UDP-glucose: β-(1-3)-glucan (paramylon) synthase from *Euglena gracilis*

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Thesis presented in partial fulfillment of the
requirements for the degree of
Magister Scientiae
in
Plant Biotechnology
in the
Faculty of Science
Stellenbosch University

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December 2007

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:..... Date:.....

Vir Ma en Pa

Abstract

The photosynthetic protist *Euglena gracilis* synthesizes a storage carbohydrate named paramylon, a glucan consisting only of β-(1-3)-glycosidic linkages. The enzyme that produces paramylon is a glycosyltransferase commonly known as paramylon synthase (EC 2.4.1.34; UDP-glucose: 1,3-β-D-glucan 3-β-D-glucosyl transferase). This enzyme uses UDP-glucose as its main substrate. In 2001, Bäumer *et al*. isolated and partially purified paramylon synthase, but never presented any sequence information. Hence, the main aim of this project was to isolate and characterize the gene(s) coding for the paramylon synthase.

Different approaches were taken in order to isolate and characterize the gene(s). In the first part of the study molecular techniques were used to try and identify the gene. The two methods used were library screening and PCR amplification. Different libraries were screened using either functional staining or an affinity probe. The second method concentrated on the use of degenerate oligonucleotides, based on the amino acid sequences of conserved regions from known β-(1-3)-glucan synthase genes from various organisms, to PCR amplify the gene sequence from *Euglena*. These approaches were not successful in the isolation of the gene(s).

In the second part of the study protein purification techniques were used in an attempt to obtain *de novo* protein sequence from the purified paramylon synthase enzyme. Several protein purification techniques were tried with the most successful being preparative ultra centrifugation followed either by sucrose density centrifugation or product entrapment (a type of affinity purification). These resulted in partial purification of the paramylon synthase protein. The partially purified proteins were separated using polyacrylamide gel electrophoresis, and the polypeptides able to bind the precursor, UDP-glucose, were identified using a radiolabeled isotope of UDP-glucose. These polypeptides were subjected to LC-MS-MS in order to obtain sequence information from them. One tryptic fragment showed high homology to β-(1,3)-glucan synthase genes from different yeasts.

Opsomming

Die fotosinterende protist *Euglena gracilis* sintetiseer ‘n bergings koolhidraat bekend as paramylon, ‘n glukaan wat slegs uit β-(1-3)-glikosidiese verbindings bestaan. Die ensiem wat paramylon produseer is ‘n glikosieltransferase algemeen bekend as paramylon sintase (EC 2.4.1.34; UDP-glukose: 1,3-β-D-glukaan 3-β-D-glukosiel transferase). Die ensiem gebruik UDP-glukose as hoofsubstraat. In 2001 het Bäumer *et al*. die paramylon sintase geïsoleer en gedeeltelik gesuiwer, maar geen geenvolgorde is uit die werk verkry nie. Daarom was die hoofdoel van die projek om die geen/gene wat kodeer vir paramylon sintase te isoleer en te karaktiseer.

Verskillende benaderings is gevolg om die geen/gene te isoleer en te karaktiseer. In die eerste deel van die studie is gekonsentreer op molekulere tegnieke in poging om die geen te identifiseer. Die twee metodes wat hier gebruik is die biblioteek keuring en PKR amplifikasie. Verskillende biblioteke is gekeur deur die gebruik van of funksionele kleuring of affiniteits probes. Die tweede metode was deur die gebruik van degeneratiewe oligonukleotiede, gebasseer op die aminosuur volgorde van gekonserveerde streke van bekende β-(1-3)-glukaan sintase gene van verskeie organismes, om die geenvolgorde te PKR amplifiseer vanuit *Euglena*. Die benadering was nie suksesvol in die isolasie van die geen/gene nie.

In die tweede deel van die studie is gebruik gemaak van proteïensuiweringstegnieke in poging om die *de novo* proteïenvolgorde van die gesuiwerde paramylon sintase te bekom. Verskeie proteïensuiweringstegnieke is probeer en die suksesvolste was preparatiewe ultrasentrifugering gevolg deur sukrosedigtheidsgradiëntsentrifugering of produkbetrapping (‘n tipe affiniteitsuiwering). Dit het die gedeeltelikke suiwering van die paramylon sintase proteïen tot gevolg gehad. Die gedeeltelik gesuiwerde proteïene is geskei met behulp van poliakrielamied gel elektroforese en die polipeptiedes wat oor die vermoë beskik om die voorganger, UDP-glukose, te bind is geïdentifiseer met behulp van ‘n radiogemerkte isotoop van UDP-glukose. Die polipeptiedes is onderhewig aan LC-MS-MS gestel om die volgorde informasie te bekom. Een triptiese fragment het homologie getoon aan β-(1,3)-glukaan sintase gene van verskillende giste.

Erkennings

Ek is dankbaar vir die geleentheid wat ek gehad het om te studeer aan die Instituut vir Plantbiotegnologie. Aan almal werksaam aan die IPB, dankie vir al die hulp en advies en julle vriendskap.

Lisa en Suereta: ek weet nie of ek dit sonder julle kon gemaak het nie. Julle was nog elke dag die laaste paar jaar daar vir my. Dankie vir al die koffies, ondersteuning en hulp en dankie dat ek altyd op julle kan staat maak!

‘n Groot dankie aan my familie, dankie dat Oupa elke week gebel het om te hoor of ek al klaar is en spesiaal dankie vir Ma, Pa, Werner, Emile, Izel en Inge, dankie vir al julle liefde, ondersteuning en geduld deur al my jare van studeer. Ek waardeer dit baie en is lief vir julle!

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List of Abbreviations

ºC	degrees centigrade
^{33}P	phosphorus-33 (radio-isotope)
bp	base pairs (nucleic acid)
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease I (EC 3.1.21.1)
dNTP	deoxyribonucleotide triphosphates
dpm	decays per minute
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequenced tag
g	gram
g	gravitational force
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl- β-D-thiogalactopyranoside
K_m	Michaelis constant
kDa	kilodalton
L	litre
µL	microlitre
mL	millilitre
LB	Luria Broth
min	minute
µM	micromolar
mM	millimolar
nm	nanometre
nmol	nanomole
NaAc	sodium acetate
mRNA	messenger ribonucleic acid

OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pfu	plaque forming unit
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	unit (enzyme)
UDP	uridine diphosphate
UTP	uridine triphosphate
UV	ultraviolet
V_{max}	maximal velocity
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

List of Tables and Figures

Chapter 1
Introduction

The South Africa health industry has shown rapid growth over the last few years and a wide variety of nutritional supplements are today available on the market. Supplements are used by a wide variety of people, such as those leading an active and demanding lifestyle, those with illnesses or with poor eating habits, and the health conscious. Supplements are even available to growing, young animals.

One of these supplements, although not yet popular in South Africa but available in the United States of America and United Kingdom, is β-(1,3)-glucan, named simply 'glucan' or 'β-glucan'. This has been found to be one of the most exciting discoveries in the field of nutritional supplements. Earlier *in vitro* studies have shown that macrophages have a specific receptor for β-(1,3)-glucans and that when the glucan binds to this receptor it stimulates a cascade of events leading the production of cytokines, soluble factors secreted by the cells of the lymphoid system that act as signals to other lymphoid cells (Czop, 1985; DiLuzio, 1983). Later, Wyde (1989) showed that orally administered β-(1,3)-glucans are also effective in stimulating the same response. The systemic effect of β-(1,3)-glucans can, therefore, be described as non-specific immune stimulation. In addition, β-(1,3)-glucans increase the effectiveness of antibiotics and reduces the low density lipoprotein (LDL) cholesterol level in the body (Kogan, 2000; Stone and Clarke, 1992). The mechanism behind this effect is not yet fully understood and probably depends on many physical factors, such as the specific molecular structure of the glucan, its solubility in water, the molecular weight, branching, the presence of charged residues and conformational features (Freimund *et al*., 2003; Yadomae and Ohno, 1996).

The β-(1,3)-glucans used for nutritional supplements are currently obtained from baker's yeast (*Saccharomyces cerevisae),* but in nature β-(1,3)-glucans are widespread, being found in higher plants, bacteria, fungi, and algae. In plants β-(1,3)-glucans are part of the cell wall and are usually synthesized as a wound response. Some fungi and algae, on the other hand,

produce β-(1,3)-glucans as storage carbohydrates, and in bacteria they are usually excreted as exopolysaccharides.

Over the past years, efforts have been directed towards the characterization of the properties and functions of β-(1,3)-glucan synthases from these organisms. β-(1,3)-glucan synthase has been extensively studied to date because of its ubiquity in higher plant tissues (Lawson *et al*., 1989). Most of the β-(1,3)-glucan synthases are membrane-associated complexes and are difficult to purify and characterize due to their inherent instability after solubilization from their native membrane environment (Drake *et al*., 1992). The protein complexes in which β-(1,3)-glucan synthases are usually present are very large, consisting of several protein subunits.

All of the enzyme complexes producing β-(1,3)-glucans in plants, bacteria and yeast are known to be present in the plasma membrane and secrete the β-(1,3)-glucans either into the cell wall, or outside the cell. Their utilization by the biotechnology industry is, therefore, difficult as such proteins are often only active when present within a membrane rather than when in the soluble fraction. The protist *Euglena gracilis,* however*,* manufactures an intracellular granular reserve β-(1,3)-glucan in high amounts (Barsanti e*t al*., 2001). Because this reserve carbohydrate is granular it might be expected (in analogy with the plant granular carbohydrate storage polysaccharide starch) that the enzymes synthesizing it would be soluble, rather than present in a membrane. If this were the case, then these glucan synthases would be easier to use from a biotechnological point of view. They could be expressed in microorganisms and the carbohydrate isolated after growth in bio-reactors, or they could be used to manufacture transgenic plants that would then manufacture β-(1,3)-glucans in their cytosol.

The most detailed recent study on the biosynthesis of paramylon was performed in 2001 by Bäumer and co-workers. This group isolated and partially purified paramylon synthase from *Euglena gracilis*. They found that, like other β-(1,3)-glucan synthases, it was present in a large complex with several subunits, of which two were shown to have the ability to bind UDP-glucose. What was also demonstrated, however, was that the enzymes present within this complex were bound into granula membranes. The primary aim of this study, therefore, was to isolate the paramylon synthase gene from *E. gracilis* and by doing so confirm the localization of the enzyme complex.

In Chapter 2 an overview of the protist *E. gracilis* is given, as well as purification techniques used in isolating the β-(1,3)-glucans synthase protein from different organisms. In this study, as an initial attempt cDNA libraries were screened for the cDNAs that code for paramylon synthase (Chapter 3). Secondly, by means of protein purification it was further attempted to purify paramylon synthase in order to obtain *de novo* protein sequence from this synthase (Chapter 4).

Chapter 2
Review of Literature

2.1. Introduction

Since the 1980's various attempts have been made to study β-glucan synthases in different organisms, from higher plants to yeast, protists and bacteria. Much progress has been made in identifying the genes coding for these synthases, especially those that manufacture β-glucans as a component of cell walls. Far less progress has been made, however, in studying their activities. The reason for this is that the β-glucan synthases are often present in membranes, and are part of larger protein complexes. As such they are extremely difficult to purify in their native state. The protist *Euglena gracilis* is unusual as it accumulates a pure β-(1,3)-glucan in granular form that acts as a storage carbohydrate and is commonly known as paramylon. Very little is known about paramylon synthesis in *E. gracilis*, as the genes coding for the synthases have not been identified.

Apart from a brief overview on the taxonomy and morphology of the protist *E. gracilis*, this overview discusses the difficulty of isolating membrane-bound enzyme complexes and the development of novel techniques that enable the isolation of these complexes while maintaining their function. In addition, the methods used to isolate these enzyme complexes from different organisms, including *E. gracilis,* will be described.

2.2. *Euglena gracilis*

2.2.1. Genus

The first cells of the family *Euglena* were discovered by C.G. Ehrenberg (1795 - 1876) in 1830. *Euglena* is a group of single-celled organisms and belongs to the kingdom Protista that includes unicellular algae, simple fungi and protozoa. This genus demonstrates some

plant-like, as well as animal-like characteristics, and presented a taxonomy problem to early scientists. Cavalier-Smith (1993) decided to classify *Euglena* in a separate phylum, Euglenozoa, to avoid further confusion. Phylogenetically *Euglena* was found to be closely related to *Trypanosoma* (Cavalier-Smith, 1993) although with a very different metabolism. Apparently an evolutionary ancestor of *Euglena* acquired chloroplasts, probably from an endosymbiotic relationship with algae. All known species of *Euglena* live in fresh water environments and reproduce asexually.

Felski (2004) has reported that, to date, a total of 250 different *Euglena* species have been described. However, the possibility exists that the total number of species is smaller because the size and form of a species can depend on different factors such as nutritional conditions, the cells growth phase, and environmental factors. It is also possible, therefore, that some of the described species are only variations of others.

2.2.2. Euglena gracilis

E. gracilis was one of the first photosynthetic, eukaryotic microorganisms used for laboratory purposes. The reason for this is that *Euglena* is easily cultivated under laboratory conditions. The genus was found to be biochemically, physiologically and morphologically flexible. The fact that it is also easily studied under a light or electron microscope added to its popularity (Felski, 2004).

The *Euglena* cell can vary in size from 10 μm to almost 500 μm in length and is normally elongated and cylindrical, with most cells having a spindle-like shape (Figure 1). *Euglena* cells have a pellicle surrounding them and no cell wall. This pellicle is quite flexible, so its shape changes considerably during euglenoid movement. For its movement, *Euglena* has a striking flagellum that is continually mobile from base to tip, and is held either in front, or laterally, of the moving cell. An eyespot (stigma) is present in most euglenoids, ensheathing the neck of the reservoir, and contains β-carotene derivatives and other carotenoid pigments. Unlike plant cells that contain an eyespot, the eyespot in euglenoids is not a specialized region containing chloroplasts. *E. gracilis* does contain chloroplasts, however, and they contain chlorophylls *a* and *b*, β-carotene, antheroxanthin, neoxanthin, and small amounts of other carotenoids and quinones (Leedale, 1967). Despite this, many other characteristics indicate that the euglenoids are not closely related to the land plants.

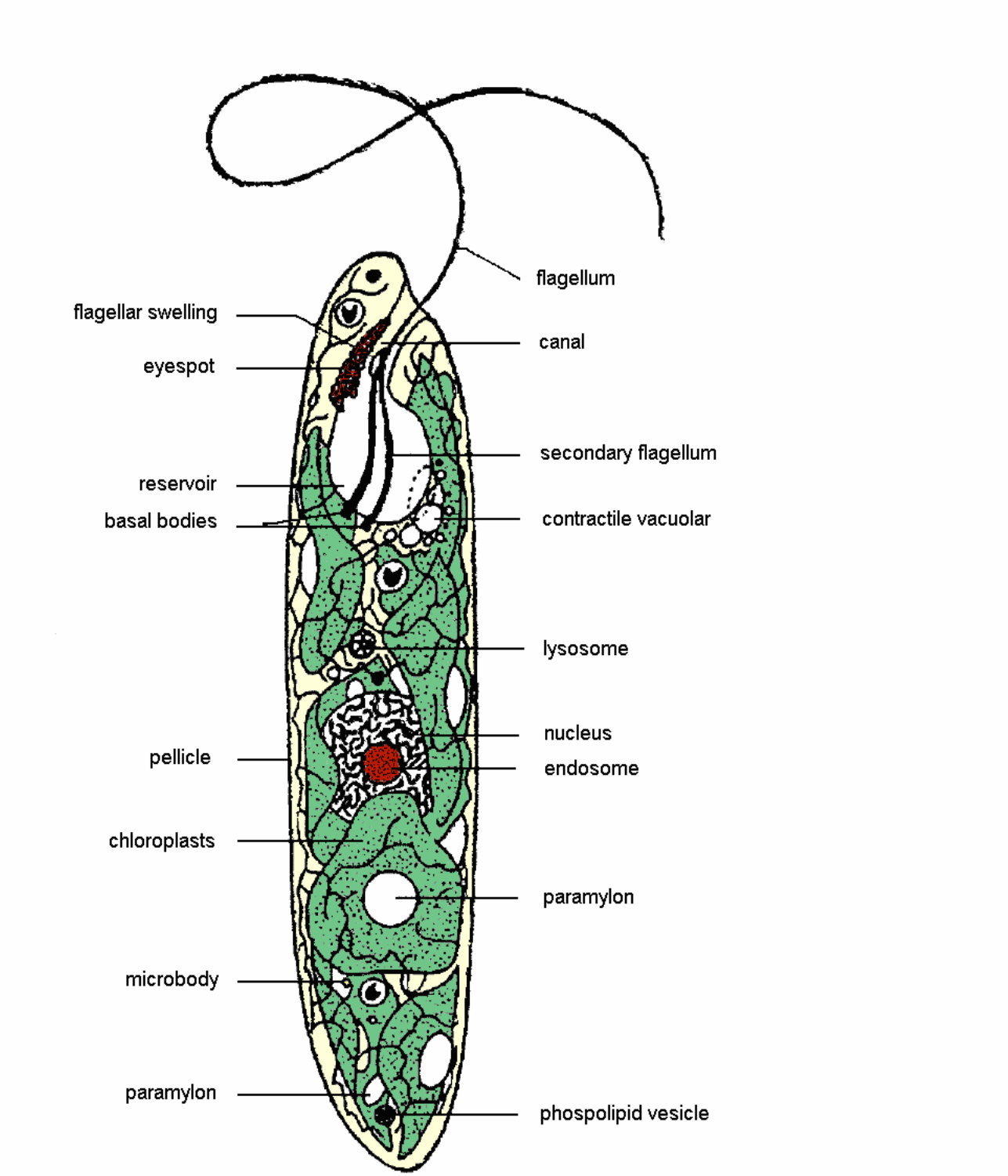

Figure 1. Schematic diagram of *Euglena gracilis* showing typical structures (Adapted from Buetow, 1999).

2.2.3. Growth conditions of E. gracilis

Members of the family *Euglena* are widespread in nature. The species are free-living and settle in fresh water washbasins, storage tanks and seas. No saltwater forms have yet been discovered, even though *Euglena* is found in the marine sediment. *Euglena* species are generally aerobic, but some tolerate anaerobic conditions under unfavourable conditions. *E. gracilis* can survive extreme conditions such as temperatures ranging from 1 up to 38 °C (Felski, 2004) and pH values ranging from 2.3 to 11 (Felski, 2004; Yamane *et al*., 2001).

Several factors are important in determining the rate of cell growth in *Euglena*. One of these is the history of the cells. The previous nutritional conditions influence the rate of adaptation of *Euglena* when transferred to a new medium (Yaden, 1965). If cultivation took place under favourable conditions, and the cells were not stressed, they will adapt fast when transferred to a new medium. From this point the growth phase and the external carbohydrate source will determine the cultivation rates of the cells (Pringsheim, 1955).

Another factor determining growth rate is the cultivation method of the cells. It was shown that *Euglena* could grow photoautotrophically, using light and CO_2 as the inorganic carbon source, heterotrophically using various organic carbon sources, or mixotrophically in the presence of light and organic carbon (Ogbonna *et al*., 1998). In the past *E. gracilis* cells have been grown successfully on different carbon sources, including glutamate / malate, DL-lactate, D-glucose, and ethanol (Jasso-Chávez *et al*., 2003). During growth it was shown that *E. gracilis* could only utilize the energy from available light in the presence of an organic carbon source (Yamane *et al*., 2001).

In a comparative study of carbon sources, glucose was found to be the best in terms of cell growth under heterotrophic conditions (Ogbonna *et al*., 1998). The glucose concentration in the medium plays an important role in the successful growth of *E. gracilis*, with glucose concentrations of less than 0.2% [w/v] no growth can be determined, but with the concentration increased to 3% [w/v] growth can be observed (Barras and Stone, 1965). Furthermore, Ono *et al*. (1995) showed that *Euglena* also has the unique ability to produce carbohydrates from ethanol. It was shown that the content of the carbohydrate paramylon in the cells increased as the ethanol is utilized from the medium. Hence, *Euglena* could

efficiently use ethanol for the generation of ATP in conjunction with the electron transport chain on the mitochondrial inner membrane.

Studies showed that the pH value plays another important role when glucose is used as the carbon source for cell growth. Glucose is easily utilized when pH values are around 4.5, but not when the pH value is raised to between 6.8 and 7.0 (Cook and Heinrich, 1965; Hurlberg and Rittenberg, 1962; Barry, 1962). *Euglena gracilis* can grow under extremely low pH conditions such as pH 2.5 – 3.5 (Yamane *et al*., 2001) and when glucose and $(NH_4)_2SO_4$ are used as respective carbon and nitrogen sources the media becomes further acidified to as low as pH 1.8 – 2.0 due to the SO_4^{2-} accumulation caused by the ammonium uptake (Yamane *et al*., 2001). Under these conditions, high growth rate and yield can be obtained despite the heavily acidic pH throughout the culture period. Yamane *et al*. (2001) observed that these conditions were found to lead to a 15% increase in growth obtained under mixotrophic conditions in comparison with the heterotrophic conditions.

2.2.4. The reserve carbohydrate

The carbohydrate reserve product formed in *E. gracilis* cells is the linear β-(1,3)-glucan, paramylon. Gottlieb (1850) was the first to isolate the paramylon granules and showed that they were composed of a carbohydrate that did not stain by iodine, although being isomeric with starch.

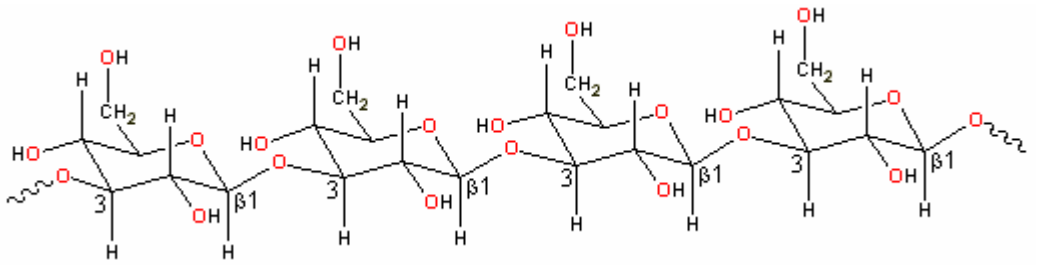

Figure 2. β-(1,3)-linkages of glucose molecules. One part of a paramylon molecule (www.lsbu.ac.uk/water/images/curdlan.gif).

Later studies showed that the β-(1,3)-linkages of paramylon (Figure 2), in comparison with the α-(1,4) and α-(1,6)-linkages present in starch, was the reason for this observation (Booy *et al*., 1981; Clarke and Stone, 1960; Kreger and Meeuse, 1952).

In *Euglena* paramylon is synthesized as a storage carbohydrate in granular form with a highly crystalline and complex fibrillar structure that is surrounded by a single membrane (Bäumer *et al*., 2001). Paramylon granules can be distributed widely in the cytoplasm, can form cups over the pyrenoids, can be massed together, or be few but large and be located in a fairly constant position (Barsanti *et al*., 2001). *E. gracilis* can accumulate large quantities of paramylon when grown in the presence of an utilizable carbon source under both light and dark conditions.

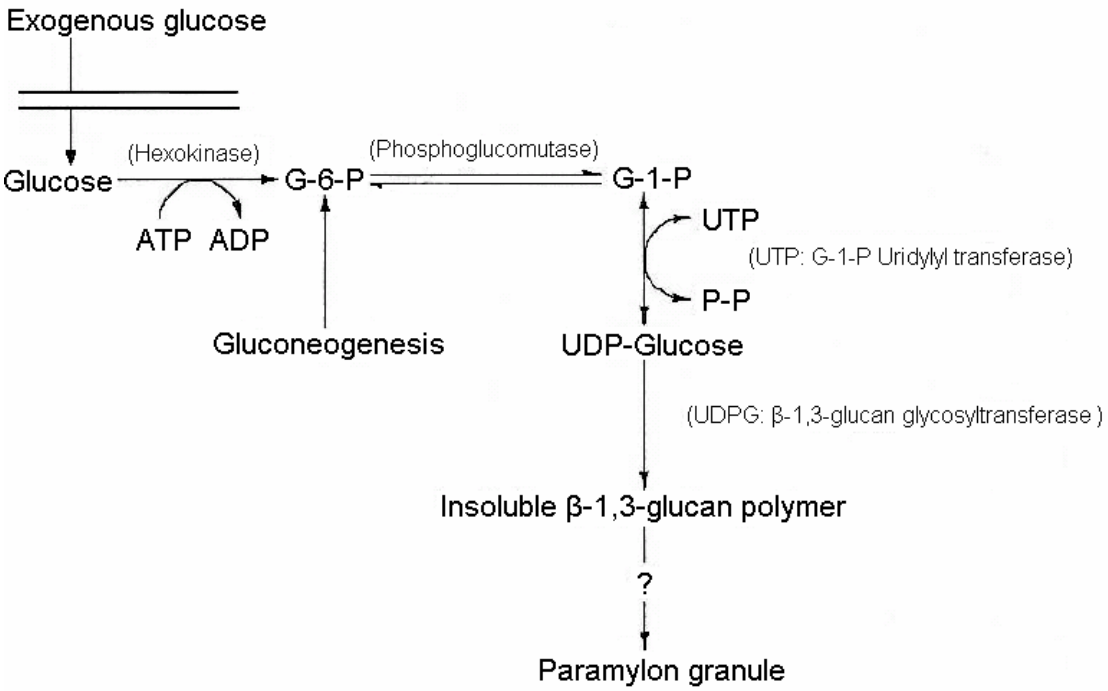

Figure 3. Outline of the reactions leading to the synthesis of paramylon (Barras and Stone, 1965). Through the action of hexokinase, glucose is converted to glucose-6-phosphate (G-6-P) with the reduction of one adenosintriphosphate (ATP) to adenosindiphosphate (ADP). Glucose-1-phosphate (G-1-P) is subsequently formed and converted to UDP-glucose by the enzyme uridinetriphosphate (UTP): glucose-1-phosphate uridylyl transferase. Then, through the action of the UDP-glucose (UTPG): β-(1,3)-glucan glycosyltransferase the insoluble β-(1,3)-glucan is formed from UDP-glucose.

Very little is known about the synthesis of paramylon, even though some aspects of its biochemistry and biology are well understood. In Figure 3 the outline of the reactions leading to paramylon synthesis according to our present understanding is shown.

2.3. β-(1,3)-glucans

Higher plants, as well as yeasts, protists and bacteria, contain β-glucans. These polysaccharides can serve as structural scaffolds in a similar way to cellulose in plants and chitin in animals, or as storage carbohydrates like in algae (Freimund *et al*., 2003).

In plants, the glucan callose is a linear β-(1,3)-glucan with some (1,6)-branches and is synthesized in several locations within the plant, forming part of specialized walls or wall-associated structures (Verma and Hong, 2001). It is also synthesized rapidly, in response to wounding, pathogen attack and mechanical pressure and it is deposited locally to form a protective barrier in association with other components (Stone and Clarke, 1991).

Prokaryotic organisms synthesize a variety of polysaccharides that include β-(1,3)-glucans, β-(1,2)-glucans, and cellulose (Stone and Clarke, 1992). Glucans containing (1,3)-glucosidic linkages are important components of cell walls in fungi like *Saprolegnia* (Bulone *et al*., 1990). They are often present as an inner wall layer and sometimes associated covalently with other wall polymers, particularly polysaccharides such as cellulose (Bulone *et al*., 1990). Curdlan, produced as an exopolymer in species from the bacterium genera *Agrobacterium* and *Alcaligens*, also consists of β-(1,3)-glucan chains (Lee, 2004).

β-(1,3)-glucans serve as a storage carbohydrate in brown algae (laminarin), euglenoids (paramylon), chrysophytes (leucosin) and fungi (cellulin, mycolaminarin, pachyman) (Stone and Clarke, 1992). Because of their high crystallinity (approaching 90%), paramylon granules are unique among the carbohydrate storage products in plants and algae. The high level of crystallinity is comparable to that of *Valonia* cellulose, the most crystalline cellulosic material yet known (Baker *et al*., 1997; Marchessault and Deslandes, 1979). These β-(1,3)-glucans are usually water insoluble but a few water soluble β-(1,3)-glucans have

been reported. Interestingly, these soluble β-(1,3)-glucans contain some (1,6)-linkages or a mannitol residue on each chain (Howard *et al*., 1976).

2.4. Glucan synthases

The synthesis of β-(1,3)-glucans *in vivo* is catalyzed by the enzyme β-(1,3)-glucan synthase (EC 2.4.1.34: UDP-glucose: (1→3)-β-glucan 3-β-D-glucosyl transferase) and this is the glycosyl transferase that has been the most extensively studied (Lawson *et al*., 1989).

The earliest investigations of *in vitro* β-glucan synthesis were conducted in order to elucidate cellulose synthesis in plants, but protein preparations from different plants catalyzed the incorporation of labeled glucose from UDP-glucose into a water insoluble polymer, later identified as the β-(1,3)-glucan, callose (Delmer, 1987; 1999). β-(1,3)-glucan synthases have been characterized in a number of plant species including *Lolium multiflorum*, *Gossypium hirsutum*, *Daucus carota*, *Beta vulgaris*, *Pisum sativum*, in the fungi *Caulerpa simpliciuscula* and *Aspergillus nidulans* and the bacterium *Agrobacterium tumefaciens,* amongst others.

Initial genetic approaches led to the discovery of genes necessary for glucan synthesis but not coding for catalytic enzymes, these being either nuclear factors (Enderlin and Selitrennikoff, 1994) or enzymes which modify the synthases post-translationally (Inoue *et al*., 1999; Díaz *et al*., 1993). More recently, putative glucan synthase genes of *Saccharomyces cerevisiae* were identified using genetic approaches. The first of these was Douglas *et al*. (1994), who showed that a mutation 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 found, 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. After disruption of this gene, significant reduction in *in vitro* β-(1,3)-glucan synthase activity, amongst other phenotypic changes, was observed. In a different study, Ram *et al*. (1995) cloned the gene CWH53 which was found to be identical to *FKS1* and *ETG1*.

The biochemical approach followed by Inoue *et al*. (1995) provided more information about the protein. After purification a 200 kDa protein was obtained and the sequencing information was used to clone two genes that showed homology to *ETG1*/*FSC1*/*CWH53*. Hydropathy profiles of both proteins suggest that these genes encode integral membrane proteins that can be assumed to have approximately 16 transmembrane domains (Inoue *et al*., 1995).

Selitrennikoff (1995) pointed out that, in these cases, the glucan synthases are apparently integral transmembrane enzymes, 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 of several components, has made purification by monitoring enzyme activity even more challenging.

2.5. Purification methods for glucan synthases

Membrane bound glucan synthases have proven difficult to purify since these enzymes are very unstable (Bulone *et al*., 1990). In all cases the first attempt to purify these complexes to homogeneity never succeeded. The purification of β-(1,3)-glucans synthases from all organisms, using membrane dissociating agents followed by chromatography and electrophoresis proved to be insufficient (Meikle *et al*., 1991), since all these attempts resulted in a significant loss of activity during the consecutive purification steps.

Consequently, several different approaches have been taken toward identification of the polypeptides that comprise the enzyme complex. With the use of dissociating agents, like non-ionic (e.g. digitonin, Triton X and Tween) and/or zwitterionic detergents (e.g. CHAPS (Figure 4) and CHAPSO), the complex is released from the membrane.

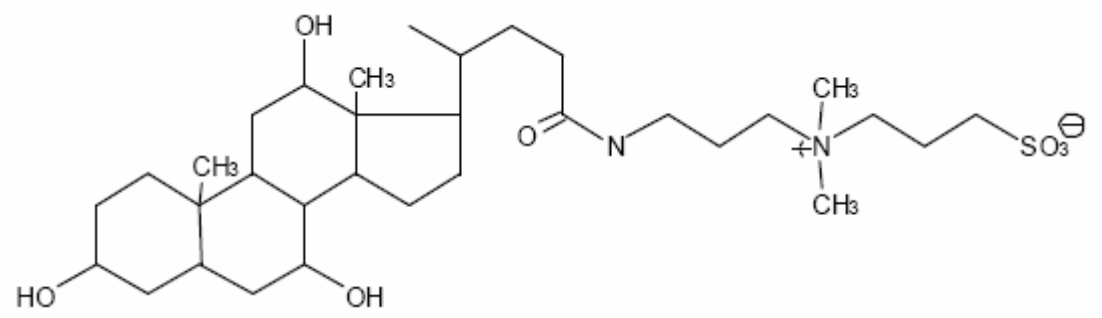

Figure 4. Chemical structure of the detergent CHAPS used for β-glucan synthase solubilization (Luis, A., 2002).

After solubilization with these dissociating agents the enzymes retain high activity, and normally up to 50% of the total enzymatic activities were recovered in the soluble fraction (Bulone *et al*., 1990). Hence these methods were found to be effective as initial purification steps for polysaccharide synthases and this allows for further purification and the study of the polypeptides, their molecular masses, and functions as well as the catalytic subunits.

Two methods that gained widespread popularity during isolation of β-(1,3)-glucans synthases are gradient density centrifugation and product entrapment. Both methods, with or without a combination of other protein purification techniques, have made the work somewhat easier when it came to isolation of these enzyme complexes.

Gradient density centrifugation is an effective method for the separation of β-(1,3)-glucan synthases from the bulk of solubilized membrane proteins. In density gradient centrifugation the proteins pass through the gradient and are separated according to their different sedimentation coefficients. Bulone *et al*. (1990), however, found that during the gradient density centrifugation of a β-(1,3)-glucan synthases activity was lost, something that should be kept in mind during experimental design.

Product entrapment was first described by Kang *et al*. (1984) and is a type of affinity purification in which the affinity matrix is generated by the synthase itself. The solubilized enzyme preparation is incubated with its substrates and effectors, and the synthase associated with the insoluble reaction product is concentrated by low-speed centrifugation

and recovered. Later Wu and co-workers (1991) incubated the preparation with an enzyme-assay mixture that differed from the medium in which the enzymes were solubilized and demonstrated that the enzyme was released from the pellet after centrifugation. This demonstrates that it is possible to precipitate the enzyme from the assay mixture without formation of the product. One disadvantage of this, however, is the non-specific precipitation of proteins during the course of the product entrapment reduces the degree of purification obtained (Frost *et al*., 1990).

2.6. Polypeptides from β-(1,3)-glucan synthase complexes

Glucan synthases have been shown to be organized as large complexes (> 450 kDa) and that these complexes all consist of different subunits of which several may be involved in glucan synthesis (Bulone *et al*., 1990; Lin *et al*., 1990; Eiberger and Wasserman, 1987; Read and Delmer, 1987).

Compared to other β-(1,3)-glucan synthase complexes with known or estimated molecular masses, the paramylon synthase complex from *E. gracilis* is the largest reported to date, being in the order of 670 kDa (Bäumer *et al*., 1991). This and other examples across species are presented in Table 1, which is by no means complete. The examples in Table 1 also indicate that β-(1,3)-glucan synthase complexes across species always consist of a number of polypeptides.

Attempts to isolate β-(1,3)-glucan synthases were supplemented by photoaffinity labelling of the polypeptides that might be involved in the synthesis of the glucan. Photoaffinity analogs of UDP-glucose have been utilized in the study of plant, fungal, bacterial and yeast β-(1,3)-glucan synthases. The major advantage of photoaffinity labelling is that the size of the subunits involved in the glucan synthesis can be determined without them being first purified to homogeneity (Drake and Elbein, 1992).

Drake *et al*. (1992) gave the basic criteria that have to be met to demonstrate the effectiveness of photoaffinity labelling: 1) saturation of active site photo-incorporation; 2) inhibition of photo-incorporation by the natural substrate; and 3) demonstration of specific photo-incorporation in crude enzyme preparations.

For direct photolabelling of UDP-glucose-binding polypeptides for callose synthases (Delmer *et al*., 1990) and paramylon synthases, α-[^{32}P]-UDP-glucose was used in conjunction with UV irradiation (Bäumer *et al*., 2001). After photoaffinity labelling with α-[^{32}P]-UDP-glucose, Bäumer *et al*. (2001) identified two proteins of sizes 37 and 54 kDa that were likely to be the UDP-glucose binding polypeptides of the paramylon synthase complex.

Table 1. Examples of β-(1,3)-glucans from plant, fungus and protist species showing the presence of multiple polypeptides within the β-(1,3)-glucan synthase complex across species

Source	β-glucan	Complex size	Polypeptides binding UDP-glucose	Polypeptides in complex	Literature
Plants					
Beta vulgaris	callose	>500 kDa			Sloan *et al.* (1986)
			57 kDa		Frost *et al.*(1990)
				27,29/31,35,43,57,70,83,92 kDa	Wu *et al.* (1991)
Gossypium	callose			26,29,34,46,52,58,66,84 kDa	Delmer *et al.* (1991)
			34,35 kDa		Shin *et al.* (1995)
			52 kDa		Li *et al.* (1993)
Pisum sativum	callose		55 kDa		Dhugga *et al.* (1991)
Daucus carota	callose			43,57,150 kDa	Lawson *et al.* (1989)
Fungi					
Saprolegnia	β-(1,3)-glucan			25,30,32,34,48,50,80,90,100 kDa	Bulone *et al.* (1990)
Protist					
Euglena gracilis	paramylon	670 kDa	34, 54 kDa		Bäumer *et al.* (2001)

Delmer *et al*. (1991) and Li *et al*. (1993) showed that the labelling of developing *Gossypium* fibre callose synthase resulted in a 52 kDa subunit likely to be the catalytic subunit for β-(1,3)-glucan synthase. Interaction with the labelled probe requires Ca^{2+}, a specific activator for callose synthases, which is known to lower the K_m of higher plant callose synthases for the substrate UDP-glucose (Hayashi *et al*., 1987). It is interesting to note that

the polypeptides were labelled by incubation of solubilized membrane proteins with $MgCl_2$ and [^{14}C]-UDP-glucose, but that these same polypeptides did not label with [^{32}P]-UDP-glucose. This possibly means that the polypeptides labelled with [^{14}C]-UDP-glucose serve only as acceptors of the glucose moiety (Delmer *et al*., 1991).

Shin and Brown (1995) also identified and characterized polypeptides involved in β-(1,3) and β-(1,4)-glucan synthesis from *Gossypium* after purification and subsequent photoaffinity labelling with azido-[^{32}P]-UDP-glucose. Two polypeptides of sizes 34 and 35 kDa were enriched by product entrapment. Photoaffinity studies showed that these two polypeptides bind UDP-glucose *in vitro*. Amino acid analysis of these two polypeptides showed that they have a conserved sequence domain identical to plant annexins, implying calcium-regulated membrane-binding properties of the 34 and 35 kDa polypeptides *in vivo*. This suggested that these two polypeptides could function as regulators of cellulose and callose synthesis in cotton fibre (Shin and Brown, 1995).

Other photoaffinity analogs of UDP-glucose have also been used. Lawson *et al*. (1989) showed that in *Daucus carota*, polypeptides of sizes 43, 57 and 150 kDa were labelled after photoaffinity labelling with 5-azidouridine 5'-β-[^{32}P]-diphosphate glucose ($5N_3$[^{32}P]-UDP-glucose). It is suspected that the 57 or 150 kDa or both subunits are responsible for binding UDP-glucose. The UDP-binding polypeptides of callose synthase from *Beta vulgaris* was identified by Frost *et al*. (1990), also using the photoaffinity probe $5N_3$[^{32}P]-UDP-glucose. A 57 kDa polypeptide was a strong candidate for the UDP-glucose binding polypeptide. The 57 kDa UDP-glucose binding subunit does not seem to be unique to beetroot, since the same was observed in plasma membrane fractions from carrots.

With the use of photoaffinity labelling, the polypeptides involved in β-glucan synthesis have been identified and characterized over a range of species during the past years. Photoaffinity labelling is valuable tool for identifying UDP-glucose binding proteins, especially when the binding pattern can be shown to correlate with kinetic properties and enrichment of enzyme activities upon purification. After these studies there is still no evidence that these UDP-glucose binding polypeptides from β-(1,3)-glucan synthase complexes are structurally or functionally related, thus meaningful comparisons must await availability of their amino acid sequences (Meikle *et al*., 1991).

2.7. Kinetics of β-1,3-glucan synthases

Hayashi *et al*. (1987) studied β-(1,3)-glucan synthases from mung bean and cotton. A stimulation of synthase activity was observed after the addition of micromolar concentrations of Ca^{2+} and millimolar concentrations of β-glycosides. These effectors act by raising the V_{max} of the enzyme and by lowering the K_m for UDP-glucose from just over 1 millimolar to 0.2 and 0.3 millimolar. It was found that Mg^{2+} enhances the affinity of the mung bean enzyme for Ca^{2+}, but not for β-glycoside. Saturated Ca^{2+} and Mg^{2+} concentrations resulted in only a slight stimulation in the further production of β-(1,3)-glucan.

For paramylon synthase (Bäumer *et al*., 2001) the pH optimum was reported as being between 7.4 and 8.0, while the temperature optimum was found to be 20 ºC. It was found that 1 mM Ca^{2+} also led to an activation of the enzyme. The K_m value for the substrate UDP-glucose was found to be 12.5 μM and the V_{max} value for the β-(1,3)-glucan formation can reach 0.24 $nmol.min^{-1}$ protein. Immunological detection of the paramylon synthase complex confirmed the activity measurements.

In another example, Morrow and Lucas (1986) isolated and solubilized β-(1,3)-glucan synthases from sugar beet petiole tissue. Activity was also stimulated by Ca^{2+} and it was found that activation was nearly saturated at 100 μM. Enzyme activity was also activated by β-glycosides and digitonin. The fluorochrome from aniline blue, sirofluor, inhibited enzyme activity at a concentration of 1 mM. The pH optimum was found to be 7.5, which falls into the pH range in which Bäumer *et al*. (2001) found paramylon synthases to be most active.

It is clear from this that all β-(1,3)-glucan synthases show similar qualities. All β-(1,3)-glucan synthases are activated by Ca^{2+} with K_m values of UDP-glucose in the millimolar range (Hayashi *et al*., 1987). Interestingly, β-(1,4)-glucan synthases in higher plants are Mg^{2+} dependent and not Ca^{2+} dependent (Delmer *et al*., 1991).

Chapter 3
Attempts to Isolate cDNAs Coding for Paramylon Synthases

3.1. Introduction

The protist *Euglena gracilis* produces a storage carbohydrate that consists entirely of β-(1,3)-linkages. It is present as granules within the cell of the *Euglena*, and is known as paramylon. Although genes coding for β-(1,3)-glucan synthase proteins have been identified from many species, the ones coding for proteins responsible for synthesizing paramylon have yet to be identified. It might be possible to identify these cDNAs by screening a *Euglena* cDNA library using a heterologous DNA probe coding for a β-(1,3)-glucan synthase from another species. However, given the evolutionary distance between *Euglena* and the other species from which such cDNAs have been identified it was decided that these probes might not have sufficient homology to allow such an approach to succeed and therefore it was decided to also try and establish other screening methods. In this chapter an approach will be described for identifying the paramylon synthase cDNAs by utilizing *E. gracilis* cDNA libraries in a variety of screens based on the function of the protein.

All eukaryotic β-(1,3)-glucan synthase genes identified to date code for proteins which are targeted to the plasma membrane where they produce β-(1,3)-glucan either for inclusion in the cell wall, or to be excreted. The cDNAs coding for these enzymes generally do not produce active proteins when expressed in *E. coli* as the proteins are unlikely to be targeted to membranes, which would be necessary for them to be active. Since paramylon is produced in the cytosol, it was hoped that the enzymes that produce this β-(1,3)-glucan are soluble. If that is the case then it is more likely that if a cDNA coding for paramylon synthases is expressed in *E. coli*, it will produce an active protein and this could be used to identify the cDNA coding for it. This would be done by transforming the library into *E. coli* and identifying colonies that manufacture β-(1,3)-glucans by using dyes which specifically stain β-(1,3)-glucans. One problem with this approach could be that if the dye cannot enter the bacterium it would not be able to stain for the β-(1,3)-glucan.

One other property of paramylon synthases that might allow for a screen to be developed to isolate their cDNAs from a cDNA library is that, like other β-(1,3)-glucan synthases, they bind UDP-glucose (Bäumer *et al*., 2001). Radiolabelled UDP-glucose has been used to examine the molecular size of various β-(1,3)-glucan synthases, including paramylon synthase. As a second approach, then, a phage cDNA library could be used to infect *E. coli* cells. The bacteria could be allowed to grow in top agar containing glucose as the carbon source. Plaques where the paramylon synthase is expressed might transfer glucose onto the paramylon leading to an extension in the chain length. Plaque lifts will allow the transfer of protein from the library, and if the membranes are then incubated with radiolabeled UDP-glucose it should be bound by the paramylon synthases that are present on the membrane and this could be detected using autoradiography.

In a different strategy, PCR oligonucleotides, some degenerative and some not, were designed against known β-(1,3)-glucan synthase sequences. It has been shown that degenerate oligonucleotides are useful for amplifying homologous sequences and the hope was that this approach would help to identify the paramylon synthase from *Euglena*. Regions were identified in aligned known sequences and from this oligonucleotides, some with low degeneracy, were designed.

The two different approaches described here are the molecular techniques that were used to attempt to identify the paramylon synthase genes. The advantages of using these approaches are that they are relatively quick, and that they have a good chance of leading to the isolation of a full-length cDNA clone or amplification of the target sequence. Difficulties could be encountered, however, if the protein is not active in the screens described, either because it is not present within a membrane, or because other proteins from *Euglena* are necessary for its activity. Further difficulties could be encountered if the paramylon synthase gene does not contain the homologous sequences that the oligonucleotides are designed for.

3.2. Materials

3.2.1. Organisms

3.2.1.1. Euglena gracilis

Euglena gracilis strain Z Klebs SAG 1224-5/25 was obtained from the Algensammlung, Göttingen, Germany.

3.2.1.2. Escherichia coli *strains*

SOLR™ strain: e14⁻(McrA⁻) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ uvrC umuC::*Tn*5* (Kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R [F′ *proAB lacI*^{q}*Z*Δ*M15*] Su⁻ (nonsuppressing), (Stratagene).

XL1-Blue MRF′ strain: Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*Δ*M15* Tn*10* (Tet^r)], (Stratagene).

DH5α strain: F'/*endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lac*ZYA-*argF*) deoR (Φ80d*lac*Δ(*lacZ*)*M15*), (Promega).

3.2.2. Chemicals and Kits

All chemicals, enzymes and kits were obtained from Sigma (St. Louis, Missouri, USA), Roche Diagnostics (Mannheim, Germany), Promega (Madison, Wisconsin, USA), Stratagene (La Jolla, California, USA), Invitrogen (Carlsbad, California, USA), CalBiochem (Merck Biosciences, Darmstadt, Germany), or QIAGEN (Hilden, Germany).

3.2.3. Plasmids

Uni-ZAP® XR: Insertion vector that allows for the *in vivo* excision of the pBluescript® phagemid that includes an ampicillin resistance gene (Stratagene).

3.2.4. cDNA library

Euglena gracilis cDNA library was obtained from Dr Meike Hoffmeister (Institut für Botanik III, Heinrich-Heine-Universität, Düsseldorf, Germany).

3.2.5. Web-based programs

A list of bioinformatics tools used in the analysis of different sequences coding for β-(1,3)-glucan synthases is given in Table 2.

Table 2. Web-based bioinformatics tools used in sequence analysis

Search Engine/Database	http Site	Reference
Block Maker	bioinformatics.weizmann.ac.il/blocks/blockmkr/www/make_blocks.html	Henikoff *et al*. (1995)
CLUSTALW	www2.ebi.ac.uk/clustalw	Thompson *et al.* (1994)
CODEHOP	bioinformatics.weizmann.ac.il/blocks/codehop.html	Rose *et al.* (1998)
DNASIS	www.oligo.net/dnasis.htm	-
Euglena EST database	tbestdb.bcm.umontreal.ca/searches/organism.php?orgID=EL	O'Brien *et al.* (2007)
ExPasy Tools	www.expasy.ch/tools	-
NCBI BLAST	www.ncbi.nlm.nih.gov/BLAST/	Altschul *et al.* (1990)
Pfam database	www.sanger.ac.uk/Software/Pfam/	Finn *et al.* (2006)
Primer3	frodo.wi.mit.edu/	Rozen *et al.* (2000)

3.2.6. Oligonucleotides

Details of oligonucleotides designed from conserved regions in fungal and yeast β-(1,3)-glucan synthase sequences are given in Tables 3 and 4.

Table 3. Oligonucleotides designed against conserved regions in fungal β-(1,3)-glucan synthase sequences

Name	Sequence 5´→ 3´
1-3 GS Fw1	GGGACTCCATGCGAAATATG
1-3 GS Rev1	TCTGAATGAGAGTGGCAACG
1-3 GS Fw2	AAGTCTGCCGCTCCTGAATA
1-3 GS Rev2	GATACGACCACCTCGAAGGA

Table 4. Degenerate oligonucleotides designed against conserved regions in the *Saccharomyces cerevisae* sequences

Name	Sequence 5´→ 3´
PM Deg Rev1	GGTTGAAGAAGTCGGGGTGNCCRTA
PS Deg Rev2	GGTTGAAGCCCACGTCGSKNCCYTTN
PM Deg Fw1	CGACGGCAAGCCCGANAAYCARAA

N = G, A, T or C; R = A or G; S = C or G; K = G or T; Y = C or T.

3.3. Methods

3.3.1. Screening of cDNA library on aniline blue-containing media

As a first attempt in obtaining the cDNA for the β-(1,3)-glucan synthase, a *Euglena gracilis* cDNA library was obtained and mass excision was performed according to the method outlined in the ZAP-cDNA synthesis kit (Stratagene). The required volume of titered phage was combined with 200 µL SOLR™ cells and 100 µL was plated out on LB agar plates containing 1% [w/v] aniline blue. These were incubated overnight at 37 ˚C and blue staining colonies were used to inoculate an overnight culture in LB media. Plasmid DNA was isolated from the culture using the GenElute™ Plasmid Miniprep kit (Sigma) and was then subjected to sequencing (section 3.3.17.) from both ends using the T3 and T7 oligonucleotide primers.

3.3.2. Euglena gracilis *cultures*

3.3.2.1. Growth in liquid medium

Euglena gracilis was grown for 2 weeks in the dark at RT in media containing (per L) 1.0 g NaAc, 1.0 g beef extract, 2.0 g tryptone, 2.0 g yeast extract, 0.2 g KNO_3, 0.01 g $(NH_4)_2HPO_4$, 0.01 g $CaCl_2$ and 20.0 g glucose.

3.3.3. Total RNA extraction and purification

Total RNA was extracted at RT according to a method modified from Bugos *et al*. (1995). Cells were centrifuged at 5000 *g* for 10 min and the supernatant was discarded, after which 10 mL of homogenization buffer (0.1 M Tris-HCl, pH 7.5; 1 mM EDTA; 0.1 M NaCl; 1% [w/v] SDS) and 10 mL of 25:24 [v/v] phenol : chloroform was added. Cells were homogenized using a vortex mixer at high speed for 1 min. After the addition of 700 µL 3 M NaAc (pH 5.2), cells were further homogenized for 30 s followed by incubation on ice for 15 min. After centrifugation at 12000 *g* for 15 min at 4 ˚C, the upper aqueous phase was transferred to a new tube containing 3 volumes of ethanol and 0.1 volumes of 3 M NaAc in order to precipitate the RNA. The tube was incubated for 2 h at - 20 ˚C and then centrifuged at 10 000 *g* for 10 min to recover the precipitated RNA. The supernatant was discarded and the RNA pellets were washed with 70% [v/v] ethanol, centrifuged at 10 000 *g* for 5 min and then dried. After resuspension in 500 µL double distilled water, any remaining insoluble material was removed by centrifugation at 10 000 *g* for 5 min at 4 ˚C and the supernatant transferred into a fresh microfuge tube. RNA was treated with RQ1 RNase-Free DNase (Promega) to remove genomic DNA contamination. RNA was stored at – 80 ˚C.

3.3.4. Messenger RNA isolation

Messenger RNA (mRNA) was isolating by utilizing the PolyATtract® mRNA Isolation System (Promega) according to the manufactures guidelines.

3.3.5. RNA quantification and purity determination

Concentrations of RNA were calculated from spectrophotometric absorbance measurements at 260 nm (Power wave$_x$ microplate scanning spectrophotometer, Bio-Tek Instruments, Winooski, Vermont, USA). RNA purity was expressed as the ratio of absorbance measurements (260:280 nm) and confirmed by agarose gel electrophoresis on a 1% agarose gel and run in TE buffer.

3.3.6. Synthesis of α-[^{33}P]-UDP-glucose

In order to synthesize α-[^{33}P]-UDP-glucose, 250 µCi α-[^{33}P]-UTP (PerkinElmer Life Sciences, Boston, MA, USA), was dried in a Speed Vac® Plus SC110A (Savant Instruments, Inc., Holbrook, NY, USA) and then resuspended in 250 µL of 50 mM HEPES-KOH (pH 7.3), 8 mM $MgCl_2$, 0.8 mM EDTA, 13 mM glucose-1-phosphate, 3.5 U UDP-glucose pyrophosphorylase, and 4 U pyrophosphatase. Following incubation for 30 min at 30 ºC the synthesized α-[^{33}P]-UDP-glucose was stored at - 20 ºC.

3.3.7. Generation of cDNA library

The ZAP-cDNA synthesis kit (Stratagene) was utilized to produce cDNA according to the manufactures guidelines. Synthesized cDNA was fractionated using the drip column provided and subsequently ligated into the UNI-ZAP XR vector, according to the manufacturer's instructions.

3.3.8. In vitro *packaging of ligated cDNA*

The ligated DNA was packaged using the Packagene® Lambda DNA Package System (Promega) according to the manufacturer's guidelines.

3.3.9. Screening of the phage library

Escherichia coli XL1-Blue cells suspended in 10 mM $MgSO_4$ (OD_{600} 0.5) were infected with titered Lambda ZAP phage (Stratagene) and plated out in top agar (LB with 0.7% agarose) on LB plates. The plates were incubated at 37 ˚C until the phage lysed the bacterial

lawn to form plaques. Before performing plaque lifts, nitrocellulose membranes were soaked in 10 mM IPTG and then air-dried over Whatman paper (chromatography paper). The nitrocellulose membranes were laid onto the plates and incubated for another 4 h at 37 ˚C, after which the plates were chilled to 4 ˚C in order to prevent the agar from peeling off upon lifting. After removal from the plates, the membranes were incubated with shaking in 10 mL 250 mM Tris-HCl buffer containing 10 µL α-[^{33}P]-UDP-glucose (produced as described in Section 3.3.6.) for 1 h and then air-dried on Whatman paper. The membranes were then placed in a sealed plastic bag and exposed overnight on a Supersensitive Cyclone Phosphor screen (Packard). The hybridization was visualized by means of a phosphorimager (Cyclone TM Storage Phosphor System, Packard Instrument Co., Meriden, USA). Plaques appearing to bind UDP-glucose were isolated from the agar plate and transferred to 250 µL of SM buffer containing 20 µL of chloroform. The tube was centrifuged to ensure the agar piece was suspended and then stored at 4 ˚C until retransformation was performed. Steps were repeated for the second and third rounds of screening.

3.3.10. Amplification and mass excision of cDNA library

The library was amplified followed by a mass excision with Ex Assist helper phage according to the method provided in the ZAP-cDNA synthesis kit (Stratagene). The amplified library was titered before mass excision and contained 5.1 x 10^9 pfu/mL. The excised phagemids were titered each time before usage.

3.3.11. Functional screen of cDNA library

After titering, phagemids were combined with 200 µL of SOLR™ *E. coli* cells and incubated for 15 min at 37 ˚C. These were plated out in top agar on LB plates supplemented with 1% [w/v] glucose. The bacterial library was allowed to grow at RT for a week before it was subjected to staining. Colonies were stained with either 0.5% [w/v] Fluorescent Brightener 28 (Calcofluor) or 1% [w/v] aniline blue for 10 min, followed by destaining with 1 M NaCl for 30 min. Colonies staining darkly or that were fluorescing were picked and streaked out on a LB plate containing IPTG and X-Gal and incubated at 37 ˚C overnight. The colonies that contained inserts were cultured in LB medium overnight. To confirm positive staining, the experiment was repeated using plasmid DNA obtained from the GenElute™ Plasmid

Miniprep kit (Sigma) according to the manufactures guidelines. The plasmid DNA was transformed into *E. coli* DH5α and the staining was repeated as described.

3.3.12. Extraction of RNA and cDNA synthesis

RNA extraction was performed as described in Section 3.3.3. and quantified as described in Section 3.3.5. Superscript III Reverse Transcriptase (Invitrogen) was utilized to generate single stranded cDNA fragments from total RNA. Before cDNA synthesis, mRNA was diluted to 5 μg/μL with RNase free water.

3.3.13. Design of PCR oligonucleotides

Several available β-(1,3)-glucan synthase sequences from fungal species (GenBank accession numbers XM716336.1; D88815.1; AF102882.1; AF027295.1; AY254574.1) were aligned using the web-based bio-informatics tool, DNASIS (Hitachi Software Engineering Co., Yokohama, Japan). Oligonucleotides were designed from the consensus sequence using the web-based program Primer3.

A β-(1,3)-glucan synthase motif is present in the web-based Pfam database (Finn *et al*., 2006). To design degenerate oligonucleotides all 123 β-(1,3)-glucan motif sequences present in the Pfam database were converted into blocks using the web-based Block Maker program (Henikoff *et al*., 1995). The blocks were used to design degenerate oligonucleotides by the web-based program, CODEHOP (Rose *et al*., 1998), using *Euglena* codon usage.

3.3.14. PCR

The PCR conditions were as follows: 3 min denaturation at 94 °C; 35 cycles of 45 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C; final elongation step of 72 °C for 10 min.

3.3.15. DNA purification from agarose gels

Following electrophoresis in agarose gels (Section 3.3.5.), DNA bands were excised from the gel and DNA purification was performed using QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacture's guidelines.

3.3.16. Cloning of PCR products

The DNA was ligated into the pGEM®-T Easy vector (Promega) according to the manufacturer's guidelines. Inserts were confirmed after transformation of competent *E. coli* DH5α cells by blue / white colony screening. The colonies that contained inserts were used to grow overnight cultures in LB and these were then subjected to plasmid DNA isolation using GenElute™ Plasmid Miniprep kit (Sigma) according to the manufacturer's guidelines.

3.3.17. DNA sequencing

DNA was sequenced by the DNA sequencing facility (Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa) with an Applied Biosystems ABI Prism 373 Genetic Analyser using an ABI BigDye™ terminator cycle sequencing ready reaction kit according to the manufacturer's guidelines (Perkin-Elmer, Boston, Massachusetts, USA).

3.4. Results and Discussion

3.4.1. Construction of cDNA library

Euglena gracilis cultures were grown in a liquid media supplemented with glucose for 3 to 4 weeks before RNA was extracted. Different RNA extraction methods were evaluated, with the protocol described above providing the best quality RNA. Good quality mRNA was obtained and used for the construction of the cDNA library. Restriction analysis of randomly selected clones showed that around 50% of the clones contained inserts and showed a good distribution of the various insert sizes. Sizes ranged from 900 - 1 980 bp, with the average

insert being 1 000 bp. Thus, assuming 1 400 bp as the coding sequence for an average length protein (Makalowski and Boguski, 1998) this library is a good representation of the RNA present in the *E. gracilis* culture at the time of isolation.

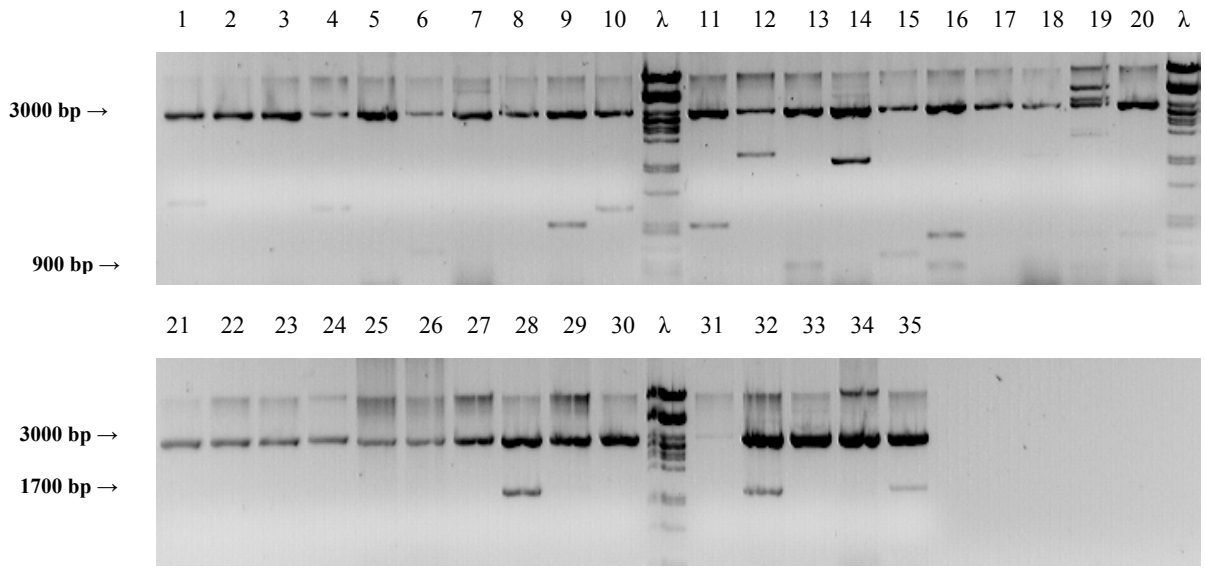

Figure 5. Restriction enzyme analysis of 35 clones to determine insert size. In total 17 of the 35 selected clones contained inserts. pBluescript SK- was cut with KpnI and SacI that cut on both sides of the multiple cloning site on the vector. pBluescript SK- is 3 000 bp in size and this is clearly visible after restriction analysis of the vector. The smallest fractions on both gels were 900 and 1 700 bp, respectively. DNA marker Lambda (λ) digested with PstI was used (Promega).

3.4.2. Library screening methods

For screening purposes, two cDNA libraries were used. One *E. gracilis* library was manufactured as described above, whilst the other was donated by Dr Meike Hoffmeister. The libraries were screened by two separate methods.

3.4.2.1. Functional screen

In an attempt to clone the cDNA(s) that encode β-(1,3)-glucan synthase from *E. gracilis,* a phage cDNA library was obtained. In addition, a similar phage library was produced using RNA isolated from *Euglena*. These were converted to plasmid libraries and transformed into *E. coli*. Firstly, several rounds of screening were performed on LB plates that contained the β-(1,3)-glucan specific stain, aniline blue. Aniline blue was added to the medium at 1% [w/v] and some colonies were obtained that showed a faint blue colour.

Staining for β-(1,3)-glucan activity is very useful since it is sensitive and easy to perform. The organisms can be grown within or on agar at high densities and the substrate can be incorporated into the growth medium or applied in the top agar. Staining can be performed at any time once the cells have grown sufficiently. The β-(1-3)-glucans specifically bind the triphenylmethane dye, aniline blue (Nakanishi *et al.* 1974), and to a benzophenone fluorochrome found in the dye (Evans *et al*. 1984). Fluorescent Brightener 28 (Calcofluor) and Congo Red also bind to β-(1,3)-glucans and induce fluorescence, but these dyes are not specific for β-(1,3)-glucans (Nakanishi *et al.* 1974).

All the colonies seem to take up some of the blue dye and all had a faint blue colour to them. Some colonies did in fact seem "bluer" and were bigger in every case when compared to the other colonies on the plate. These were then subjected to plasmid DNA isolation and subsequently sequenced. BLAST results indicted that these clones never contained the cDNA of interest. A summary of the nucleotide sequences that showed homology to the various clones is presented in Table 5.

In further attempts, a cDNA library was constructed as described and screened. Screening was repeated several times with the different stains. Initially, aniline blue was the only stain that produced a visual difference in the staining of colonies. Colonies that stained positive for the production of paramylon were subjected to blue / white screening and it seemed that almost half of these did not contain any inserts. Only those colonies that were shown to contain inserts were analyzed further.

Table 5. Nucleotide sequences found to produce significant alignments with clone sequences isolated during functional screening of the cDNA library

GenBank Accession no.	Name
ATH5682	*Arabidopsis thaliana* mRNA for inositol 1,4,5-trisphosphate 5-phosphatase
NTY14032	*Nicotiana tabacum* mRNA for ferrodoxin-NADP reductase
MARIREDM2	*M.auratus* mRNA for ribonucleotide reductase M2 subunit
AJ901879.1	*Trichoderma atroviride* mRNA for for Epl1 protein (epl1 gene), clone
MMPHEREC2	*Mus musculus* mRNA for pheromone receptor 2
PFL17188	*Platichthys flesus* Ki-ras gene (exons 1 to 4)
PFL17187	*Platichthys flesus* Ki-ras gene (exons 1, 2, 3, and 4b)

The insert size of those plasmids that contained inserts was determined and had an average insert size of 1 000 bp. After subsequent retransformation into *E.coli* DH5α, the newly transformed cells were streaked out next to the empty vector and no differences in staining were observed. Some selected colonies were still sequenced from both 5' and 3' ends, but after running a BLAST search, no significant homologies were found at either the nucleotide or amino acid levels. Since this staining method has previously been used successfully in our laboratory, it would appear that the problem might lie with the use of *E. coli* SOLR™ in the initial screening steps. The *E. coli* SOLR™ strain is normally used for phage infection, whereas *E. coli* DH5α is normally used for cloning and amplification. It is possible that a polymer present in the *E. coli* SOLR™ cell wall accounts for the blue staining and is therefore responsible for producing the false positive results.

Screening did not lead to the identification of the paramylon synthases from *E. gracilis* and several factors could have contributed to this lack of success. Firstly, it is possible that the donated library was partly degraded during transport and therefore decreasing the chances of isolating the paramylon synthase. A second possibility is that the cDNA for paramylon synthase was not present in either of the libraries used. If RNA was extracted under conditions where the paramylon synthase was not active, the cDNA would possibly not be present. Once again this cannot be confirmed for the donated cDNA library, but care was taken when constructing the second library to grow the *E. gracilis* culture under conditions conducive to the production of paramylon synthase. Thirdly, if the cDNA for the paramylon

synthase was present in the libraries, the possibility exists that the enzyme requires specific metabolites or modifying enzymes, such as protein kinases, to be active. These might not have been present in *E. coli* and therefore the enzymes would not be active, even if present. Finally, if the paramylon synthase is membrane bound it is possibly not targeted to the right part of the *E. coli* cell. Once again, this leads to the enzyme being inactive and might therefore explain the unsuccessful screening attempts.

3.4.2.2. Phage plaque screening

Another approach to identify cDNAs coding for paramylon synthesizing enzymes using the phage cDNA library was also attempted. An *E. coli* culture was infected with phage and, following cell lysis, plaque lifts were performed using nitrocellulose membranes. The membranes were then incubated with α-[^{33}P]-UDP-glucose. This is the substrate for the paramylon synthase reaction and it has been demonstrated by Bäumer *et al*. (2001) that it becomes bound to the synthase. The primary screening procedure of approximately 500 000 plaques yielded 46 potentially positive signals. Another round of screening was performed in order to purify the clones further (Figure 6), so during the next round of screening 21 positive colonies from the first round were used and more positive staining was observed. Both positive and negative plaques from each plate were selected and used in the next round of screening. The negative plaques were considered as a negative control. At this point it became clear that there was a great deal of non-specific binding, with similar numbers of positive plaques on the negative control plates as on the positive plates. The screening procedure was, therefore, abandoned.

3.4.3. Amplification of paramylon synthase through PCR

Several oligonucleotides were designed for conserved regions within known β-(1,3)-glucan synthase sequences. Following DNA amplification with the different oligonucleotides under varying conditions no PCR products could be obtained.

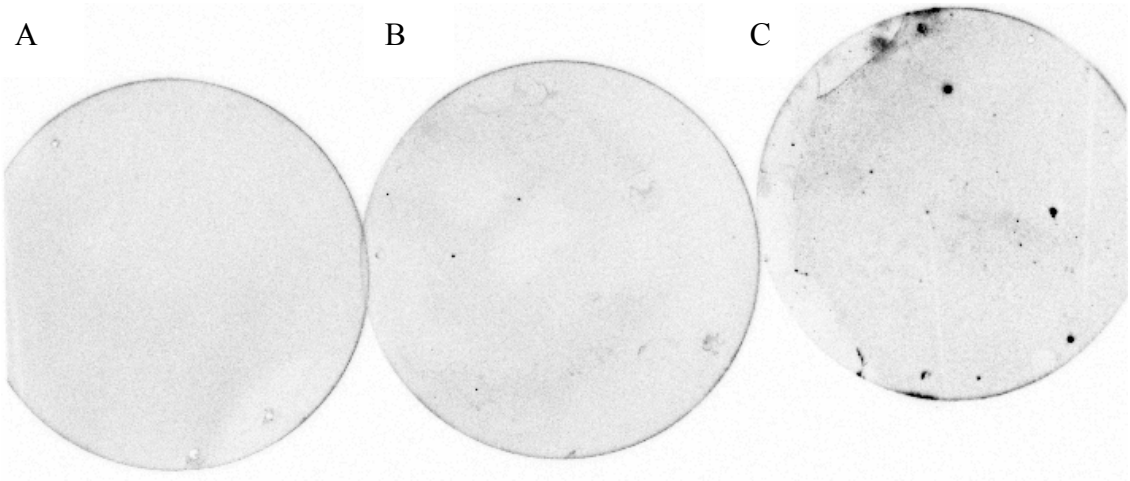

Figure 6. Three nitrocellulose membranes from the second round of screening after hybridization and visualization on a phosphoimager. All three membranes were from plates that contained positive plaques from the first round of screening. During the second round of screening A gave no positive signals, some signals showed up on B, but was eliminated and C gave definite positive signals.

The non-degenerate oligonucleotides were designed from the β-(1,3)-glucan synthase sequences available from fungal species. To test these oligonucleotides, DNA amplification was performed using cDNA from the fungus *Aspergillus niger* (donated by Dr Ibo Eduardo, Institute for Plant Biotechnology, Stellenbosch University, Stellenbosch, South Africa). Following sequencing, it was confirmed that the PCR product obtained was the β-(1,3)-glucan synthase from *Aspergillus niger*. The lack of amplification from the *Euglena gracilis* cDNA suggests that fungi and protists are evolutionarily too distant from each other and that the conserved regions were not present in the *Euglena* β-(1,3)-glucan synthase sequence.

The degenerate oligonucleotides derived from *Saccharomyces cerevisae* sequences also did not yield any glucan synthase sequences. High level of degeneracy was built into the oligonucleotides, which could lead to lower affinity of the oligonucleotide primers for the DNA template.

Other reasons why this screening attempt may not have resulted in the β-(1,3)-glucan synthase being identified are that the PCR conditions were probably not correct, or that the

cDNA did not contain the gene of interest. It could also mean that the β-(1,3)-glucan synthase genes, even though they have a similar function, do not contain the same conserved domains. With the genetic information about glucan synthesis that has been generated from *Saccharomyces cerevisae* and some fungi, specific genetic probes can be designed in order to assist with the identification of these genes in other species and possibly in *Euglena*. This information could also be combined with EST sequences in the future in order to identify genes involved in glucan synthesis.

Chapter 4
Identification of the UDP-glucose: β-(1-3)-glucan (Paramylon) Synthase from *Euglena gracilis*

4.1. Introduction

The β-(1,3)-glucan synthases are usually membrane-bound enzyme complexes which utilize UDP-glucose as their substrate. Such complexes have been found in many diverse species, from higher plants to yeasts, protists and bacteria. In the classes of Heterokontophyta, Phaeophyceae, Chrysophyceae and Euglenidae β-(1,3)-glucans was found to serve as storage carbohydrates (Kiss and Triemer, 1998; Hoek *et al*., 1995; De Madariaga, 1992; Kreeger and Van der Veer, 1970). On the other hand, all green algae store starch, a storage carbohydrate containing mostly α-(1,4)-linkages (Van den Hoek *et al*. 1995).

One of the objectives in the study of paramylon synthases is to isolate and characterize the polypeptides of this enzyme complex that are responsible for the synthesis of the glucan, paramylon. Many protein subunits are present within the paramylon synthase complex, and two have been demonstrated to bind UDP-glucose (Bäumer *et al*., 2001). These subunits could be priming molecules, in analogy to glycogenin, the reversibly glycosylated protein essential for glycogen biosynthesis, or they might be responsible for synthesizing the glucan. This second hypothesis seems highly likely, given that UDP-glucose binding subunits have been partially purified and shown to demonstrate synthetic activity (Bäumer *et al.*, 2001).

As a second approach in this study, classical protein purification methods as well as other published methods were utilized in an attempt to purify the paramylon synthase protein from *Euglena gracilis*.

4.2. Materials

4.2.1. Organisms

4.2.1.1. Euglena gracilis

Euglena gracilis strain Z Klebs SAG 1224-5/25 was obtained from the Algensammlung, Göttingen, Germany.

4.2.1.2. Escherichia coli *strains*

DH5α strain: F'/*endA1 hsdR17*($r_k^- m_k^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lac*ZYA-*argF*) deoR (Φ80d*lac*Δ(*lacZ*)*M15*), (Promega).

4.2.2. Chemicals and Kits

All chemicals, enzymes and kits were obtained from Sigma (St. Louis, Missouri, USA), Roche Diagnostics (Mannheim, Germany), Promega (Madison, Wisconsin, USA), Stratagene (La Jolla, California, USA), Invitrogen (Carlsbad, California, USA), CalBiochem (Merck Biosciences, Darmstadt, Germany), or QIAGEN (Hilden, Germany).

4.2.3. Plasmids

pGEM®-T Easy: Vector for cloning PCR products in *E. coli* (Promega).

4.2.4. Web-based programs

Table 6. Web-based bioinformatics tools used in sequence analysis

Search Engine/Database	http Site	Reference
Euglena EST database	tbestdb.bcm.umontreal.ca/searches/organism.php?orgID=EL	O'Brien *et al.* (2007)
Mascot	www.matrixscience.com/	Hirosawa *et al.* (1993)
NCBI BLAST	www.ncbi.nlm.nih.gov/BLAST/	Altschul *et al.* (1990)

4.2.5. Oligonucleotides

Table 7. Degenerate and non-degenerate oligonucleotides designed from the obtained protein sequence

Name	Sequence 5′→ 3′
Para Deg FWD	CGATGTGCTTYACNCARGG
Para NonDeg FWD	ATGTGCTTTACCCAAGGGTACT

N = G, A, T or C; R = A or G; S = C or G; K = G or T; Y = C or T.

Table 8. Oligonucleotides designed for cDNA cloning and 3' RACE

Name	Sequence 5′→ 3′
cDNA cloning	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTV
3' RACE	GGCCACGCGTCGACTAGTAC

4.3. Methods

4.3.1. Culture conditions

4.3.1.1. Growth on solid medium

Euglena gracilis was grown at RT in Petri dishes on media containing (per L): 15.0 g bacteriological agar, 1.0 g NaAc, 1.0 g beef extract, 2.0 g tryptone, 2.0 g yeast extract, 0.2 g KNO_3, 0.01 g $(NH_4)_2HPO_4$, 0.01 g $CaCl_2$ and 20.0 g glucose. Every two weeks cells were transferred to a new Petri dish with a wire loop under aseptic conditions. Stock plates were kept at RT and used to inoculate liquid media.

4.3.1.2. Growth in liquid medium

For growth in liquid media, the same recipe as for solid medium was used, but with the agar omitted. Cultures were grown in the dark and supplemented once a week with 2% [w/v] glucose per L.

4.3.2. Enzyme isolation and solubilization

Cells were harvested from the cultures and all further steps were carried out at 4 ºC. Cells were centrifuged at 5 000 *g* for 10 min, washed twice with distilled H_2O, and resuspended in buffer A (Bäumer *et al*., 2001) consisting of 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 3 mM EDTA, 0.04% [v/v] β-mercaptoethanol and modified with 1 mM PMSF instead of Pefabloc SC. Following sonication, the disrupted cells were centrifuged at 1 500 *g* for 5 min. The pellet was washed three times with buffer A, centrifuged again at 1 500 *g* for 5 min, and then resuspended in buffer B (Bäumer *et al*., 2001) consisting of 25 mM Tris-HCl (pH 7.4), 20% [w/v] sucrose, and 1 mM β-mercaptoethanol. The detergent CHAPS was added to a final concentration of 0.15% [w/v] and glycerol added to a final concentration of 15% [v/v]. The paramylon synthase was solubilized overnight at 4 ºC with shaking. The enzyme extract was stored at – 80 ºC, with only minor loss of activity being observed over time.

4.3.3. β-(1,3)-glucan synthase activity measurements

4.3.3.1. [^{14}C]-UDP-glucose assay

The incorporation of radioactivity from [^{14}C]-UDP-glucose into an acid-insoluble product was measured according to the method of Bäumer *et al*. (2001). Standard incubation mixture contained (in 70 µL): 16.8 mM Tris-acetate (pH 8.0), 1 mM $CaCl_2$, 1 µM [^{14}C]-UDP-glucose (20 µCi/mL), 2 µL 0.8% [w/v] paramylon as primer, and 50 µL enzyme extract or 50 µL heat inactivated enzyme extract as control. The mixture was incubated at 25 ºC for 15 min, after which 0.5 mL 5% [v/v] TCA was added to terminate the reaction. This was followed by heating to 100 ºC for 1 min and the addition of 1.5 mL 98% [v/v] ethanol and precipitated overnight at 4 ºC. The mixture was centrifuged at 3 000 *g* for 3 min and the pellet washed four times with 70% [v/v] ethanol. The precipitate was resuspended in 1 mL 100% [v/v] ethanol. Following transfer to a scintillation vial, 5 mL scintillation fluid was added and the radioactivity was measured in a Tri-Carb® Scintillation Analyzer (PerkinElmer Life Sciences, Boston, MA, USA).

4.3.3.2. In gel assay

Electrophoresis was performed under native conditions as described in section 4.3.8.2.. For detection of *in situ* enzyme activity, the method of Thelen and Delmer (1986) was used with some modifications. Following electrophoresis, gels were rinsed in buffer containing 10 mM Tris-HCl (pH 7.5) for 30 min with one change of buffer. Gels were incubated for enzyme activity at RT with shaking in 50 mM Tris-acetate (pH 8.0), 5 mM $CaCl_2$, 5 mM UDP-glucose, and 200 µL of 0.8% [w/v] paramylon as primer for 18 to 42 h. After this time, gels were transferred to 50 mL of 0.1% [w/v] Fluorescent Brightener 28 (Calcofluor) and incubated in the dark with shaking for 30 min at RT. This was followed by 2 h in 10 mM Tris-HCl (pH 7.5) for destaining. Gels were left shaking in distilled H_2O, if necessary, until the background staining had disappeared.

4.3.4. Solubilization of paramylon

Paramylon granules were purchased from Fluka (Sigma) and dissolved according to the method of Trudel *et al.* (1998). Dissolved paramylon was used as a primer in assays. One

gram of paramylon was dissolved in 100 mL of 0.5 M NaOH. This was precipitated with 2 volumes of cold 98% [v/v] ethanol and recovered by centrifugation at 12 000 *g* for 10 min at 4 ºC. The pellet was dissolved in 40 mL of distilled H_2O. This was again precipitated by cold 98% [v/v] ethanol and the pellet resuspended in 30 mL of distilled H_2O. The pH was adjusted to 7.0 with 2 M HCl. The volume was brought to 100 mL with distilled H_2O. From 1 g of starting material it was predicted that 20% was lost during dissolving process and, hence, the final concentration was estimated to be closer to 0.8 g per 100 mL. Dissolved paramylon was stored at 4 ºC.

4.3.5. Renaturing of proteins in gel

SDS-PAGE was performed as described in Section 4.3.8.1., with slight modifications. The CHAPS-solubilized protein extracts to be loaded onto the gel were either denatured at 95 ºC or were not denatured at all. Following separation, the gel was incubated in 100 mL of 25 mM Tris-HCl (pH 7.4) and 1% [v/v] Triton X-100 for 24 h with 5 changes of buffer. Gels were further treated as described for the in gel assay in Section 4.3.3.2., being first incubated with UDP-glucose and then the stained with Fluorescent Brightener 28 (Calcofluor).

4.3.6. Protein purification

4.3.6.1. Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed overnight at 4 ºC using different concentrations of ammonium sulfate, ranging from 25 - 100% [w/v] in 25% increments. The protein pellets obtained after centrifugation were resuspended in 25 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol, or in Laemmli loading buffer for separation on PAGE gels. When necessary the enzyme activity was determined afterwards as described in Section 4.3.3..

4.3.6.2. Anion exchange chromatography

Chromatography on a preparative scale was carried out using a DEAE anion exchange Hi Trap 20 mL column on an ÄKTA*prime* FPLC-system (Amersham Biosciences, Uppsala,

Sweden). The CHAPS-solubilized protein extract was loaded onto a column equilibrated with buffer A (50 mM Tris-HCl (pH 8.0); 1 mM EDTA; 5 mM $MgCl_2$). The column was then washed with 25 mL of buffer A. Elution of the active fraction was carried out using a 75 mL gradient up to 1 M KCl in buffer B (50 mM Tris-HCl (pH 8.0); 1 M KCl) and a flow rate of 0.3 mL/min. Elution of proteins was monitored using UV absorbance at 280 nm. Five millilitre fractions were collected and paramylon synthase activity was estimated as described in Section 4.3.3. The fractions containing the enzyme activity were pooled for further purification and activity measurements.

4.3.6.3. Size exclusion chromatography

A 10 mL Sephadex G-200 column was prepared in buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol. One millilitre of CHAPS-solubilized protein extract was loaded onto the column and the proteins eluted using the column buffer. Fractions of 0.5 mL were collected and paramylon synthase activity was estimated as described in Section 4.3.3.

4.3.6.4. Ultra-centrifugation

Ultra-centrifugation of the CHAPS-solubilized protein extract was performed at 140 000 *g* for 2 h at 4 ºC in a Beckman Preparative Ultra-centrifuge (Beckman Instruments, Palo Alto, California, USA). The subsequent pellet was resuspended in 25 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol. This was subjected to protein content and activity measurements as described in Sections 4.3.7. and 4.3.3., respectively. When necessary the pellet was used in further purification methods as described below.

4.3.6.5. Sucrose density gradient centrifugation

Linear sucrose gradients of 20 – 55% [w/v] sucrose were poured on a 65% sucrose bed. All sucrose solutions were prepared with buffer containing 25 mM Tris-HCl (pH 7.4) and 1 mM β-mercaptoethanol with the addition of 0.15% [w/v] CHAPS. Approximately 1 - 2 mL of the CHAPS-solubilized protein extract or protein from other purified pools was layered on top of the gradient and ultra-centrifuged at 140 000 *g* for 18 h at 4 ºC. After centrifugation, 0.5 mL aliquots were collected from the top of the gradient. Fractions were assayed for

protein content and paramylon synthase activity as described in Sections 4.3.7. and 4.3.3., respectively.

4.3.6.6. Product entrapment

The CHAPS-solubilized protein extract or protein from other purified pools (200 µL) was incubated with 16.8 mM Tris-acetate (pH 8.0), 1 mM $CaCl_2$, 1 mM UDP-glucose, and 15 µL 0.8% [w/v] paramylon for 30 min at RT, followed by 60 min on ice. The synthesized paramylon, containing the entrapped protein, was collected by centrifugation at 4 000 *g* for 30 min at 4 ºC. The pellet was resuspended in 50 µL buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM $CaCl_2$, and 1 mM β-mercaptoethanol. Entrapped proteins were assayed for protein content and paramylon synthase activity as described in Sections 4.3.7. and 4.3.3., respectively.

4.3.7. Protein determination

Protein content was determined by the method of Bradford (1976). BioRad protein assay reagent was used with Bovine Serum Albumin (BSA) as the standard. Absorbance was measured at 595 nm with a Power $wave_x$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA).

4.3.8. Electrophoresis

4.3.8.1. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Laemmli (1970) was used to separate proteins. Gels were usually poured to a size of 7 x 11 cm and 0.75 mm thickness, using a BioRad Protean minigel apparatus (Bio-Rad Laboratories GmbH, Munich, Germany). Liquid samples to be analyzed by SDS-PAGE were mixed with 0.25 volumes of 5x Laemmli loading buffer (5 ml glycerol, 1 g SDS, 2.56 ml β-mercaptoethanol, 2.13 ml 0.5 M Tris-HCl (pH 6.8), trace of bromophenol blue). Pellets to be analyzed were resuspended in 1x Laemmli loading buffer. All the samples were denatured for 5 min at 95 °C and loaded onto the gel. Electrophoresis was performed at RT and run with 1x SDS-PAGE running buffer (5x buffer: 30 g/L Tris,

144 g/L glycine, 10 g/L SDS) at 120 V. Pre-stained protein marker (SDS 7B2, Sigma) was used as standard.

4.3.8.2. Native polyacrylamide gel electrophoresis (Native PAGE)

Native PAGE was performed as for SDS-PAGE (Section 4.3.8.1.), but without the addition of SDS in the gels and buffers. Additionally, samples were not denatured at 95 ºC and the gels were run at 4 ºC.

4.3.9. Staining of PAGE gels

4.3.9.1. Colloidal Blue staining

Gels were stained with the Colloidal Coomassie Blue Staining Kit (Invitrogen) according to the manufacturer's guidelines and subsequently destained in distilled H_2O.

4.3.9.2. Silver staining

Gels were stained with the PlusOne™ Silver Staining Kit (Amersham Biosciences) according to the manufacturer's guidelines.

4.3.10. Identification of UDP-glucose binding polypeptides

4.3.10.1. Synthesis of α-[^{33}P]-UDP-glucose

In order to synthesize α-[^{33}P]-UDP-glucose, 250 µCi α-[^{33}P]-UTP (PerkinElmer Life Sciences, Boston, MA, USA) was dried in a Speed Vac® Plus SC110A (Savant Instruments, Holbrook, NY, USA) and then resuspended in 250 µL of 50 mM HEPES-KOH (pH 7.3), 8 mM $MgCl_2$, 0.8 mM EDTA, 13 mM glucose-1-phosphate, 3.5 U UDP-glucose pyrophosphorylase, and 4 U pyrophosphatase. Following incubation for 30 min at 30 ºC the synthesized α-[^{33}P]-UDP-glucose was stored at - 20 ºC.

4.3.10.2. Photoaffinity-labelling α-[^{33}P]-UDP-glucose

Samples were incubated, in a reaction mixture of 70 µL, with 16.8 mM Tris-acetate (pH 8.0), 1 mM $CaCl_2$, and 10 µL α-[^{33}P]-UDP-glucose, for 20 min at 4 ºC under illumination of UV light (254 nm) in a cross-linker (Ultra Lum, Carson, California, USA). Before separation on SDS-PAGE, the samples were concentrated using the methanol-chloroform-water precipitation method described by Wessel and Flügge (1984).

4.3.11. In gel tryptic digestion of proteins and sequencing

The Proteo extract All-in-One Trypsin Digestion Kit (CalBiochem) was used to digest proteins into peptides for sequencing by LC-MS-MS (Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa).

4.3.12. Extraction of RNA and cDNA synthesis

RNA extraction was performed as described in Section 3.3.3 and was quantified as described in Section 3.3.5. Superscript III Reverse Transcriptase (Invitrogen) was used to generate single stranded cDNA fragments from total RNA according to manufacturer's guidelines. Before cDNA synthesis, mRNA was diluted to 5 µg/µL.

4.3.13. PCR

The polymerase chain reaction was used to amplify cDNA using custom-made oligonucleotides. The cDNA cloning oligonucleotide was used to attach a homopolymeric tail to the cDNA template. The oligonucleotides designed against the obtained β-(1,3)-glucan sequence were each used in conjunction with the 3' RACE oligonucleotide. The "hot-start" PCR protocol from the *Taq* DNA polymerase (Promega) was used. The PCR conditions were as follows: 3 min denaturation at 94 °C; 30 s at 80 °C; 35 cycles of 45 s at 94 °C, 30 s at 55 °C, 90 s at 72 °C; final elongation step of 72 °C for 10 min. Annealing temperatures ranging from 50 – 60 ºC were used in following PCRs.

4.3.14. DNA purification from agarose gels

After visualizing the DNA in an agarose gel, the band to be purified was excised from the gel and DNA purification was performed using QIAquick® Gel extraction kit (QIAGEN) according to the manufacturer's guidelines.

4.3.15. Ligation of PCR products

The DNA was ligated into the pGEM®-T Easy vector (Promega) according to the manufacturer's guidelines. Colonies containing inserts were grown overnight in LB media and these were then subjected to plasmid DNA isolation using GenElute™ Plasmid Miniprep kit (Sigma) according to the manufacturer's guidelines.

4.3.16. DNA sequencing

DNA was sequenced by the DNA sequencing facility (Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa) with an Applied Biosystems ABI Prism 373 Genetic Analyser using an ABI BigDye™ terminator cycle sequencing ready reaction kit according to the manufacturer's guidelines (Perkin-Elmer, Boston, Massachusetts, USA).

4.4. Results and Discussion

4.4.1. Enzyme isolation

Cells were harvested from dark-grown cultures by centrifugation. Following sonication, paramylon granules were separated from the cell debris by low speed centrifugation. It was found that, under these conditions, paramylon granules would stick to the side of the centrifugation tube. After the removal of the supernatant, the granules were washed from the sides without disturbing the pellet. The paramylon synthase activity was solubilized by incubation of the granules with CHAPS, however the activity was found to vary between

experiments. Consequently, before further purification was attempted the presence of activity was normally first determined by native gel electrophoresis and activity staining.

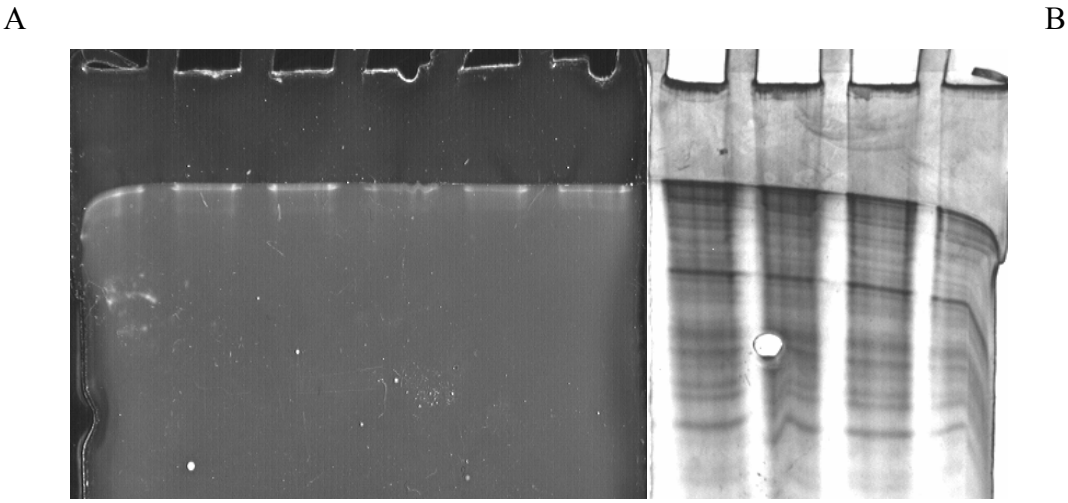

Figure 7. Electrophoresis of CHAPS solubilized enzyme extracts under native conditions. On the 10% PAGE gel all the lanes contain the same volume of the CHAPS-solubilized enzyme extract. The gel on the right (B) was stained with Coomassie Colloidal Blue and the gel on the left (A) with Fluorescent Brightener 28. It is clear from the gel that the bigger enzyme complexes aggregate at the top of the resolving gel. This is also where the product is formed, visible after the in gel assay as seen in gel A.

4.4.2. Detection of glucan synthase activity

Following solubilization of the glucan synthase activity, two methods were used to detect the activity, either by a solution assay or by an in gel assay. By using [^{14}C]-UDP-glucose, the incorporation of radioactivity into the product can be measured according to the method by Bäumer *et al*. (2001). This is a relatively accurate way to measure activity. For the in gel assay, it was shown that glucan synthases remain active in the gel for an extended period of time (Thelen and Delmer, 1986) and can be detected by characterization of the product (Figure 7). As found previously (Kudlicka and Brown, 1997; Shin and Brown, 1995), glucan synthase activity is concentrated at the top of the separating gel and sometimes can also be found in the loading well. Therefore this seems to follow a trend across species. This method

is not sensitive to low levels of enzyme activity, but it is an easy way to analyze a large number of samples quickly without using radioactivity.

It has been demonstrated that membrane-bound enzymes of higher plants synthesize callose, a β-(1,3)-glucan, in response to wounding, physiological stress, or infection (Delmer, 1987). Callose synthase is activated by micromolar levels of Ca^{2+} (Delmer, 1987; Delmer *et al*., 1991). It was also found that the enzyme requires Ca^{2+} for its activity *in vitro* (Bulone *et al*., 1999). In the previous study by Bäumer *et al*. (2001) it was found that Ca^{2+} is also essential for paramylon synthase activity in *E. gracilis* and it was therefore included in all assays.

β-glucosides are reported to be activators of Ca^{2+}-dependent callose synthase (Delmer, 1991). They have also been shown to activate other glucan synthases, however the reason for this dependence are not clear. Cellobiose was not included in the paramylon synthase assay, but rather dissolved paramylon. Therefore in further work it might be useful to test the effect of cellobiose on paramylon synthase activity, since this was not assessed. Since, in these experiments, solubilized paramylon was used as a primer a more in depth look at the effect of paramylon on the synthase itself should follow.

4.4.3. Renaturation of proteins in gel

As initial studies on the paramylon synthase complex, attempts were focused on the identification of the UDP-glucose-binding polypeptides of the complex. As an initial approach, CHAPS-solubilized enzyme extracts were first separated on an SDS-PAGE gel, followed by renaturation with Triton-X. The protein samples were either denatured by heating at 95ºC, or by incubation at RT for 10 min. The gels were then incubated with UDP-glucose and paramylon in order to see how many polypeptides retain paramylon synthase activity. Gel staining was performed by Fluorescent Brightener 28 (Calcofluor) that specifically stains β-(1,3)-glucans. This demonstrated that four definite activity bands were present and that possibly all these polypeptides are involved in paramylon synthesis.

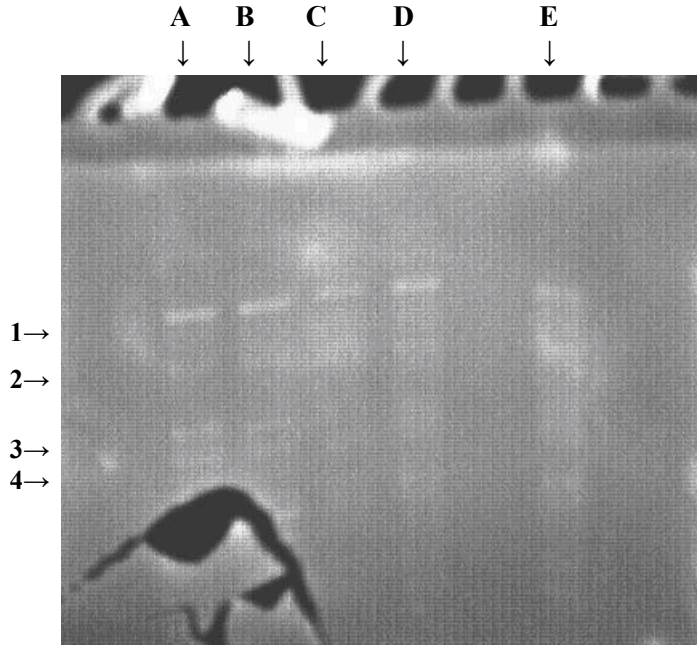

Figure 8. Renaturing of CHAPS-solubilized proteins in an SDS-PAGE gel. In the 10% gel all the lanes contain the same volume of the CHAPS-solubilized enzyme extract. Lanes A – B were denatured at 95 °C and lanes C – E were not heat denatured. After renaturing of proteins with Triton-X the gel was incubated with UDP-glucose to allow for paramylon formation. Four definite bands were visible in lanes A and B, indicated here by arrows 1 to 4.

The fact that the sample that was not subjected to heat denaturing did not show these four bands cannot be explained at this moment. With the fluorescent stain no marker was visible on the gel and the sizes of these bands cannot be confirmed. It can be speculated that bands 3 and 4 are the same bands showed by Bäumer *et al*. (2001) to bind UDP-glucose. These bands were reported to be of sizes 37 and 54 kDa. The two bands (1 and 2) at the top might be denatured protein or the same proteins as the bottom two (3 and 4) with other proteins still attached. From this data, it may be concluded that the paramylon synthase complex consists of different subunits, of which at least two have the ability to bind UDP-glucose.

4.4.4. Protein purification

Different strategies for protein purification were attempted for the solubilized paramylon synthase of *E. gracilis*. Some classic biochemical approaches, such as anion exchange, showed no increase in activity and were therefore abandoned as purification methods. A similar result was observed by Bäumer *et al*. (2001). Other methods such as sucrose density gradient centrifugation led to good protein purification.

4.4.4.1. Ammonium sulfate precipitation

In order to evaluate ammonium sulfate precipitation as a method for concentrating and partially purifying glucan synthase activity, precipitation experiments were performed. Ammonium sulfate precipitation was performed in 25% increments and stirred overnight at 4 ºC. The pellets from 25, 50, 75 and 100% [w/v] ammonium sulfate were subjected to radioactive activity measurements and it was found that the highest paramylon synthase activity was obtained after precipitation with 50% ammonium sulfate. The activity was twice that from the 75% [w/v] pellet, but this was found not to be much higher than the activity obtained in the 25% [w/v] pellet. Ammonium sulfate fractions were also subjected to in gel activity measurements and this confirmed the results. During these initial experiments, the protein concentration was not determined and should be determined before considering this method as a protein concentration method in future studies. It may be expected that, in conjunction with other purification methods, this method may be suitable for concentration of proteins with little loss of activity.

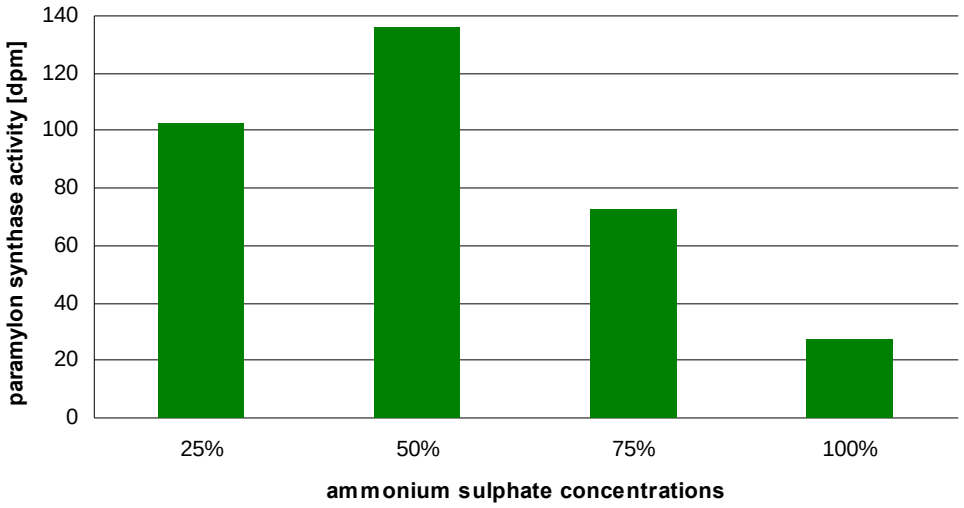

Figure 9. Paramylon synthase activities following ammonium sulfate concentration of the CHAPS-solubilized protein extract. Highest activity was obtained after precipitation with 50% ammonium sulfate and the second highest activity was obtained in the 25% ammonium sulfate fraction.

4.4.4.2. Anion exchange chromatography

Proteins bind to ion exchange resin by electrostatic forces between protein surface charges and charged groups on the exchanger resin. Anion exchangers have positively charged groups and therefore attract negatively charged groups on the protein. Elution from the resin is performed by increasing the ionic strength and weakening the electrostatic interaction between the protein and adsorbent. In the first anion exchange experiment, protein detection by UV showed two large peaks of protein eluting. The corresponding fractions where pooled into 11 samples and the activities were measured with the results shown in Figure 10. It is clear from this that the proteins corresponding to paramylon synthase were concentrated in samples 2 (fraction 16 and 17) and 3 (fraction 18 and 19). However, it was not possible to demonstrate any activity in the individual fractions following these measurements, probably because enzyme activity was lost during storage of the fractions. Further attempts at anion exchange in order to separate the proteins always resulted in the loss of activity. These first results were, therefore, not reproducible and it can be concluded that this method is not a valuable purification step.

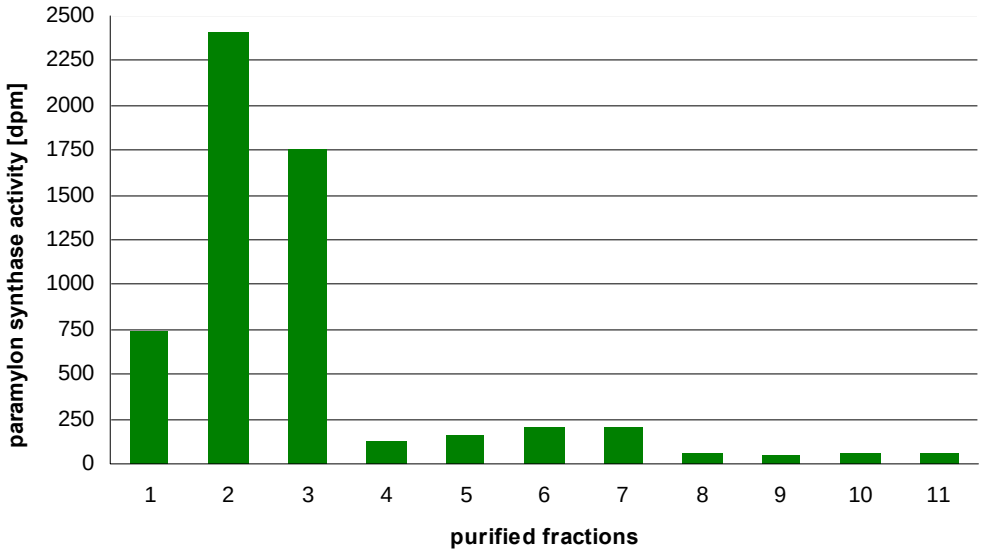

Figure 10. Activity measurements of the fractions obtained after anion exchange of the CHAPS-solubilized protein extract. Activity was found concentrated in the first samples, but could not be confirmed.

4.4.4.3. Size exclusion chromatography

Size exclusion chromatography separates proteins on the basis of their molecular size and shape and since the paramylon protein complex was estimated at 670 kDa (Bäumer *et al*., 2001) it would be expected to be separated from smaller enzyme complexes following passage through a Sephadex G-200 column. As a positive control, CHAPS-solubilized protein extracts were used and the first eight fractions obtained were separated out on a native gel and in gel activity measurements performed as in Section 4.3.3.2.. None of the expected paramylon synthase activity was observed in these expermiments. During the initial chromatography experiments the protein concentration of the individual fractions was not determined. If protein concentration had been determined, it would have helped to shed some light on why these almost always resulted in a loss of activity.

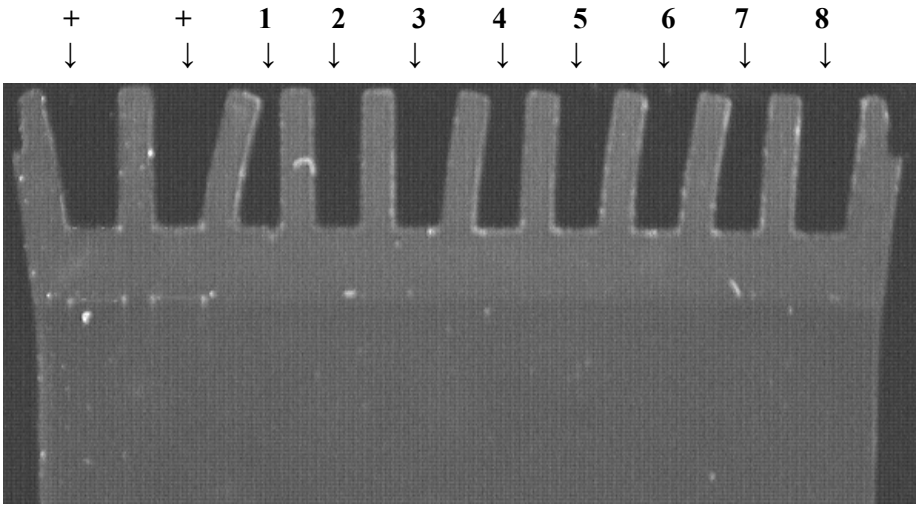

Figure 11. Native gel electrophoresis of CHAPS-solubilized paramylon synthase after size exclusion chromatography. The product, paramylon, was stained by the use of Fluorescent Brightener 28 (Calcofluor). From left to right: Lanes 1 and 2 (labelled +) contain the positive controls, the rest of the lanes contain fractions 1 to 8 as collected from the column.

Size exclusion chromatography was performed and, technically, the glucan synthase activity should elute in the void volume. Bäumer *et al*. (2001) demonstrated this previously for the paramylon synthase complex. It is known that other glucan synthases are composed of large multi-subunit complexes, which was also shown for paramylon synthase by Bäumer *et al*. (2001). Size exclusion did not result in purification, but it is advised for future studies to determine the protein concentration and to do the radioactive assay which is a more sensitive assay for paramylon synthase activity. It can then be decided if this method is suitable as a pre-purification step. One problem is that the loading capacity of the column is very low and, therefore, only a small amount of the sample can be loaded onto the column. It might be more valuable in future to attempt other methods of size exclusion, such as spin columns.

4.4.4.4. Ultra-centrifugation

Preparative ultra-centrifugation is an effective method for separation of macromolecules. It was found that the paramylon synthase activity pelleted after 2 h of ultra-centrifugation at 140 000 *g*. Compared with the CHAPS-solubilized protein extracts loaded into the

centrifuge tube, the pellet contained a 2.5-fold increase in β-glucan synthase activity, with little loss of protein. Ultr-centrifugation is, therefore, the best method tested as an initial purification step.

In Figure 12, the purification of the paramylon synthase enzyme complex is indicated by an increase in activity. The cell free homogenate always showed very low activity and up to a 50% increase could be seen by separation of the paramylon granules. CHAPS-solubilization of the proteins further increased the activity, clearly indicating that the enzymes are associated with the granule. Purification was further increased by ultra-centrifugation.

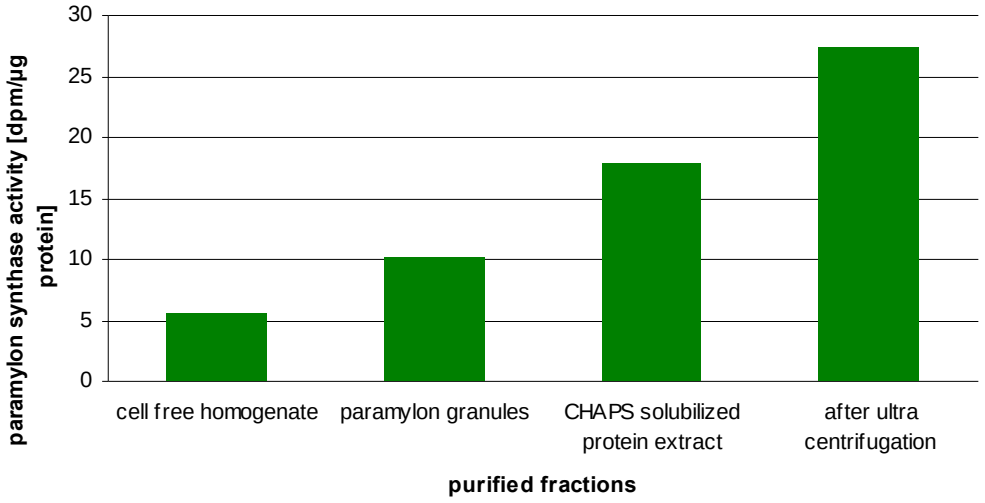

Figure 12. Preparative ultra-centrifugation of CHAPS-solubilized protein extract. After paramylon granules were treated with CHAPS, an increase in activity was observed. This was further increased by ultra-centrifugation of the CHAPS-solubilized protein extracts. In total, a 2.5-fold increase in activity was observed with little loss of protein.

4.4.4.5. Sucrose density centrifugation

In density gradient centrifugation, the proteins pass through the gradient and are separated according to their different sedimentation coefficients. Sucrose gradient centrifugation was performed with the CHAPS-solubilized protein extract. The specific activity of the most active fraction (fraction 11 on Figure 13) was increased 3-fold compared with the crude solubilized fraction, while the total recovery was 60%.

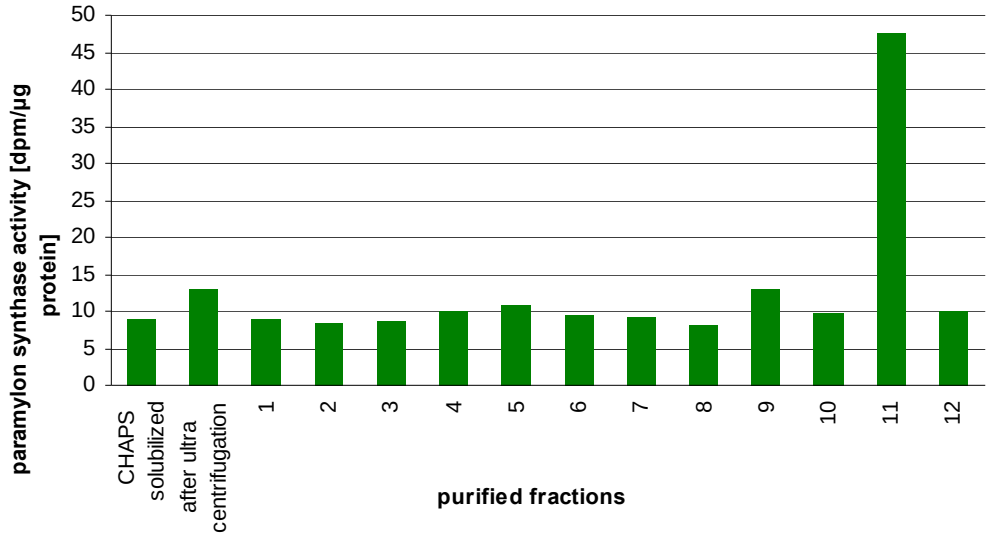

Figure 13. Sucrose gradient centrifugation of concentrated paramylon synthase obtained by preparative ultra-centrifugation. Activity was increased 4-fold but with low recovery of protein.

4.4.4.6. Product entrapment

A method successfully used in the past for the purification of glucan synthases is product entrapment. Product entrapment was used for the first time by Kang *et al*. (1984) for the purification of chitin synthase. 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 centrifugation. During this study, protein content was not determined due to the small volumes obtained after entrapment. Product entrapment performed with a fraction showing the highest activity after sucrose density centrifugation led to a 50% increase in activity.

This by itself was found to be insufficient as a purification step since after sucrose density centrifugation very little protein was recovered and activity was found to decreases with storage. Another reason for the instability of the enzymes could be that, after sucrose gradient centrifugation, other proteins and phospholipids are eliminated that might be needed for full enzyme activity. Preparative product entrapment was also carried out directly with CHAPS- solubilized protein extracts. A 50% increase in activity was also observed. This can therefore be considered as a purification step in future studies since protein recovery is expected to be very good.

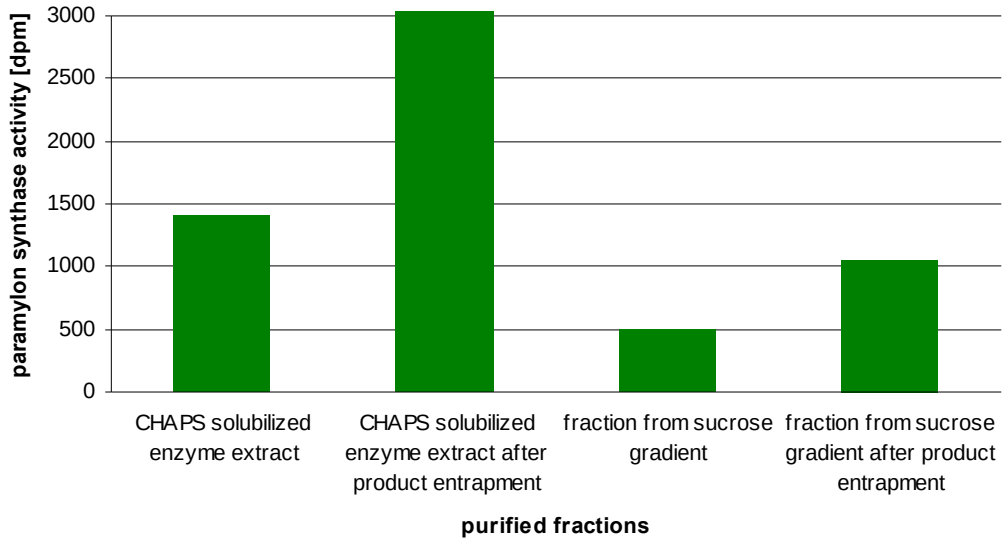

Figure 14. Product entrapment of CHAPS-solubilized enzyme extracts. The CHAPS-solubilized enzyme extract were subjected to product entrapment with a great increase in activity observed. Another partially purified fraction obtained from the sucrose gradient was also subjected to product entrapment in a separate experiment and the same increase in activity was observed. In each experiment, activity was increased by 50% after product entrapment of the enzyme.

4.4.4.7. Photoaffinity labelling α-[^{33}P]-UDP-glucose

As a method to identify the UDP-glucose binding polypeptides of the glucan synthases, various groups have used photoaffinity labelling. A range of UDP-glucose labels were used and were able to identify certain UDP-glucose binding polypeptides from different organisms (Bäumer *et al*., 2001; Shin and Brown, 1995; Drake *et al*., 1992; Meikle *et al*., 1991; Frost *et al*., 1990; Delmer *et al*., 1990; Lin et al., 1990; Lawson *et al*., 1988). The fractions obtained from the sucrose gradient showing the highest activity were incubated with α-[^{33}P]-UDP-glucose as described, in order to identify the UDP-glucose binding subunits of the paramylon synthase complex. From Figure 12, it is clear that there are two subunits of sizes 37 and 54 kDa that showed affinity to the substrate. The two polypeptides from the paramylon synthase that bind UDP-glucose to fall into the size range reported for other organisms. In the wells of the gel A more labeled protein is visible, probably due to overloading of protein.

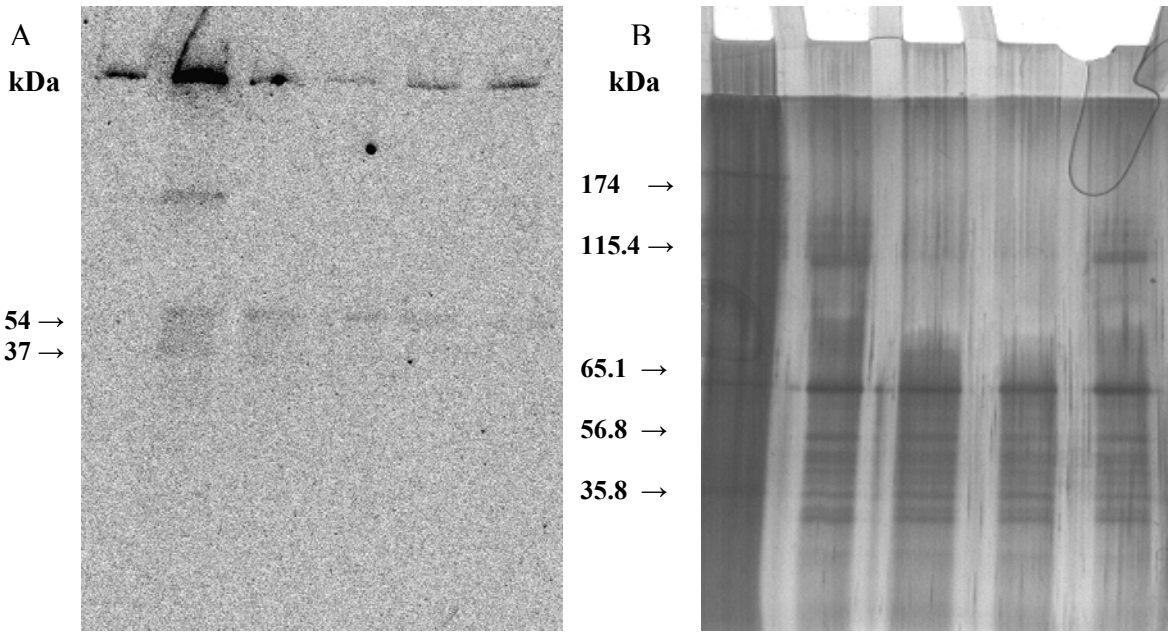

Figure 15. Radiolabeled proteins from the sucrose gradient fraction. In the silver-stained gel (B), the presence of the 54 kDa polypeptide is very clear. This corresponds to the darker band visible on the photo obtained from the phosphoimager (A). The 37 kDa band is not as clearly visible on the silver-stained gel and was also detected in low concentrations by the phosphoimager.

4.4.4.8. Peptides from protein sequencing

Following separation of the protein extract that was incubated with α-[^{33}P]-UDP-glucose, the 37 and 54 kDa polypeptide bands were subjected to in gel tryptic digestion. The peptides were sequenced by LC-MS-MS and no significant peptide sequences where obtained from the 54 kDa polypeptide. The top peptide sequences obtained for the 37 kDa polypeptide are shown in Table 9. From these peptides, one 13 amino acid peptide from the 37 kDa polypeptide showed homology to the β-(1,3)-glucan synthase from various yeasts (Table 9).

Table 9. Peptide sequences from the 37 kDa polypeptide and the proteins that match these peptide sequences. These peptides obtained the highest scores through the Mascot web-based search engine (Hirosawa *et al*., 1993). The 13 amino acid peptide showed homology to a glucan synthase subunit from yeasts

Peptide	Proteins matching peptide
M.EGGEEEVER.I	YHR028C - *Saccharomyces cerevisiae* (Baker's yeast)
R.THAHAAAVRR.D	Rv0922-like protein - *Mycobacterium celatum*
R.VAMAMAEK.A	Similar to Cytosine/adenosine deaminases - *Shewanella sp*. MR-4
R.AGPGEKAPR.I	Sexual cell division-inducing pheromone - *Closterium ehrenbergii*
K.MCFTQGYLEFSAR.L	CaKRE6 *Candida albicans CaKRE6 Glucan synthase subunit* - *Debaryomyces hansenii* (Yeast) (*Torulaspora hansenii*)
K.MCFTQGYLEFSAR.L	P32486 *Saccharomyces cerevisiae* YPR159w KRE6 Glucan synthase subunit - *Debaryomyces hansenii* (Yeast) (*Torulaspora hansenii*)

Degenerate and non-degenerate oligonucleotides were designed against the one peptide (as described in Section 4.2.4.) showing homology to a glucan synthase subunit. At the same time, oligonucleotides for 3' RACE were designed. Following 3' RACE with the non-degenerate oligonucleotide, a 448 bp PCR product was amplified and then following amplification of that 448 bp PCR product another band of 468 bp was also visible on the gel (Figure 16). To investigate both products obtained, both were sequenced and the nucleotide sequence obtained from the 468 bp product showed high homology to *Euglena* ferrodoxin (Table 10). No homology was seen for the 448 bp product. The PCRs were repeated with the same results.

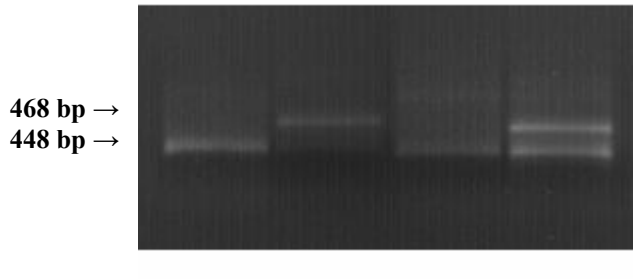

Figure 16. PCR products of 448 and 460 bp obtained after 3' RACE and amplification of the product respectively.

Table 10. Nucleotide sequences obtained from 3' RACE PCR product

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Accesion no: P22341

Ferrodoxin from *Euglena viridis*

```
Query  442  D*YILDAAEAAGIDLPYSXRAGACSSCTGVVKTGTVDNSDQSFLDDDQLGKGFVXTCTAY  263
            D YILDAAE AGIDLPYS RAGACSSCTG+VK GTVD SDQSFLDDDQ+ KGF  TCT Y
Sbjct  21   DQYILDAAEDAGIDLPYSCRAGACSSCTGIVKEGTVDQSDQSFLDDDQMAKGFCLTCTTY  80
Query  262  PTSDCTIETXKEEDLF  215
            PTS+CTIET KE+DLF
Sbjct  81   PTSNCTIETHKEDDLF  96
```

Accesion no: AAW79313.1

Chloroplast ferrodoxin from *Acetabularia acetabulum*

```
Query  478  DXYXXDAAEXAGXDLPYSXRAGXCSXCTGVVKXGTVDNSXQSFLXXXXLGKGFVXTXXAX  299
            D Y  DAAE  G DLPYS RAG CS CTGVVK GT+D S QSFL    +G GFV T  A
Sbjct  62   DVYILDAAEEEGIDLPYSCRAGSCSSCTGVVKSGTIDQSDQSFLDDDQMGNGFVLTCVAY  121
Query  298  PTSXCXXETHKEEXL  254
            PTS C  ETHKEE L
Sbjct  122  PTSDCTIETHKEEEL  136
```

There are many possible reasons as to why the PCRs did not yield the glucan synthase cDNA. The quality of the RNA obtained and the conditions under which the cells were grown play the most important role. Total RNA was used for the cDNA synthesis, but mRNA could also have been used. The amount and quality of the cDNA yielded and the oligonucleotide that was designed for this reaction also play an important role. Also the conditions under which the PCR was performed can have a significant effect on the products obtained. Since degeneracy was introduced into one oligonucleotide, this would lead to a lower affinity of the oligonucleotide primer for the DNA template and a lack of success in obtaining any PCR products. It would be worthwhile to repeat the PCR experiment after designing new oligonucleotides. PCR can be performed under varying conditions and the use of nested PCRs can also be considered.

Chapter 5
General discussion and conclusion

The main aim of the work presented in this thesis was to clone the paramylon synthase genes from the protist *Euglena gracilis*. Paramylon, a linear β-(1,3)-glucan, is produced as the storage carbohydrate in the *Euglena* cells. UDP-glucose was shown to be the substrate for paramylon synthesis by Marechal and Goldemberg (1964). In 2001, Bäumer and co-workers showed that paramylon synthase was a multi-subunit enzyme complex and the protein was estimated at 670 kDa after partial purification. Bäumer also showed that the enzyme complex has two polypeptides with the ability to bind UDP-glucose. The paramylon synthase was, however, only partially purified and sequence information was never obtained.

As a first attempt to clone paramylon synthase, a number of molecular biology techniques were utilized. Different libraries were screened, one was a cDNA library obtained from Dr Meike Hoffmeister and the other cDNA library was constructed for this study. This, as far as can be determined, was the first attempt to screen a cDNA library of *E. gracilis* for the paramylon synthase gene. Unfortunately, these attempts to clone the paramylon synthase cDNA from an *E. gracilis* library were unsuccessful. Following the functional screen of the libraries, results indicated that the enzyme complex might indeed be membrane-bound as found by Bäumer *et al*. (2001). More information about the properties of the paramylon synthase complex would also shed some light on why the screening did not work.

In further attempts, oligonucleotide primers were designed against conserved regions from known β-(1,3)-glucan synthase sequences from *Sacchoromyces cerevisae* as well as fungal species. Even if these species contain conserved regions between their protein sequences, the same could not be shown in *Euglena* since no DNA sequence could be obtained from the oligonucleotides. This might also possibly be due to incorrect PCR conditions being used.

All molecular attempts were therefore unsuccessful and for this reason the focus of the project was shifted toward protein purification, as done by Bäumer *et al*. (2001), in order to

identify the paramylon synthase. Bäumer *et al*. (2001) found that classic purification methods were insufficient to purify paramylon synthase. The paramylon synthases were very unstable and activity was lost during these attempts. These findings were confirmed by initial studies in which classic protein purification methods like ammonium sulfate precipitation, anion exchange and size exclusion were tested as purification steps.

The enzyme activity was pelleted by preparative ultra-centrifugation and this was a valuable first purification step since very little protein was lost during this step. As a second purification step, both sucrose density centrifugation and product entrapment showed an increase in activity. In the work done by Bäumer *et al*. (2001) ultra-centrifugation was also used as the intial step after solibilization, followed by sucrose density centrifugation and anion exchange. From this study and the work done by Bäumer *et al*. (2001) it was clear that anion exchange is not an option when purifying paramylon synthase and hence product entrapment is advised as a purification step.

From the purification studies it became clear that the paramylon synthase complex is indeed associated with the granula membranes. An increase in activity was observed following the addition of CHAPS to the paramylon granula fraction. This detergent is normally used to dissociate complexes from membranes and the increase in activity observed indicates that the paramylon synthase activity was released from the membrane. This then confirms the observation made by Bäumer *et al*. (2001) that the paramylon synthase are localized on the granula membrane. It was possible to characterize other β-(1,3)-glucan synthases from different organisms using protein purification and knowing that the paramylon synthase is bound to the granula membrane it should be advised that in further attempts to purify and characterize this enzyme all attempts should rather be focused on protein purification.

The UDP-glucose binding polypeptides of the complex were found to be 37 and 54 kDa and this was within the range of other β-(1,3)-glucan synthesizing subunits. Sequence information from these peptides did show some homology to yeast β-(1,3)-glucan subunits. This is then the first time that some sequence information is available for the *Euglena gracilis* β-(1,3)-glucan synthases.

In conclusion, the paramylon synthase enzyme from *E. gracilis* was partially purified and some sequence information was obtained from the protein. Further studies should be

directed towards obtaining more sequence information in order to help with the cloning of paramylon synthase.

Literature Cited

Aharoni, A., Griffiths, A.D. and Tawfik, D.S. (2005) High-throughput screens and selections of enzyme-encoding genes. *Current Opinion in Chemical Biology* 9: 210 –216.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215: 403 – 410.

Baker, A.A., Helbert, W., Sugiyama, J., and Miles, M.J. (1997) High-Resolution Atomic Force Microscopy of Native *Valonia* Cellulose I Microcrystals. *Journal of Structural Biology* 119: 129 - 138.

Barras, D.R. and Stone, B.A. (1965) Carbohydrate composition and metabolism in *Euglena*. The Biology of *Euglena*, Vol. II, Buetow, D. E., ed., 149 - 187. Academic Press, New York, USA.

Barsanti, L., Vismara, R., Passarelli, V. and Gualtieri, P. (2001) Paramylon (β-1,3-glucan) content in wild type and WZSL mutant of *Euglena gracilis*. Effects of growth conditions. *Journal of Applied Phycology* 13: 59 – 65.

Bäumer, D., Preisfeld, A. and Ruppel, H.G. (2001) Isolation and characterization of paramylon synthase from *Euglena gracilis* (Euglenophyceae). *Journal of Phycology* 37: 38 - 46.

Beaulieu, D.J., Tang, S., Yan, B., Vessels, J.M., Radding, J.A. and Parr (Jr), T.R. (1994) Characterization and cilofungin inhibition of solubilized *Aspergillus fumigatus* (1,3)-β-D-glucan synthase. *Antimicrobial Agents and Chemotherapy* 38: 937 - 944.

Booy, F.P., Chanzy, H. and Boudet, A. (1981) An electron diffraction study of paramylon storage granules from *Euglena gracilis*. *Journal of Microscopy* 121: 133 - 140.

Buetow, D.E. (1968) The biology of *Euglena*, Vol. II, Biochemistry, 159 – 161, Academic Press, New York and London.

Bulone, V., Girard, V. and Fevre, M. (1990) Separation and partia1 purification of β-1,3- and β-1,4-glucan synthases from *Saprolegnia. Plant Physiology* 94: 1 748 – 1 755.

Clarke, A.E. and Stone, B.A. (1960) Structure of paramylon from *Euglena gracilis*. *Biochimica Biophysica Acta* 44: 161 - 163.

Côté, F., Letarte, J., Grenier, J., Trudel, J. and Asselin, A. (1989) Detection of β-1,3-glucanase activity after native polyacrylamide gel electrophoresis: application to tobacco pathogenesis-related proteins. *Electrophoresis* 10: 527 – 529.

Czop, J.K. and Austen, K.F. (1985) A beta-glucan inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway. *Journal of Immunology* 134: 2 588 – 2 593.

Delmer, D.P. (1987) Cellulose biosynthesis. *Annual Review of Plant Physiology* 38: 259 - 290.

Delmer, D.P. Solomon, M. and Read, S.M. (1990) Direct photolabeling with [^{32}P]UDP-glucose for identification of a subunit of cotton fiber callose synthase. *Plant Physiology* 95: 556 – 563.

Delmer, D.P. (1999) Cellulose biosynthesis: exciting times for a difficult field of study. *Annual Review of Plant Physiology Plant Molecular Biology* 50: 245 - 276.

DeLuzio, N.R. (1983) Immunopharmacology of glucan: a broad spectrum enhancer of host defence mechanisms. *Trends in Pharmacological Sciences* 4: 344 - 347.

De Madariaga, I. (1992) Interspecific differences in the photosynthetic carbon metabolism of marine phytoplancton. *Marine Biology* 114: 509 – 515.

Díaz, M., Sanchez, Y., Bennett, T., Sun, C. R., Godoy, C., Tamanoi, F., Duran, A. and Perez, P. (1993) The *Schizosaccharomyces pombe* cwg2+ gene codes for the β–subunit of a geranylgeranyltransferase type I required for β-glucan synthesis. *The EMBO Journal* 12: 5 245 – 5 254.

Drake, R.R., Li, L. and Brown (Jr.), R.M. (1992) Photoaffinity labeling of cellulose synthases. Proceedings of Cotton Fiber Cellulose: Structure, Function and Utilization Conference, Savannah, Georgia, USA.

Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Basinsky, W., Li, W., El-Sherbeini, M., Clemas, J. A., Mandala, S. M., Frommer, B.R. and Kurtz, M. (1994) The *Saccharomyces cerevisiae* FKS1 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-β-glucan synthase. *Proceedings of the National Academy of Science USA* 91: 12 907 – 12 911.

Eiberger, L.L. and Wasserman, P.B. (1987) Partial purification of digitonin-solubilized 1,3-β-glucan synthase from red beet root. *Plant Physiology* 83: 982 – 987.

Enderlin, C. S. and Selitrennikoff, C. P. (1994) Cloning and characterization of a *Neurospora crassa* gene required for (1,3)-β-glucan synthase activity and cell wall formation. *Proceedings of the National Academy of Science USA* 91: 9 500 – 9 504.

Evans, N.A., Hoyne, P.A., and Stone, B.A. (1984) Characteristics and specificity of the interaction of a fluorochrome from aniline blue (Sirofluor) with polysaccharides. *Carbohydrate Polymer* 4: 215 – 230.

Felski, M. (2004) Fermentative Gewinnung von Paramylon durch *Euglena gracilis* in konditioniertem Kartoffelfruchtwasser. PhD Thesis. Universität Bielefeld, Germany.

Finn, R.D., Mistry, J., Schuster-Böckler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khanna, A., Durbin, R., Eddy, S.R., Sonnhammer, E.L.L. and Bateman, A. (2006) Pfam: clans, web tools and services. *Nucleic Acids Research* 34 (Database Issue): 247 - 251.

Freimund, S., Sauter, M., Käppeli, O. and Dutler, H. (2003) A new non-degrading isolation process for 1,3- β-D-glucan of high purity from baker's yeast Saccharomyces cerevisiae. *Carbohydrate Polymers* 54: 159 – 171.

Frost, D.J., Read, S.M., Drake, R.R., Haley, B.E. and Wasserman, B.P. (1990) Identification of the UDP-glucose-binding polypeptide of callose synthase from *Beta vulgaris* L. by photoaffinity labeling with 5-azido-UDP-glucose. *The Journal of Biological Chemistry* 265: 2162 – 2167.

Grenier, J. and Asselin, A. (1993) Detection of β-1,3-glucanase activity in gels containing alkali-soluble yeast glucan. *Analytical Biochemistry* 212: 301 – 302.

Gottlieb, J. (1850) Ueber eine neue, mit Starkmehl isomere Substanz. *Annalen der Chemie und Pharmacie* 75: 51 - 61.

Henikoff, S., Henikoff, J.G., Alford, W.J. and Pietrokovski, S. (1995) Automated construction and graphical presentation of protein blocks from unaligned sequences. Gene-COMBIS, *Gene* 163: GC 17 - 26.

Hirosawa, M., Hoshida, M., Ishikawa, M. and Toya, T. (1993) MASCOT: multiple alignment system for protein sequences based on three-way dynamic programming. *Bioinformatics* 9: 161 – 167.

Howard, R.J., Wright, S.W. and Grant, B.R. (1976) Structure and some properties of soluble 1,3-β-glucan isolated from the green alga *Caulerpa simpliciuscula*. *Plant Physiology* 58: 459 - 463.

Internet: www.lsbu.ac.uk/water/images/curdlan.gif (November 2007).

Internet: www.oligo.net/dnasis.htm, Hitachi Software Engineering Co., Yokohama, Japan (December 2006).

Internet: http://expasy.org/tools/ (December 2006).

Inoue, S. B., Takewake, N., Takasuka, T., Mio, T., Adachi, M., Fujii, Y., Miyamoto, C., Arisawa, M., Furuichi, Y., and Watanabe, T. (1995) Characterization and gene cloning of 1,3-β-D-glucan synthase from *Saccharomyces cerevisiae*. *European Journal of Biochemistry* 231: 845 - 854.

Inoue, S. B., Qadota, H., Arisawa, M., Watanabe, T. and Ohya, Y. (1999) Prenylation of Rho1p is required for activation of yeast 1,3-glucan synthase. *Journal of Biological Chemistry* 274: 38 119 – 38 124.

Jasso-Chávez, R. and Moreno-Sánchez, R. (2003) Cytosol-mitochondria transfer of reducing equivalents by a lactate shuttle in heterotrophic *Euglena*. *European Journal of Biochemistry* 270: 4 942 – 4 951.

Kiss, J.Z. and Triemer, R.E. (1998) A comparitive study of the storage carbohydrate granules from *Euglena* (Euglenida) and *Pavlova* (Prymnesiida). *Journal of Protozoology* 35: 237 – 241.

Kreeger, D.R. and Meeuse, B.J.D. (1952) X-ray diagrams of *Euglena*-paramylon, of the acid insoluble glucan of yeast cell walls and of laminarin. *Biochimica Biophisica Acta* 9: 699 - 700.

Kreeger, D.R. and Van der Veer, J. (1970) Paramylon in a chrysophyte. *Acta Botanica Neerlandica* 19: 401 - 402.

Kudlicka, K. and Brown, R.M. (1997) Cellulose and callose biosynthesis in higher plants. I. Solubilization and separation of (1-3)- and (1-4)-β-glucan activities from mung bean. *Plant Physiology* 115: 643 – 656.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680 - 685.

Lawson, S.G., Mason, T.L., Sabin, R.D., Sloan, M.E., Drake, R.R., Haley, B.E. and Wasserman, B.P. (1988) UDP-glucose: (1,3)-β-glucan synthase from *Daucus carota* L. *Plant Physiology* 90: 101 – 108.

Leedale, G.F. (1967) *Euglenoid Flagellates*, ed. W.D. Mcelroy and C.P. Swanson, Prentice-Hall, Inc., Englewood Cliffs, NJ.

Lin, F.C., Brown, R.M. Jr, Drake, R.D. and Haley, B.E. (1990) Identification of the uridine 5'-diphosphoglucose (UDP-glucose) binding subunit of cellulose synthase in *Acetobacter xylinum* using the photoaffinity probe 5-azido-UDP-glucose. *Journal of Biological Chemistry* 265: 4 782 – 4 784.

Luis, A. (2002) Glucan synthase of *Phytophthora sojae*. Characterization, purification and attempts of molecular cloning. PhD Thesis. Ludwig-Maximilians-Universität, München, Germany.

Makalowski, W. and Boguski, M. S. (1998). Evolutionary parameters of the transcribed mammalian genome: an analysis of 2820 orthologous rodent and human sequences. *Proceedings of the National Academy of Science USA* 95: 9 407 – 9 412.

Marchessault, R.H. and Deslandes, Y. (1979) Fine structure of (1,3)-β-D-glucans: Curdlan and Paramylon. *Carbohydrate Research* 75: 231 - 242.

Marechal, L.R. and Goldemberg, S.H. (1964) Uridine diphosphate glucose-β-1,3-glucan β-3-glucosyltransferase from *Euglena gracilis*. *The Journal of Biological Chemistry* 239: 3 163 – 3 167.

Meikle, P.J., Ng, K.F., Johnson, E., Hoogenraad, N.J. and Stone, B.A. (1991) The β-glucan synthase from *Lolium multiflorum*. *The Journal of Biological Chemistry* 266: 22 569 – 22 581.

Nakanishi, I., Kimura, K., Kusui, S. and Yamazaki, E. (1974) Complex formation of gel-forming bacterial (1–3)-β-D-glucans (curdlan type polysaccharides) with dyes in aqueous solution. *Carbohydrate Research* 32: 47 – 52.

O'Brien, E.A., Koski, L.B., Zhang, Y., Yang, L., Wang, E., Gray, M.W., Burger, G. and Lang, B.F. (2007) TBestDB: a taxonomically broad database of expressed sequence tags (ESTs) *Nucleic Acids Research* (Database issue) 35: 445 - 451.

Ogbonna, J.C., Tomiyama, S. and Tanaka, H. (1998) Heterotrophic cultivation of *Euglena gracilis* Z for efficient production of α-tocopherol. *Journal of applied Phycology* 10: 67 – 74.

Ono, K., Kawanaka, Y., Izumi, Y., Inui, H., Miyatake, K., Kitaoka, S. and Nakano, Y. (1995) Mitochondrial alcohol dehydrogenase from ethanol-grown *Euglena gracilis*. *Journal of Biochemistry* 117: 1 178 – 1 182.

Pringsheim, E.G. (1955) Über *Ochromonas danica* n. sp. und andere Arten der Gattung. *Archiv fur Mikrobiologie* 23: 181 - 192.

Ram, A. F. J., Brekelmans, S. S. C., Oehlen, L. J. W. M. and Klis, F. M. (1995) Identification of two cell cycle regulated genes affecting the β-1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. *FEBS Letters* 358: 165 - 170.

Read, S.M. and Delmer, D.P. (1987) Inhibition of mung bean UDP-glucose 1,3-β-glucan synthase by UDP-pyridoxal. Evidence for an active site amino group. *Plant Physiology* 85: 1 008 – 1 015.

Rose, T.M., Schultz, E.R., Henikoff, J.G., McCallum, C.M. and Henikoff, S. (1998) Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly-related sequences. *Nucleic Acids Research* 26: 1 628 – 1 635.

Rozen, S. and Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S. and Misener, S. (eds), 365 - 386 *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ.

Selitrennikoff, C. P. (1995) *Antifungal drugs: (1,3)- β-glucan synthase inhibitors*. R.G. Landes Company. Austin, USA.

Shedletzky, E., Unger, C. and Delmer, D.P. (1997) A microtiter-based fluorescent assay for (1,3)-glucan sythases. *Analytical biochemistry* 249: 88 – 93.

Shin, H. and Brown, R.M. Jr (1995) *A biochemical study on β-glucan synthesis in the cotton fiber.* ASPP poster, Charlotte, North Carolina, USA.

Stasinopoulos, S.J., Fisher, P.R., Stone, B.A. and Stanisich, V.A. (1999) Detection of two loci involved in (1->3)-[beta]-glucan (curdlan) biosynthesis by Agrobacterium sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. *Glycobiology* 9: 31 – 41.

Stone, B. S. and Clarke, A. E. (1992) *Chemistry and biology of (1→3)-β-glucans*. La Trobe University Press, Melbourne, Australia.

Theather, R.M. and Wood, P.J. (1982) Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied Environmental Microbiology* 43: 777 - 780.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4 673 - 4 680.

Trudel, J., Grenier, J. and Asselin, A. (1998) Detection of enzymes active on various β-1,3-glucans after denaturing polyacrylamide gel electrophoresis. *Electrophoresis* 19: 1 788 – 1 792.

Van den Hoek, C., Mann, D.G. and Jahns, H.M. (1995) *Algae. An introduction to phylogeny*. Cambridge University Press, Cambridge, UK.

Wessel, D. and Flügge, U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the prescence of detergents and lipids. *Analytical Biochemistry* 138: 141 - 143.

Wu, A., Harriman, R.W., Frost, D.J., Read, S.M. and Wasserman, B.P. (1991) Rapid enrichment of CHAPS-solubilized UDP-glucose: (l,3)-β-glucan (callose) synthase from *Beta vulgaris* L. by product entrapment. Entrapment mechanism and polypeptide characterization. *Plant Physiology* 97: 684 – 692.

Wyde, P. (1989) *Beta-(1,3)-glucan activity in mice: intraperitoneal and oral applications.* Baylor College of Medicine. Research summary.

Yaden, (1965) PhD Thesis, University of Minnesota, Minneapolis, Minnesota, USA.

Yadomae, T. and Ohno, N. (1996) Structure-activity relationship of immunomodulating 1,3-β-D-glucans. *Recent Research Developments in Chemical and Pharmalogical Sciences* 1: 23 - 33.

Yamane, Y., Utsunomiya, T., Watanabe, M. and Sasaki, K. (2001) Biomass production in mixotrophic culture of *Euglena gracilis* under acidic conditions and its growth energetics. *Biotechnology Letters* 23: 1 223 – 1 228.