CHAPTER 8

IN VITRO SYNTHESIS AND ANALYSIS OF PLANT (1→3)-β-D-GLUCANS AND CELLULOSE: A KEY STEP TOWARDS THE CHARACTERIZATION OF GLUCAN SYNTHASES

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Abstract

The isolation and characterization of plant mutants affected in cellulose biogenesis have allowed the identification of a family of genes involved in this fundamental process. A specific attention has been given in the last years to genetic and molecular biology approaches, mainly because of the difficulty to assay and purify to homogeneity cellulose synthase complexes. At this stage, it is necessary to reconsider the importance of biochemical approaches, not only to firmly demonstrate *in vitro* that the proteins coded by the isolated genes are indeed able to catalyze cellulose synthesis, but also to isolate and identify directly the different components of the synthesizing machinery. The recent progress made in the field of *in vitro* synthesis of cellulose is promising and it will certainly allow the purification and biochemical characterization of plant cellulose synthases in the near future. In this review, a particular attention is given to the description and critical analysis of the *in vitro* approaches that have been developed for the study of plant cellulose synthases and the related enzymes callose synthases. An important problem raised by these biochemical investigations is the analysis of the *in vitro* polysaccharides. This aspect is integrated in the discussion, with a presentation of strategies and methods for high-throughput assays of β-glucan synthases and detailed structural characterization of *in vitro* products.

Keywords

biochemical approaches for the study of glucan synthases, (1→3)-β-D-glucan (callose) and cellulose synthases, *in vitro* synthesis of callose and cellulose, structural and morphological characterization of *in vitro* polysaccharides.

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Abbreviations

3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), dimethylsulfoxide (DMSO), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 3-[*N*-morpholino]propanesulfonic acid (Mops), nuclear magnetic resonance (NMR), transmission electron microscopy (TEM).

1 INTRODUCTION

The biosynthesis of cellulose, which is one of the major plant cell wall polysaccharides, is still far from being completely understood despite the efforts made in the past few decades to identify the enzymes involved in this important process. The main difficulty has been to isolate in an active form the enzyme cellulose synthase which is involved in cellulose polymerization, and to characterize it directly using biochemical approaches. Cellulose synthase is a membrane-bound complex that has been observed in membranes of several organisms by electron microscopy, using freeze fracture techniques (Brown, Jr. 1996; Kimura et al. 1999). The plant enzymes are organized as hexagonal supramolecular structures designated as rosettes, with a sixfold symmetry (Brown, Jr. 1996). They are highly unstable and extractions from plasma membranes using detergents usually yield enzyme preparations that synthesize *in vitro* no or very little cellulose from the substrate UDP-glucose. Instead, the major product obtained *in vitro* from detergent extracts is callose, i.e. a linear (1→3)-β-D-glucan (Delmer 1987; Okuda et al. 1993). Interestingly, it has been shown that when callose synthase is induced *in vivo* using elicitors cellulose synthesis decreases proportionally (Delmer and Amor 1995). In addition, the first *in vitro* experiments that led to some cellulose synthesis showed that the production of (1→4)-β-D-glucans is favored over the synthesis of callose when calcium is chelated by EGTA (Okuda et al. 1993). From these results, it has been proposed that the synthesis of callose and cellulose is performed by the same enzyme that polymerizes either polysaccharides, depending on its conformation and on regulation processes that may involve divalent cations or changes in the phosphorylation state (Delmer 1999). Demonstration of such hypotheses requires the isolation of pure enzymes in an active form, an objective that has still to be achieved. Altogether, these observations clearly show that it is difficult to analyze and discuss the mechanisms of cellulose synthesis without considering the synthesis of callose. However, this does not mean that (1→3)-β-D-glucan synthesis in plants is a phenomenon of secondary importance. Actually, callose synthesis is essential in normal plant development and plays a central role in the plant defense response to various stresses (Stone and Clarke 1992). For instance, (1→3)-β-D-glucans are deposited transiently at the cell plate during cell division and are found as components of specialized cell walls such as those of pollen mother cells (Stone and Clarke 1992). They are also associated with sieve plates and plasmodesmatal canals at various stages of plant growth and development (Stone and Clarke 1992). Callose deposition is known to occur when plant tissues are stressed, e.g., after microbial infection or wounding (Stone and Clarke 1992).

As a consequence of the difficulty to apply biochemical approaches to the study of callose and cellulose biosynthesis, many groups have focused their efforts on the identification of genes that are required for the synthesis of these polysaccharides. This has allowed important progress since a number of genes that are likely to be directly responsible for callose and cellulose synthesis have been described (Delmer 1999; Doblin et al. 2002). In the case of cellulose synthase, the first genes were identified in the cotton fiber by Pear et al. (1996). Since then, numerous functional homologues designated as *CesA* have been isolated from other organisms such as *Arabidopsis thaliana* (Arioli et al. 1998; Taylor et al. 1999; Fagard et al. 2000; Taylor et al. 2000; Scheible et al. 2001), *Zea mays* (Holland et al. 2000), *Nicotiana alata* (Doblin et al. 2001), *Hordeum vulgare* (Burton et al. 2004), and *Populus tremula* × *tremuloides* (Djerbi et al. 2004). Interestingly, all the protein sequences deduced from these genes bear the consensus D,D,D,QXXRW motif common to all members of glycosyltransferase family 2, which comprises other polysaccharide synthases, like chitin and hyaluronan synthases (Saxena et al. 1995; Campbell et al. 1997; see also the Carbohydrate-Active Enzymes server http://afmb.cnrs-mrs.fr/CAZY/index.html (Coutinho and Henrissat 1999)). However, none of the *CesA* genes show any significant similarity with plant genes (Cui et al. 2001; Doblin et al. 2001; Hong et al. 2001; Li et al. 2003) homologous to *fks1* from *Saccharomyces cerevisiae* (Douglas et al. 1994), a gene proposed to encode the catalytic subunit of the yeast (1→3)-β-D-glucan synthase. The plant Fks proteins, designated GSL ("Glucan Synthase-Like"), do not have the D, D, D, QXXRW signature. They are grouped in glycosyltransferase family 48 and constitute a family of their own (Coutinho and Henrissat 1999). The most convincing biochemical data linking callose biosynthesis to a GSL protein have been obtained in barley (Li et al. 2003). In this work, the authors have shown for the first time that amino acid sequences from a highly enriched 250-kDa protein present in a fraction exhibiting (1→3)-β-D-glucan synthase activity correspond to amino acid sequences deduced from a barley *fks 1*-like gene designated *HvGSL1*. Interestingly, at least six independent *GSL* genes were identified in barley (Li et al. 2003). These genes showed sequence identity ranging from 40 to 60%. They may code for isoforms of HvGSL1 or for proteins with another yet unknown function. It is possible that some GSL proteins do not actually catalyze the polymerization of (1→3)-β-D-glucans but are involved in other processes. It is noteworthy that the function of the yeast Fks proteins is also being debated. It has been proposed that these proteins may not be involved in the catalysis of (1→3)-β-D-glucan synthesis, but in other mechanisms such as, for instance, the transport of β-glucans (Eng et al. 1994; Garrett-Engele et al. 1995; Cabib et al. 2001; Dijkgraaf et al. 2002). This idea is further supported by the observation that, unlike the plant GSL and yeast Fks proteins, the catalytic subunit of the (1→3)-β-D-glucan synthase from an *Agrobacterium* species shares similarities with other processive glycosyltransferases (Stasinopoulos et al. 1999) and is classified in glycosyltransferase family 2 together with the *CesA* proteins (Coutinho and Henrissat 1999). Despite the progress brought by the identification of the plant

GSL genes and the biochemical characterization of the barley HvGSL1 protein, the glycosyltransferase activity of most GSL proteins and of the corresponding Fks proteins from yeast remains to be demonstrated *in vitro*. Likewise, the catalytic activity of the products of the *CesA* genes has not been proved *in vitro*. From these observations, it appears important to reconsider biochemical approaches as important tools towards the characterization of cellulose and callose synthases. Undoubtedly, *in vitro* experiments represent a key step to achieve this objective.

Even though it is still not possible to assay cellulose synthases routinely, the results obtained on the blackberry (Lai Kee Him et al. 2002) and on the cotton and mung bean enzymes (Kudlicka et al. 1995; Kudlicka et al. 1996; Kudlicka and Brown, Jr. 1997) are promising for the direct characterization of cellulose synthases using biochemical approaches. In particular, globular structures which likely correspond to the synthesizing enzyme complexes have been found associated to *in vitro* cellulose microfibrils (Figure 8-1) (Kudlicka and Brown, Jr. 1997; Lai Kee Him et al. 2002).

In the best case, up to 1 mg of cellulose could be synthesized (Lai Kee Him et al. 2002). These experiments have allowed a complete characterization of the *in vitro* cellulose using physical and chemical techniques, but callose was still the major product in the reaction mixture (Lai Kee Him et al. 2002). Thus, it remains now to improve the *in vitro* procedure to isolate higher amounts of complexes composed of cellulose and synthesizing enzymes in order to be able to identify the different proteins required for cellulose polymerization. Promising models for such improvement are the recently established cell suspension cultures of hybrid aspen (*Populus tremula* × *tremuloides*) (Ohlsson et al. 2006). The first

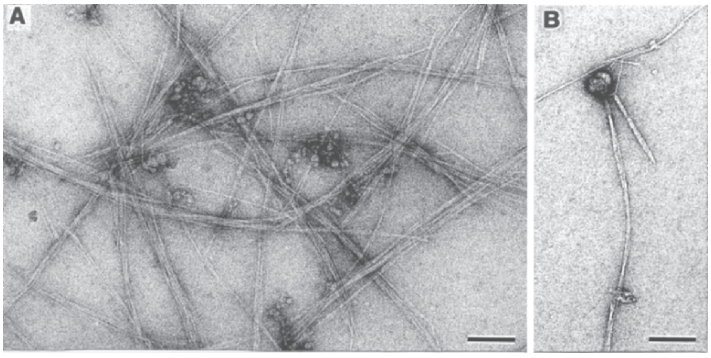

Figure 8-1. TEM images of the cellulose synthesized *in vitro* by the blackberry cellulose synthase extracted with taurocholate. The reaction mixture was as described in Table 8-1. Examinations were made after negative staining with 2% uranyl acetate. (**a**) Purified *in vitro* cellulose treated with the Updegraff (1969) reagent. (**b**) A typical example of an *in vitro* microfibril associated with a globular structure that may correspond to the synthesizing complex. This sample was not treated with the Updegraff reagent to preserve the globular structures. Bars = 100 nm. Reproduced from Lai Kee Him et al. 2002 with permission.

in vitro experiments performed on this system indicate that up to 50% of cellulose can be synthesized when the membrane-bound enzymes are extracted from cells harvested in the stationary growth phase (Colombani et al. 2004). However, even if such biochemical approaches appear to be a crucial step for the characterization of glucan synthases, it is now clear that substantial progress can be achieved only with a multidisciplinary strategy that integrates *in vitro* experiments coupled to product characterization, proteomics, immunochemical methods, molecular biology and expression analysis of the key genes.

The next sections of this review are focused on the description of *in vitro* approaches for the study of callose and cellulose synthases. Particular attention is given to the characterization of *in vitro* products. This is an important problem since non-rigorous analyses of glucan structures based for instance on the solubility in various solvents or on the resistance of the *in vitro* products towards chemical treatments may be misleading. Also, the strategy and the techniques to be used will differ depending on the objective of the characterization. The aim is generally to demonstrate that the reaction mixtures recovered after multiple *in vitro* assays contain the expected products. In addition to the demonstration that the synthesis occurred *de novo*, it is also important to determine accurately the type of linkage present in the products, which means for β-glucan synthases to be able to distinguish between (1→3)-β- and (1→4)-β-linked glucosyl units. In other situations, more detailed structural information can help understand biosynthetic processes. This usually requires the optimization of the composition of the synthetic reaction mixtures in order to obtain sufficient amounts of product for characterization using complementary physical and chemical techniques. Some of these techniques destroy the sample or lead to a loss of the three-dimensional organization of the polymers, whereas others allow the recovery of the *in vitro* polysaccharides in their original state. Therefore, the strategy used for a detailed structural characterization should take into account not only the amount of product available, but also the effect that the techniques successively used on a given sample may have on its structure and organization.

2 *IN VITRO* APPROACHES FOR THE STUDY OF β-GLUCAN SYNTHESIS

2.1 Optimization of the conditions for callose and cellulose synthesis

Protocols used for *in vitro* approaches have been described using several plant models such as, for instance, the cotton fiber (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996), *Lolium multiflorum* (Bulone et al. 1995), *A. thaliana* (Lai Kee Him et al. 2001), blackberry (Lai Kee Him et al. 2002) and recently the hybrid aspen (Colombani et al. 2004). The optimization of the conditions for *in vitro* synthesis of callose and cellulose has been discussed in more details in the report of Colombani et al. (2004), where the results obtained with the enzymes

from cell suspension cultures of the hybrid aspen are compared with those available in the literature. The assay conditions for glucan synthases have also been reviewed by Stone and Clarke (1992). Typically, these enzymes are assayed by measuring the incorporation of radioactive glucose from UDP-D-[^{14}C]glucose or UDP-D-[^{3}H]glucose into ethanol-insoluble polysaccharides. The mixture used to assay these processive glycosyltransferases usually contains 5 μM to 5 mM UDP-glucose, a membrane preparation or a detergent extract as a source of enzyme, one or several bivalent cations, and a disaccharide such as cellobiose, which activates enzymes. The pH is maintained in the range 7–8 and the temperature between 20°C and 30°C. The membrane fractions are prepared by differential centrifugation after the cells have been homogenized, usually using a mortar and pestle when the enzymes are to be isolated from plant tissues (see for instance Okuda et al. 1993 and Kudlicka and Brown, Jr. 1997), or a French press (Bulone et al. 1995; Lai Kee Him et al. 2001; Lai Kee Him et al. 2002) or a cell disrupting bomb (Colombani et al. 2004) when the starting biological material is a suspension of cells grown *in vitro*. Detergent extractions can then be performed to isolate the membrane-bound enzymes in an active form.

For the study of callose synthesis, the detergents that are commonly used are CHAPS (Sloan et al. 1987; Dhugga and Ray 1991; Wu et al. 1991; Bulone et al. 1995; Lai Kee Him et al. 2001; Li et al. 2003; Colombani et al. 2004), digitonin (Okuda et al. 1993; Kudlicka and Brown, Jr. 1997) and octylglucoside (Lai Kee Him et al. 2001). These detergents generally allow the preparation of relatively stable enzyme fractions that have a high callose synthase activity. It has been shown that detergents that belong to other families, like for instance the sulfobetain zwittergent 3–12, decanoyl-*N*-methylglucamide and glycodeoxycholate can also be efficient (Lai Kee Him et al. 2001). It seems however that these last detergents cannot be used to extract the enzyme from all plant species. For instance, we have observed that glycodeoxycholate allows the solubilization of the callose synthase from *A. thaliana*, but that it does not yield active preparations when microsomal fractions from blackberry are used as a source of enzyme (unpublished observations). Conversely, detergents that are efficient to extract the enzyme from blackberry in an active form, e.g., Brij 58, were not able to preserve the activity from *A. thaliana* cells. Also, one must be aware that some detergents may activate enzymes upon extraction and that the levels of activity recovered can be the result of both an efficient extraction and a stimulation of activity. This has been observed for instance with the detergent octylglucoside (Lai Kee Him et al. 2001). Other detergents like CHAPS, zwittergent 3–16 and lysophosphatidylcholine have been reported to activate glucan synthases from pollen tubes of *N. alata*, but in this case the activation was shown on particulate enzyme preparations and not after solubilization of the callose synthase from microsomal fractions (Li et al. 1997). These observations indicate that the level of activity recovered in detergent extracts can be dramatically affected by the nature of the detergent used for enzyme extraction. Several reports also show that the morphology and size of (1→3)-β-D-glucans synthesized *in vitro* by

fractions obtained with different detergents are affected by the nature of the detergent tested (Lai Kee Him et al. 2001; Lai Kee Him et al. 2003; Colombani et al. 2004). It was suggested that the detergents may have an effect on the general organization of the glucan synthase complexes, on the levels of activity and, indirectly, on the morphology and size of the *in vitro* products (Lai Kee Him et al. 2001). However, the mode of action of the detergents used and its consequence on enzyme activity remain to be determined at the molecular level.

The general assay mixture described above can be modified to test the effect of various components on the level of activity. When starting a study on a new plant species, it is important to test various conditions of assay for optimization. β-Glucosides, disaccharides such as cellobiose, laminaribiose or gentiobiose, and cellodextrins have been described as activators of plant callose synthases (Morrow and Lucas 1986; Hayashi et al. 1987; Li and Brown, Jr. 1993; Ng et al. 1996). In some instances, like for the enzymes from *A. thaliana* (Lai Kee Him et al. 2001) and the hybrid aspen (Colombani et al. 2004), the activation is rather low and does not exceed 10–15%. It has been proposed that β-glucosides and disaccharides act as allosteric activators of the enzymes (Morrow and Lucas 1986; Hayashi et al. 1987), although this has not been experimentally demonstrated. It has also been suggested that cellobiose may mimic an endogenous primer that would initiate polymerization of glucosyl units (MacLachlan 1982). However, the use of radioactive cellobiose showed that the activator is not incorporated in the final product during *in vitro* synthesis experiments (Hayashi et al. 1987). The mode of action of cellobiose on callose synthase activity remains to be determined, and the requirement of a primer for (1→3)-β-D-glucan synthase is still not demonstrated. Despite the fact that the effect of cellobiose is not understood at the molecular level, it is almost systematically added in *in vitro* assay mixtures.

Calcium and magnesium seem to play an important role in the regulation of callose and cellulose synthases. When doing *in vitro* experiments, it is important to test the effect of these cations on callose and cellulose synthase activities since it is possible to favor the synthesis of either polysaccharides, depending on the respective concentrations of calcium and magnesium in the medium. Calcium is required for (1→3)-β-D-glucan synthase activity from most plant species (Kauss et al. 1983; Delmer et al. 1984; Morrow and Lucas 1986; Hayashi et al. 1987; MacCormack et al. 1997; Lai Kee Him et al. 2001; Colombani et al. 2004), except for the developmentally expressed callose synthase from *N. alata* pollen tubes (Schlüpmann et al. 1993). Interestingly, in the case of enzyme preparations from cotton fiber, the addition of magnesium in the assay mixture combined with a decrease in calcium concentration favored the *in vitro* synthesis of cellulose (Okuda et al. 1993). Similar results were obtained recently using cell suspension cultures of hybrid aspen as a source of enzyme (Colombani et al. 2004). However, these results contrast with those obtained with the enzyme from blackberry (Lai Kee Him et al. 2002). In this case, the highest yields of *in vitro* cellulose were observed in the absence of cations when taurocholate was used

to extract the membrane-bound proteins, whereas the addition of magnesium (8 mM) was necessary to obtain detectable amounts of cellulose with Brij 58 extracts (Lai Kee Him et al. 2002). It seems that the presence of cations is not always a requirement for *in vitro* synthesis of cellulose by the blackberry enzyme (Lai Kee Him et al. 2002), as opposed to the situation in the cotton fiber (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996; Peng et al. 2002). It is possible that, in the case of blackberry, several isoforms of cellulose synthase with different cation requirements were extracted by the two detergents taurocholate and Brij 58. These results also indicate that the choice of the detergent to extract cellulose synthases in an active form is critical. Indeed, digitonin seems to be the best detergent for *in vitro* synthesis of cellulose with the enzymes from cotton fiber (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996) and the hybrid aspen (Colombani et al. 2004), while it is only with Brij 58 and taurocholate extracts that cellulose synthesis was possible in the case of the blackberry enzyme (Lai Kee Him et al. 2002). Furthermore, no cellulose was synthesized when taurocholate and Brij 58 extracts from *A. thaliana* were used in the conditions described for the blackberry enzyme (Lai Kee Him et al. 2002). It is likely that the cellulose synthases from various plant species have a different lipid environment and, consequently, that the extraction of active enzyme complexes from a given species depends on the structure of the detergent used. This would explain why the choice of the detergent is very critical for a successful *in vitro* synthesis of cellulose.

It seems that it is only when the rosettes are kept intact that the synthesis of cellulose I is possible *in vivo* (Arioli et al. 1998). The organization of the catalytic subunits in rosette-like structures has also been proposed as a requirement for *in vitro* synthesis of cellulose (Lai Kee Him et al. 2002). This hypothesis is supported by the occurrence at the tips of *in vitro* cellulose microfibrils of globular particles that may correspond to rosette-like structures (Figure 8-1) (Kudlicka and Brown, Jr. 1997; Lai Kee Him et al. 2002). *In vivo*, it is possible that such structures are located within membrane microdomains that have a specific lipid composition. In this hypothesis, the detergents that preserve the cellulose synthases active would extract the enzymes as intact complexes, together with structural lipids required for the cohesion of the multimeric synthases. To keep following this idea, the use of relatively strong detergents that would lead to a true solubilization of the proteins composing the enzyme complexes would also provoke the disruption of the whole machinery and, consequently, a loss or a dramatic decrease in cellulose synthesis. Interestingly, the production of lipids belonging to the sterol family has recently been shown to be crucial for cellulose synthesis as well as for cell elongation and cell wall expansion (Schrick et al. 2004). It is possible that sterols are directly involved in the stabilization of the cellulose synthase machinery, both in plasma membranes and after protein extraction with the relatively mild detergents that preserve cellulose synthase activity.

In all experiments performed by Brown, Jr. and coworkers on the cotton fiber enzyme (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996), as well as in our *in vitro* experiments on blackberry (Lai Kee Him et al. 2002) and hybrid

aspen (Colombani et al. 2004), no primer was added in the reaction mixture to achieve *in vitro* synthesis of cellulose. It is only recently that a sitosterol-β-glucoside has been identified as a putative primer for *in vitro* synthesis of cellulose in the cotton fiber (Peng et al. 2002). It is likely that any preparation obtained from plant membranes after detergent extraction contains such a primer. However, it is also possible that the *in vitro* synthesis of cellulose does not always start *de novo*, but that the polymerization of the chains occurs from preexisting (1→4)-β-D-glucan chains. It will be interesting in future experiments to see whether the addition of sitosterol-β-glucoside in reaction mixtures can lead to the synthesis of higher amounts of cellulose during *in vitro* experiments. In the next step, it will be important to demonstrate whether sitosterol-β-glucoside or any other kind of primer is really required for initiation of cellulose polymerization *in vivo*.

Altogether, these data indicate that the conditions for *in vitro* synthesis of cellulose must be optimized for each plant species and that there is no general recipe that can be applied regardless of the source of enzyme and conditions of extraction from the plasma membrane. So far, the only factor that is common to the protocols that have led to the highest *in vitro* synthesis of cellulose is the use of Mops buffer. In particular, in studies on the blackberry (Lai Kee Him et al. 2002), cotton fiber (Kudlicka et al. 1996; Peng et al. 2002) and mung bean enzymes (Kudlicka et al. 1996), as well as in our recent work on hybrid aspen (Colombani et al. 2004), Mops has been described as the buffer of choice over the previously used Tris to improve the yields of *in vitro* cellulose. The conditions that have led to successful *in vitro* syntheses of cellulose are summarized in Table 8-1.

Table 8-1. Optimal conditions for *in vitro* synthesis of cellulose. The conditions that gave the highest cellulose synthase activity with enzyme preparations from blackberry (Lai Kee Him et al. 2002), hybrid aspen (Colombani et al. 2004), cotton fiber and mung bean (Kudlicka et al. 1995 and 1996) are compared. 50 mM Mops buffer was used at a pH of 6.8 for the blackberry cellulose synthase, as opposed to 100 mM and pH 7.0 for the enzyme from hybrid aspen. For the cotton fiber and mung bean cellulose synthases, 50 mM Mops buffer was used at pH 7.5. The concentrations of detergents used to extract the enzymes in an active form are given in parentheses. Adapted from Colombani et al. 2004.

	Blackberry		Hybrid aspen	Cotton fiber, mung bean
	Brij 58 extract	*Taurocholate extract*	*Digitonin extract*	
	(0.05%)	(0.3%)	(1%)	(0.05%)
Mops buffer	+	+	+	+
Cellobiose (20 mM)	+	+	+	+
Mg^{2+} (8 mM)	+	–	+	+
Ca^{2+} (1 mM)	–	–	+	+
UDP-glucose (1 mM)	+	+	+	+
Cyclic 3′,5′-GMP (100 μM)	–	–	–	+
NaN_3 (3 mM)	–	–	–	+
Digitonin (0.05%)	–	–	–	+

2.2 Structural characterization of *in vitro* products

2.2.1 Methods adapted to high-throughput assays

The optimization of *in vitro* conditions for the synthesis of β-glucans must be validated by careful structural characterization of the products recovered in the reaction mixtures. When multiple conditions are tested, it is important to have a rapid and accurate method that will enable the distinction between (1→3) and (1→4) linked β-glucosyl residues. The use of methods that rely on differences of solubility of the *in vitro* products should be avoided or at least combined with more specific techniques. Even though (1→3)-β-D-glucans are usually described to be soluble in NaOH solutions as opposed to cellulose, their solubility in alkali is not always complete. This is particularly true for *in vitro* (1→3)-β-D-glucans of a high degree of polymerization such as those recently characterized by Pelosi et al. (2003). We have actually noticed that some (1→3)-β-D-glucans remain partially insoluble when NaOH concentrations as high as 3 M are used (unpublished observation). Also, the addition of divalent cations like magnesium in the assay mixture has been reported to lead to the synthesis of (1→3)-β-D-glucans of a lower solubility in alkali (Hayashi et al. 1987). Therefore, the isolation of a glucan synthase product that is insoluble in aqueous NaOH solutions does not prove beyond doubt that it corresponds to cellulose, especially if divalent cations have been used during *in vitro* synthesis. Crystalline cellulose is known to be resistant to the Updegraff treatment which consists of heating the polymer for 30 min at 100°C in a mixture of concentrated acetic and nitric acids (Updegraff 1969). (1→3)-β-D-Glucans are usually hydrolyzed in the Updegraff reagent and the resistance of a β-glucan to this treatment is often used to demonstrate the presence of cellulose in a given sample. Even though this method combined with incubations in NaOH solutions is very useful for isolating cellulose from a complex mixture (Lai Kee Him et al. 2002), it does not always hydrolyze completely (1→3)-β-D-glucans. Consequently, the Updegraff reagent must always be used together with more specific methods to prove that cellulose synthesis occurred. Conversely, the Updegraff reagent is so drastic that poorly crystalline (1→4)-β-D-glucans such as those synthesized by Peng et al. (2002) are usually sensitive to the treatment. Even cellulose microfibrils isolated from primary walls are partially hydrolyzed by the acid mixture (Figure 8-2) (Lai Kee Him et al. 2002). Therefore, the sensitivity of a sample towards this reagent can only demonstrate that the initial preparation does not contain crystalline cellulose, which does not mean that no (1→4)-β-D-glucans were originally present.

To date, it appears that the most rapid and reliable methods for high-throughput assays of glucan synthases rely on the use of radioactive substrate. When UDP-[^{14}C]glucose or UDP-[^{3}H]glucose are added in the reaction mixture during synthesis, hydrolysis of the *in vitro* products in the presence of specific glycoside hydrolases is usually sufficient to demonstrate the synthesis of callose and/or cellulose. This method does not only prove that the expected products were synthesized, but it also shows that the synthesis of β-glucans occurred *de novo* and that the polymers analyzed are not contaminations from the microsomal

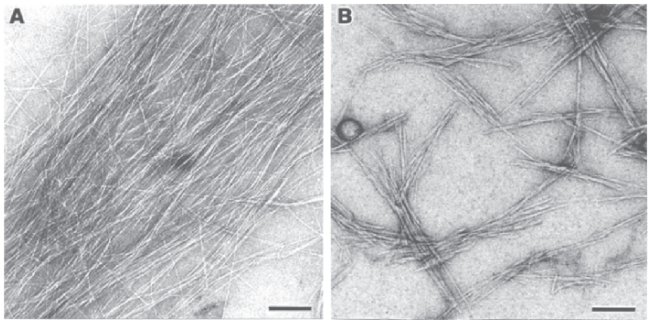

Figure 8-2. TEM images of the cellulose extracted from primary walls of blackberry cells. The samples were negatively stained with 2% uranyl acetate before observation. The cellulose microfibrils were observed before (**a**) and after (**b**) Updegraff treatment. The microfibrils treated with the Updegraff reagent were cut into fragments of 200–300 nm, indicating a low crystallinity. Bars = 100 nm. Reproduced from Lai Kee Him et al. 2002 with permission.

fraction or the cell wall. It can be completed by the analysis of oligosaccharides released upon enzymatic hydrolysis, for instance by thin layer chromatography (Okuda et al. 1993; Bulone et al. 1995). The use of glycoside hydrolases is reasonably rapid and reliable for high-throughput assays of glucan synthases. However, one should be aware that some commercial preparations of (1→3)-β-D-glucanases are contaminated with cellulases, and vice versa. Therefore, before applying this method to the identification of *in vitro* products, it is recommended to test the specificity of the hydrolase preparations on well-characterized polysaccharides.

An interesting alternative to the radioactive UDP-glucose substrates to assay callose synthases is the use of the fluorochrome from aniline blue which specifically interacts with linear (1→3)-β-D-glucans (Evans et al. 1984). A microtiter-based fluorescence assay was developed on the principle of this interaction (Shedletzky et al. 1997). Briefly, the method consists of stopping the glucan synthase reaction by adding a highly concentrated NaOH solution to the reaction mixture. The *in vitro* (1→3)-β-D-glucans are then solubilized at 80°C in the alkaline solution and incubated in the presence of the aniline blue fluorochrome. The fluorescence measured can be linked to the amount of glucose incorporated into (1→3)-β-D-glucan chains, after calibration of the assay with standard curves obtained with radioactive substrate. The major problem with this method is that it relies on the solubility of (1→3)-β-D-glucans in NaOH. Actually, the levels of callose synthase activity are underestimated when the solubility of the *in vitro* products decreases (Shedletzky et al. 1997). This is particularly true when the *in vitro* synthesis is performed in the presence of magnesium (Hayashi et al. 1987; Shedletzky et al. 1997). Therefore, the application of the method is limited to the study of *in vitro* synthesis reactions that yield (1→3)-β-D-glucans that are perfectly soluble in NaOH at 80°C. As discussed above, this is not always the case, and the solubility of the newly synthesized products depends on their degrees of polymerization and crystallinity, which

seem to vary with the conditions of assay, enzyme source and detergent used for extraction (Lai Kee Him et al. 2001; Pelosi et al. 2003). Also, even though the fluorochrome is specific for (1→3)-β-D-glucans (Evans et al. 1984), the assay does not allow the quantification of the cellulose that may be present in the sample. For these reasons, the use of radioactive UDP-glucose combined with hydrolysis experiments of *in vitro* products with specific enzymes, is by far the most commonly used method for a rapid and reliable identification of *in vitro* β-glucans.

2.2.2 Detailed structural characterization of in vitro products

The physical and chemical techniques listed in Table 8-2 provide detailed structural information that can help understand some aspects related to the polymerization and crystallization of polysaccharides. They are complementary and it is actually not possible to have a complete structural characterization of a given polymer by using only one of these techniques. Other methods based on transmission electron microscopy examinations and involving cellulases coupled

Table 8-2. Methods for the characterization of *in vitro* products synthesized by (1→3)-β-D-glucan synthases. The information that can be obtained with each method and the expected results for linear *in vitro* (1→3)-β-D-glucans such as callose are presented (after Bulone et al. 1995; Lai Kee Him et al., 2001; Pelosi et al. 2003). The average amount of product required for the different techniques as well as the possibility to recover the sample after each type of analysis are also indicated. Adapted from Colombani et al. 2004.

Method and information obtained	Expected results for *in vitro* (1→3)-β-D-glucans	Amount of product required/ possibility of sample recovery
Solubility (distinction between (1→3)-β-D-glucan and cellulose; however, this method is not completely reliable)	Insoluble in water Usually soluble in DMSO Usually soluble in 1 M NaOH	~1 mg When soluble, the sample can be recovered by dialysis against distilled water
Infrared spectroscopy (identification of linkage type/structure)	Absorption band at 889.5 cm^{-1}	~1 mg Sample recovery possible
Methylation (gas chromatography coupled to mass spectrometry) (identification of linkage type and estimation of the degree of polymerization if <100)	~100% (1→3)-linked glucose (if the degree of polymerization is higher than 100)	>100 µg Sample is hydrolyzed and cannot be recovered
^{13}C-NMR spectroscopy in DMSO (or NaOH) solution (structure)	Resonance signals at 60.9, 68.5, 72.9, 76.4, 86.2 and 103.1 ppm assigned to carbons-6, -4, -2, -5, -3 and -1 of (1→3)-β-glucan	~10 mg Sample can be recovered after dialysis against distilled water, with a loss of the native conformation

(*Continued*)

Table 8–2. (*Continued*)

Method and information obtained	Expected results for in vitro (1→3)-β-D-glucans	Amount of product required/ possibility of sample recovery
Solid state ^{13}C-NMR spectroscopy (cross-polarization/magic angle spinning NMR spectroscopy) (structure, organization of the chains, indication of degree of crystallinity)	Resonance signals at 61.1, 68.1, 74.2, 77.4, 86.8 and 103.6 ppm assigned to carbons -6, -4, -2, -5, -3 and -1 of (1→3)-β-glucan. The intensity of an additional signal at 76.0 ppm (attributed to C5) seems to be higher for (1→3)-β-D-glucans with a low molecular weight and a loose packing (Pelosi et al. 2003).	>10 mg Recovery of the sample in native state
X-ray diffraction (crystal structure and degree of crystallinity)	Organized as triple helices Usually low degree of crystallinity	~1 mg when using a conventional diffractometer Recovery of the sample in native state
Static light scattering in DMSO solutions (estimation of the degree of polymerization)	High degree of polymerization (depending on the conditions of synthesis) (Pelosi et al. 2003)	mg amounts (variable depending on the number of measurements at different concentrations and on the concentrations required to be under dilute regime conditions) Sample can be recovered after dialysis against distilled water, with a loss of the native conformation
Reaction with the aniline blue fluorochrome	Strong UV fluorescence induced (Evans et al. 1984)	<1 mg Fluorochrome difficult to remove completely
TEM (negative staining, cryo-TEM) (morphology)	Microfibrillar morphology	µg amounts Sample cannot be recovered

to gold particles have been used to distinguish microfibrils of callose and cellulose synthesized *in vitro* (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996; Kudlicka and Brown, Jr. 1997).

However, the latter methods do not provide any structural detail and it is important to couple them with physical and chemical analyses. The techniques presented in Table 8-2 have all been successfully applied to the characterization of *in vitro* (1→3)-β-D-glucans synthesized by enzymes from plants (*L. multiflorum*,

Bulone et al. 1995; *A. thaliana*, Lai Kee Him et al. 2001; *R. fruticosus*, Lai Kee Him et al. 2003 and Pelosi et al. 2003) and from the Oomycete *Saprolegnia monoica* (Pelosi et al. 2003). Some of them require relatively large amounts of *in vitro* product. It is therefore necessary to scale up the synthetic reactions to be able to use these methods to perform a detailed structural characterization. Also, some techniques like methylation analysis destroy the sample, while others like liquid NMR spectroscopy lead to a loss of the native conformation of the polysaccharide chains (Table 8-2). However, in this last case, the sample that is dissolved in dimethylsulfoxide or NaOH for the purpose of the analysis can be recovered by precipitation after extensive dialysis against distilled water. An example of a typical ^{13}C-NMR spectrum corresponding to a linear (1→3)-β-D-glucan synthesized *in vitro* by a plant enzyme is shown in Figure 8-3.

The method becomes much more sensitive when the substrate used during *in vitro* synthesis contains a sugar moiety that is enriched in ^{13}C. Recently, we have shown, with the example of a callose synthase from blackberry, that only 100 µg of (1→3)-β-D-glucan synthesized *in vitro* is sufficient for ^{13}C-NMR analysis when the synthesis reaction is performed using UDP-[U-^{13}C]glucose, i.e. a UDP-glucose molecule in which the glucosyl residue is uniformly enriched in ^{13}C (Fairweather et al. 2004). In our experiments, the UDP-[U-^{13}C]glucose was synthesized using

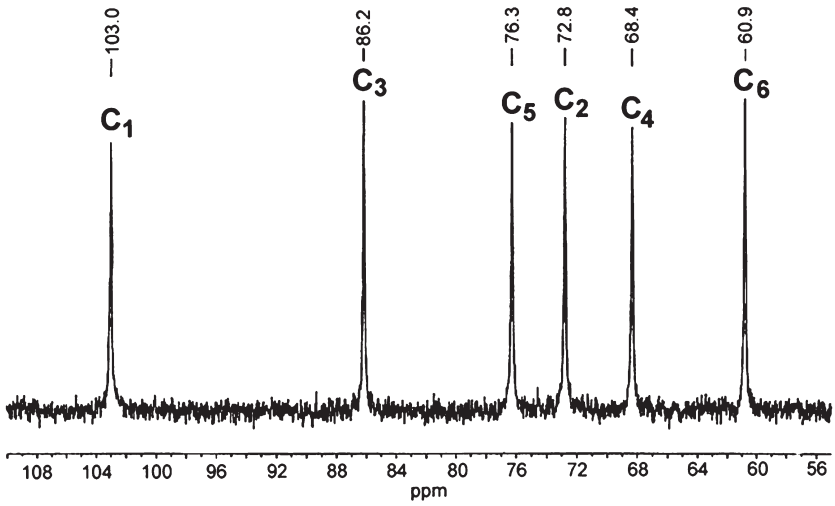

Figure 8-3. ^{13}C-NMR spectrum of the (1→3)-β-D-glucan synthesized *in vitro* by the callose synthase from blackberry. The chemical shifts at 60.9, 68.4, 72.8, 76.3, 86.2, and 103.0 ppm were measured at 75 MHz in $(CD_3)_2SO$ at 295 K, by reference to the central peak of the $(CD_3)_2SO$ multiplet (39.5 ppm). The spectrum is characteristic of a strictly linear (1→3)-β-D-glucan (Saito et al. 1977; Bulone et al. 1995; Lai Kee Him et al. 2001). Reproduced from Lai Kee Him et al. 2002 with permission.

a chemical approach, but the synthesis can also be achieved using commercially available enzymes (Ma and Stöckigt 2001). The method based on the use of ^{13}C-enriched sugar donors is so sensitive that even solid-state NMR spectroscopy can be applied to the structural characterization of *in vitro* products (Fairweather et al. 2004). It has several advantages over other analytical methods:

1. It is very sensitive and requires low amounts of *in vitro* products (~100 times less polysaccharide is required compared with the same analysis performed on a non-enriched polymer).
2. It provides a direct structural characterization of the *in vitro* product, as opposed to biochemical techniques that rely on the use of radioactive substrate and a subsequent hydrolysis of the polysaccharides with specific hydrolases. In the case of β-glucans, liquid ^{13}C-NMR analysis allows distinction between (1→3) and (1→4) linkages for products that are soluble in DMSO. For high molecular weight polymers that are insoluble in solvents commonly used for liquid ^{13}C-NMR analysis, such as cellulose, solid-state NMR spectroscopy can be used for structural characterization. In addition, solid-state NMR spectroscopy provides information on the conformation of the glucan chains in a given preparation as well as structural details that cannot be obtained with liquid NMR spectroscopy and methylation analysis. For instance, in the case of cellulose, solid-state NMR spectroscopy allows the determination of the proportions of the I_α and I_β allomorphs in the sample (Atalla and VanderHart 1984).
3. The method proves directly that the product analyzed was newly synthesized since the molecule is enriched in ^{13}C.
4. It can be applied to the study of any glycosyltransferase after synthesis of the corresponding ^{13}C-enriched sugar donor.

An alternative to NMR spectroscopy is methylation analysis. Even though this method involves an acid hydrolysis of the sample, it remains the most sensitive chemical technique for determination of linkage type when ^{13}C-enriched substrates are not available for NMR analysis of *in vitro* products. Compared with NMR spectroscopy performed on polysaccharides that are not enriched in ^{13}C, only hundreds of μg of sample (i.e. 20–30 times less sample) are required for methylation analysis. Moreover, when methylation analysis is applied to radioactively labeled polymers, it also proves that the polysaccharides analyzed were newly synthesized. The method involves several chemical modifications of the polysaccharide to be analyzed, i.e. methylation in the presence of methyl iodide, acid hydrolysis, reduction and acetylation (see for example Harris et al. 1984). Separation by gas chromatography of the resulting alditol acetates is usually sufficient to distinguish between (1→3) and (1→4) linkages, on the basis of the retention times of the derivatives on the column and by comparison with well-characterized standards. However, it is preferable to couple gas chromatography to mass spectrometry (GC/MS) to firmly identify the derivatives characteristic of (1→3) and (1→4) linkages (Figure 8-4). In some instances, the method can also allow an estimation of the degree of polymerization of a polysaccharide, especially if the starting individual chains consist of less than 100 sugar residues (100 is the lowest detection limit of

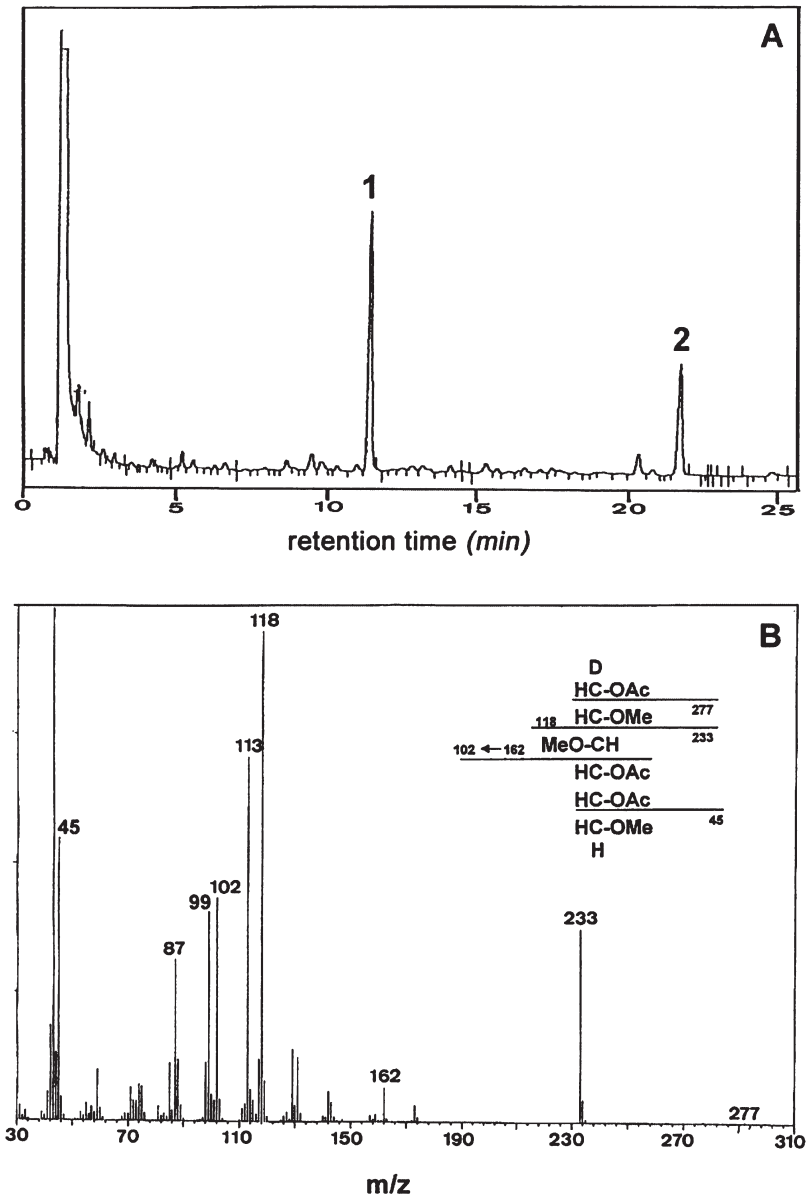

Figure 8-4. Methylation analysis of *in vitro* β-D-glucans. (**a**) Gas chromatography of the permethylated alditol acetate obtained from methylation analysis of the cellulose synthesized *in vitro* by the enzyme from blackberry. Peak 1, derivative characteristic of (1→4) linked glucosyl units. Peak 2, internal standard (*myo*-inositol). The derivative characteristic of (1→3) linked glucosyl units usually elutes 1 min before the major derivative visible in the chromatogram (not shown; see Bulone et al. 1995). (**b**) Structural characterization by electron impact mass spectrometry of the 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol derivative corresponding to peak 1 in A and characteristic of (1→4) linked glucosyl units.

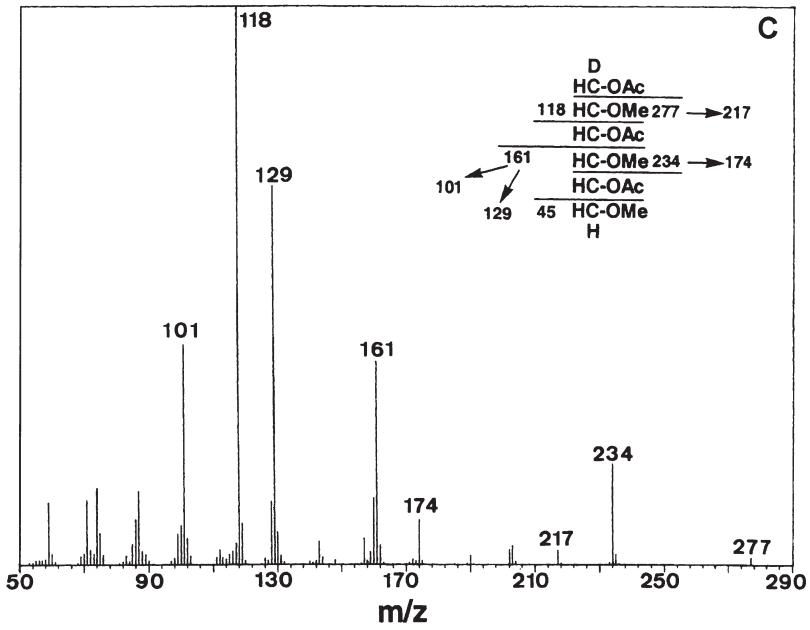

Figure 8-4. (*Continued*) (**c**) As in (**b**) but for 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol which is characteristic of (1→3) linked glucosyl units. (**a**) and (**b**) are from Lai Kee Him et al. 2002, whereas (**c**) is from Lai Kee Him et al. 2001 (reproduced with permission).

the method). The disadvantage of the methylation technique is that it does not indicate if different linkages are originally present in the same or different molecules. Also, unlike NMR spectroscopy, it does not allow the distinction between α and β linkages. In order to avoid the repetition of time-consuming large-scale *in vitro* synthesis experiments, it is preferable to perform the different analyses presented in Table 8-2 on the same sample. Thus, it is recommended, when using successively these techniques on a given preparation, to start the structural characterization with the non-destructive methods such as x-ray diffraction and solid state NMR.

The importance to achieve detailed structural characterization of *in vitro* products is illustrated by the application of the methods listed in Table 8-2 to the analysis of *in vitro* (1→3)-β-D-glucans synthesized under various conditions. For instance, it has been shown that for a given plant species the morphology and the stucture of the *in vitro* products are affected by the nature of the detergent used to extract the membrane-bound synthases (Lai Kee Him et al. 2001). Also, for a given detergent, enzymes from different plant species do not necessarily synthesize products that have the same morphology and structure (Lai Kee Him et al. 2001; Colombani et al. 2004). From these observations, it seems important

to study the lipid environment of the callose synthases from various origins. This should help understand why and how detergents affect the morphology and the structure of the *in vitro* (1→3)-β-D-glucans. In a previous study on *A. thaliana*, it was suggested that detergents may have an effect on the general organization and structure of the enzyme complex during extraction from the plasma membrane and, indirectly, on the morphology and structure of the *in vitro* (1→3)-β-D-glucans (Lai Kee Him et al. 2001). However, this hypothesis remains to be demonstrated. Interestingly, in another example where enzymes from *S. monoica* were used for the synthesis of (1→3)-β-D-glucans, it was shown that other parameters like the pH of the *in vitro* reaction mixture can influence the morphology, the degree of polymerization, the crystallinity and the structure of *in vitro* products (Pelosi et al. 2003). Even though many questions related to the mechanisms of polymerization and crystallization of polysaccharides are still unsolved, it seems that it will be possible in the near future to synthesize polysaccharides with specific properties by controlling *in vitro* reactions that involve glucan synthases from various organisms.

Some of the techniques presented in Table 8-2 have also been used to characterize the cellulose synthesized *in vitro* by cell-free extracts from blackberry (Lai Kee Him et al. 2002) and cotton fiber (Okuda et al. 1993; Kudlicka et al. 1995; Kudlicka et al. 1996). In these cases, it was possible to demonstrate that the purified *in vitro* products consisted exclusively of (1→4) β-linked glucosyl moieties, that they had a high molecular weight and that they corresponded to crystalline microfibrils that diffracted as cellulose. It is however only for the cellulose synthase studies on blackberry that multiple analyses could be performed on the same sample (Lai Kee Him et al. 2002). This was possible because one milligram of pure cellulose could be synthesized *in vitro* for the first time. Nonetheless, this amount was not sufficient to allow an accurate determination of the degree of polymerization of the β-glucan chains and to estimate, for instance by solid-state NMR spectroscopy (Atalla and VanderHart 1984), the proportions of the I_α and I_β allomorphs. It remains therefore to further improve the yields of *in vitro* cellulose to be able to obtain a more detailed structural analysis than the one described by Lai Kee Him et al. (2002). Such an achievement would also facilitate the assay of cellulose synthases and their direct characterization using biochemical approaches. A promising model for this type of biochemical studies is the suspension cultures of hybrid aspen (Ohlsson et al. 2006) from which it was possible to isolate cellulose synthase preparations that could synthesize *in vitro* up to 50% of cellulose (Colombani et al. 2004).

2.3 Purification of callose and cellulose synthases

When a reliable assay method is available for glucan synthases, and once it has been demonstrated that the *in vitro* products were synthesized *de novo* and that they correspond to the expected polysaccharides, biochemical approaches can be used to obtain enriched enzyme preparations. The reports

available in the literature on the purification of plant glucan synthases are so far almost exclusively related to callose synthases. It is only in the case of mung bean that a preliminary separation of callose and cellulose synthases has been achieved using native polyacrylamide gel electrophoresis (Kudlicka and Brown, Jr. 1997). However, none of the 12 proteins that appeared to be specifically associated with cellulose synthase activity have been characterized so far. The lack of biochemical information on plant cellulose synthases is mainly due to the difficulty of assaying these enzymes routinely. Proteins of molecular weights of 30–35 and 50–67 kDa have been described to be potentially involved in callose synthase activity. The main techniques used for these investigations were product entrapment and/or gradient centrifugation, immunochemical techniques or photoaffinity labeling with substrate analogues. For instance, proteins of 26–37 kDa were enriched in callose synthase preparations from *Beta vulgaris* (Wu et al. 1991; Wu and Wasserman 1993), *Brassica oleracea* (Fredrikson et al. 1991), *Gossypium hirsutum* (Delmer et al. 1991), *Oryza sativa* (Kuribayashi et al. 1992), *Lolium multiflorum* (Bulone et al. 1995) and hybrid aspen (Colombani et al. 2004). Monoclonal antibodies that immunoprecipitate the callose synthase from *Glycine max* were also able to recognize by Western blot a 31-kDa protein (Fink et al. 1990). Other proteins in the range of 30 kDa or 50–67 kDa have also been identified by photoaffinity labeling (Wu et al. 1991; Delmer et al. 1991; Dhugga and Ray 1991; Li and Brown, Jr. 1993; Dhugga and Ray 1994). Despite the numerous results available, there is no report that describes the sequencing of these proteins. In addition, the molecular weights of the proteins identified using the biochemical approaches described above markedly differ from the molecular weights of the proteins that were identified in *Daucus carota* (Lawson et al. 1989) and *N. alata* (Turner et al. 1998). For instance, in the latter case, a 190-kDa protein was found to copurify with enzyme activity. Also, the plant GSL proteins, which have been proposed to correspond to catalytic subunits of callose synthases, have a molecular weight in the range 220–250 kDa (Cui et al. 2001; Doblin et al. 2001; Hong et al. 2001; Li et al. 2003). It is likely that several proteins of a lower molecular weight, such as those cited above, are part of a multimeric complex and required for callose synthesis. It remains however that despite the substantial progress made with the identification of the *GSL* genes, the precise protein composition of plant callose synthase complexes and the function of each of the different subunits potentially involved in (1→3)-β-D-glucan synthesis are not known.

The development of highly sensitive proteomic methods for the characterization of membrane-bound proteins should facilitate the systematic sequencing of all the proteins that are present in fractions with high callose synthase activity. Also, the progress made on *in vitro* synthesis of cellulose is promising and proteomic analysis may also be used on fractions enriched in cellulose synthases in the near future. This approach combined with detailed structural characterization of *in vitro* products, immunochemical methods, molecular biology and gene

expression analyses should provide important information on the composition of the glucan synthase complexes, and consequently on the mechanisms of biosynthesis of callose and cellulose.

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