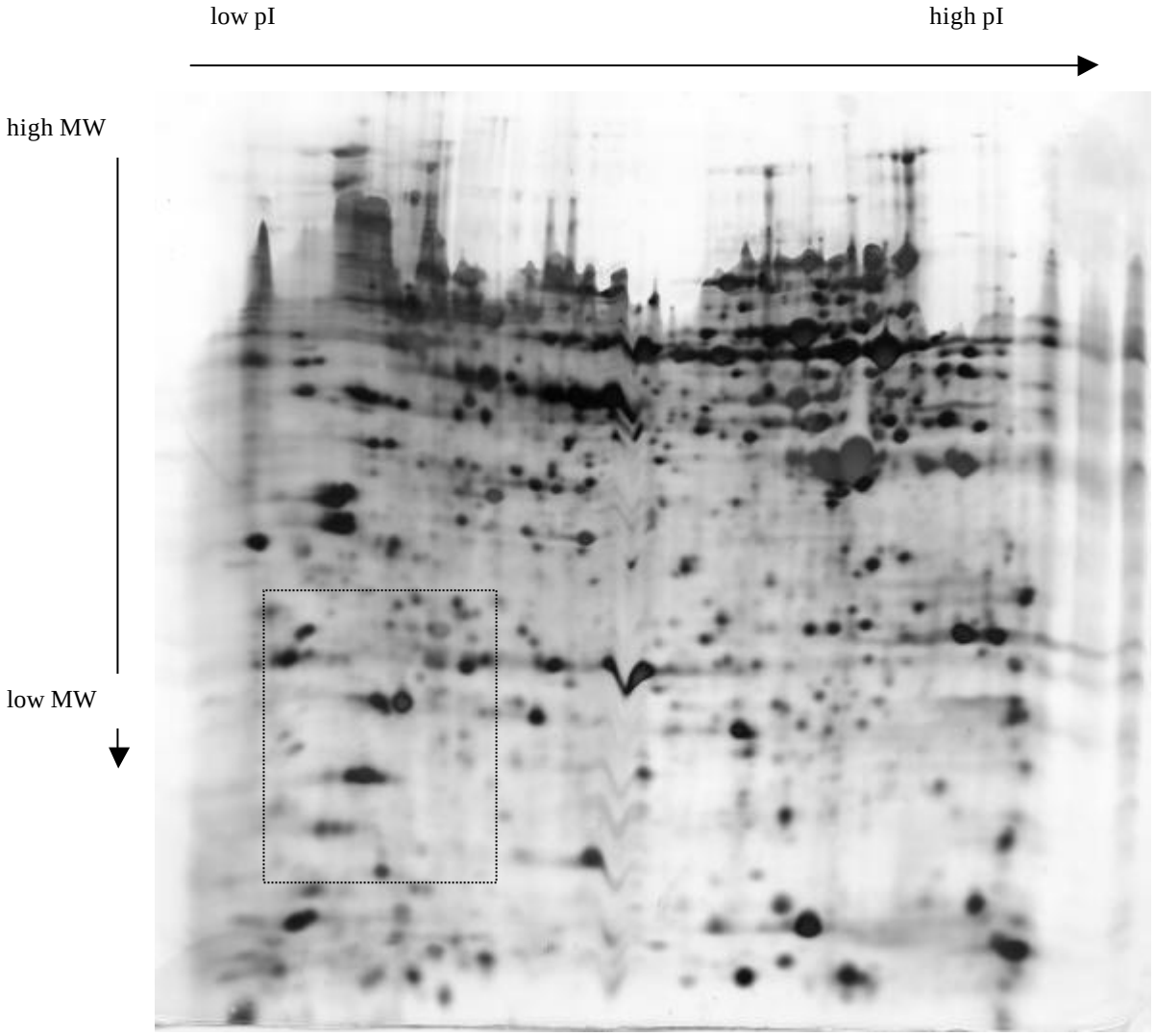

Figure 40: Two dimensional gel electrophoresis of proteins from a soluble fraction of yeast transformed with the empty vector pYES. IEF was performed at the horizontal axis and SDS-PAGE at the vertical axis. Selected area (dotted square) is presented in Figure 35, panel A.

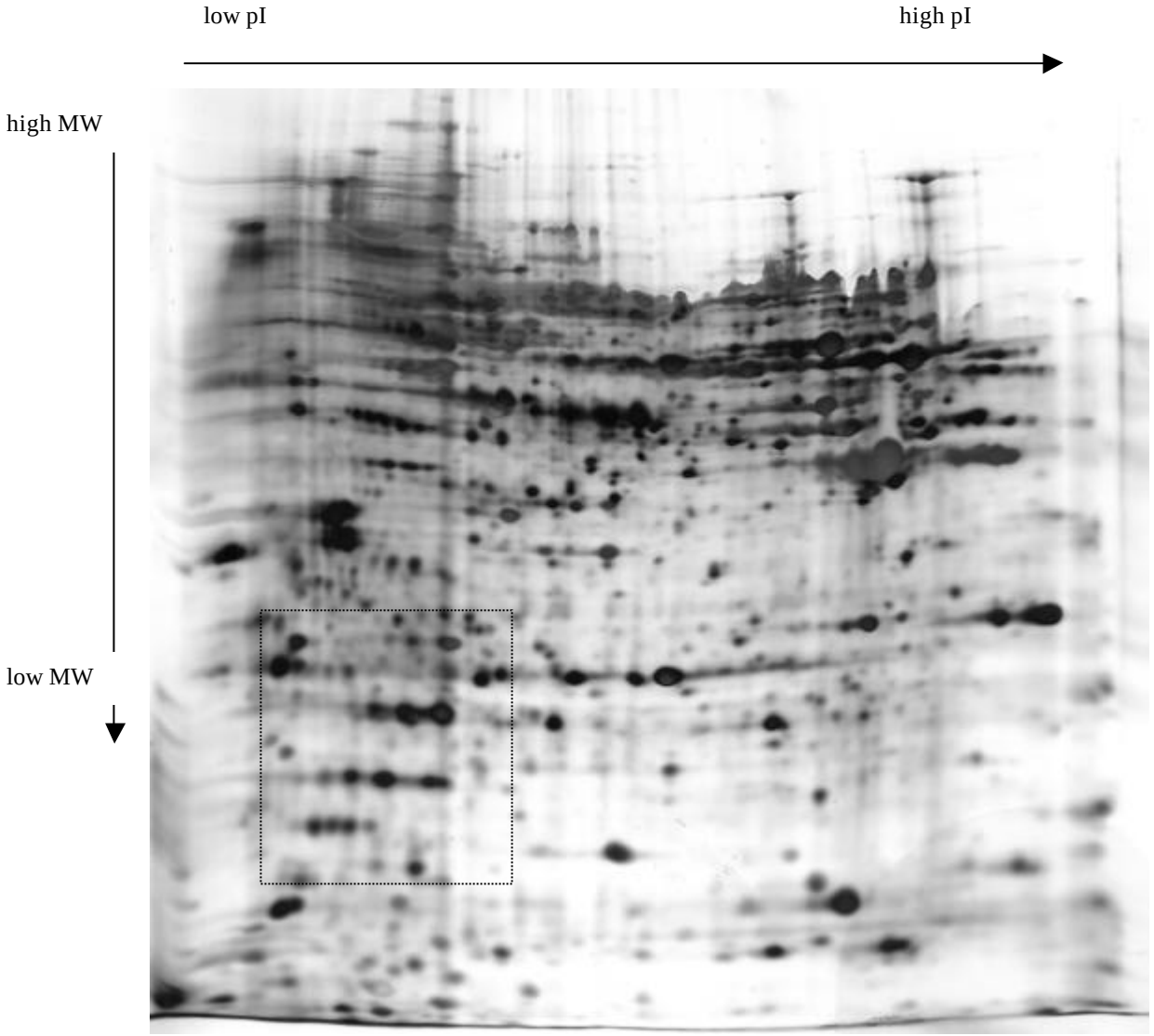

Figure 41: Two dimensional gel electrophoresis of proteins from a soluble fraction of yeast transformed with pYES-Ps-P50. IEF was performed at the horizontal axis and SDS-PAGE at the vertical axis. Selected area (dotted square) is presented in Figure 35, panel B.

8.3. Sequence alignments

```
Score = 34.6 bits (74), Expect = 3.0
Identities = 10/11 (90%), Positives = 11/11 (99%)


Ps-P50: 90  VQARVQGDAPV 100
           +QARVQGDAPV
  iqar: 3   IQARVQGDAPV 13
```

Figure 42: Alignment of the derived amino acid sequence from Ps-P50 and the "iqar" peptide.

```
Score = 49.9 bits (110), Expect = 7e-05
Identities = 14/18 (77%), Positives = 16/18 (88%)


Ps-P50: 177 KYYESHTPPEYGEFVAMD 194
           KYYE+H P EYG+FVAMD
   yen: 1   KYYENHNPLEYGDFVAMD 18
```

Figure 43: Alignment of the derived amino acid sequence from Ps-P50 and the "yen" peptide

```
Score = 21.8 bits (44), Expect = 20959
Identities = 8/13 (61%), Positives = 10/13 (76%)


Ps-P50: 320 KATNTGSGFEVDE 332
           KA NTG G EV++
   eve: 1   KARNTGVGIEVEQ 13
```

Figure 44: Alignment of the derived amino acid sequence from Ps-P50 and the "eve" peptide

Score = 15.2 bits (28), Expect = 26316
Identities = 7/14 (50%), Positives = 9/14 (64%), Gaps = 1/14 (7%)

```
Ps-P50: 38 TSTTAGSG-SSGVV 50
           +S T+  G  SGVV
   sat: 2  SSATSNPGAISGVV 15
```

Figure 45: Alignment of the derived amino acid sequence from Ps-P50 and the “sat” peptide

8.4. Detergents

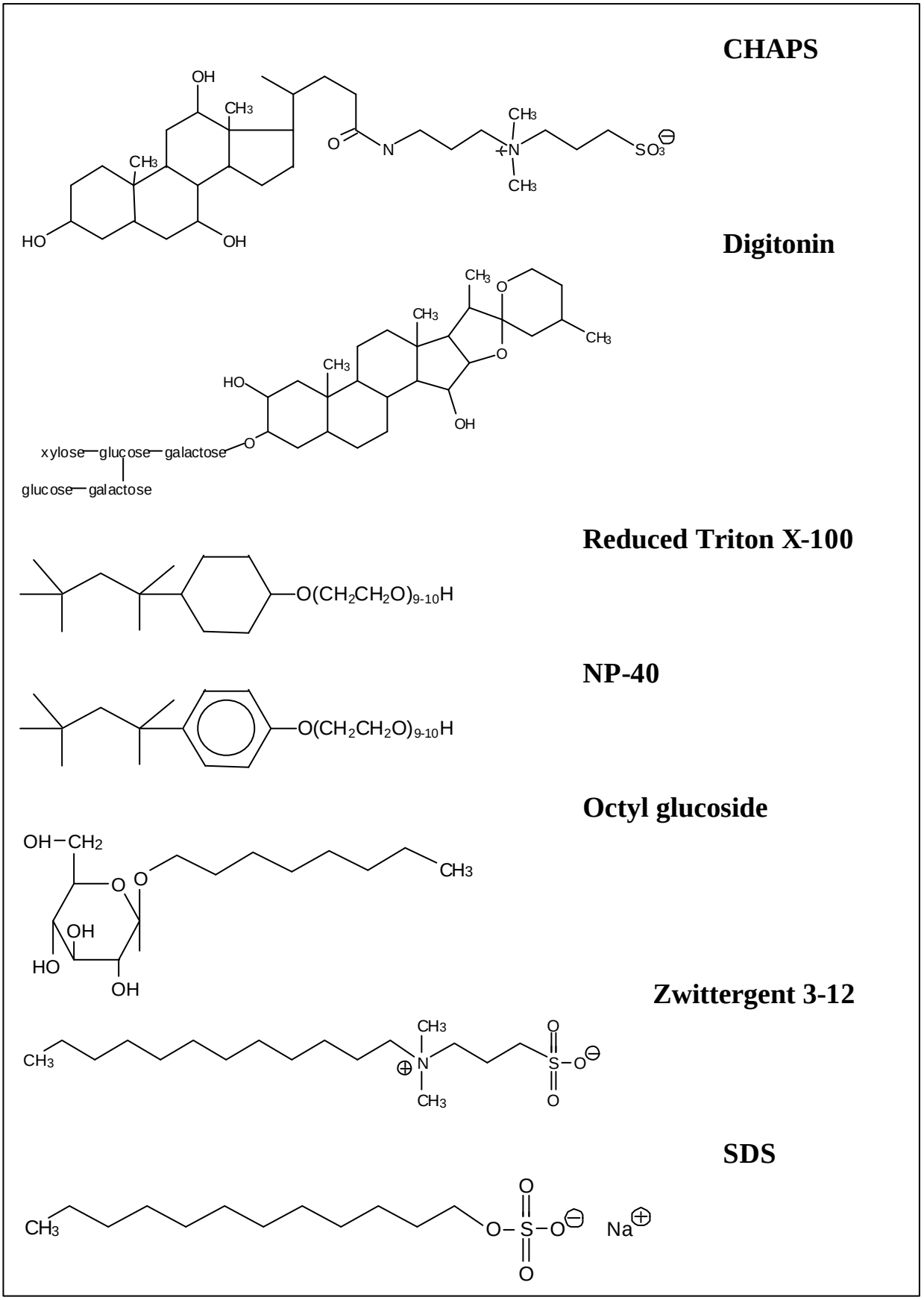

Figure 46 Chemical structures of detergents used for glucan synthase solubilization.

Lebenslauf

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Geburtsdatum:	17.02.1970
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Familienstand:	verheiratet
Staatsangehörigkeit:	**spanisch**
Erlangung der Hochschulreife:	Mai 1988, Instituto Caro Baroja, Getxo (Vizcaya), Spanien
Studium der Chemie:	Oktober 1988 – September 1994 an der Universidad Autónoma de Madrid Schwerpunkt: Biochemie und Molekularbiologie Abschluß: Diplom
anschließende Beschäftigung;	September 1994 – Dezember 1995 als wissenschaftlicher Mitarbeiter bei Dr. Eric Cosio im Centro Nacional de Biotecnología (Madrid) Januar 1996 – Dezember 1996 als wissenschaftlicher Mitarbeiter bei Prof. Dr. Jürgen Ebel im Botanischen Institut der LMU
Promotionsarbeit:	Januar 1997 – Mai 2000 bei Prof. Dr. Jürgen Ebel im Botanischen Institut der LMU
Derzeitige Beschäftigung:	seit September 2000 als wissenschaftlicher Mitarbeiter im Institut für Biotechnologie und Wirkstoff-Forschung e. V. (Kaiserslautern)

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