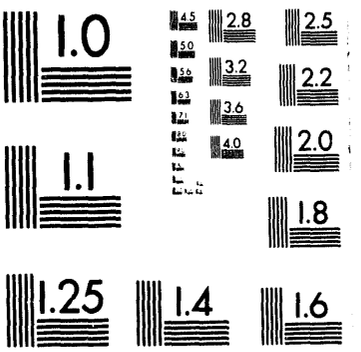
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
7.1

1 of 1

DOE/ER/20048--2

Progress Report DE-FG03-91ER20048 March 20, 1993

Enzymology and Molecular Biology of Cell Wall Biosynthesis

Peter M. Ray
Principal Investigator
Department of Biological Sciences
Stanford University
Stanford, CA 94305-5020

Tel. (415) 723-4497
FAX (415) 723-6132
email: pray@leland.stanford.edu

Introduction

In order to be able to explore the control of cell wall polysaccharide synthesis at the molecular level, which *inter alia* might eventually lead to means for useful modification of plant biomass polysaccharide production, the immediate goals of this project are to identify polypeptides responsible for wall polysaccharide synthase activities and to obtain clones of the genes that encode them. For reasons that were given in the grant proposal and were noted again in the previous year's report, we are concentrating on plasma membrane-associated (1,3)-β-glucan synthase (glucan synthase-II or GS-II) and Golgi-associated (1,4)-β-glucan synthase (glucan synthase-I or GS-I), of growing pea stem tissue. Our progress has been much more rapid with respect to GS-II than regarding GS-I, so GS-II will be considered first.

Glucan Synthase-II

We previously reported evidence that a plasma membrane-associated 55 kDa polypeptide is involved in GS-II activity (Dhugga & Ray, 1991a, 1991b). Several other laboratories have suggested that a 52-57 kDa polypeptide from various plant species is part of the GS-II system. However, in all these studies the presence in the enzyme preparations

of several to many other polypeptides (which most authors have interpreted as multiple subunits of the GS-II system) made it difficult to assign activity unambiguously to this polypeptide. Because of this, plus concern that more than one polypeptide might occur in the 55 kDa band which could lead, via an irrelevant amino acid sequence, to cloning an erroneous gene, we set out to try to purify GS-II as completely as possible.

The purification attempts required mass scale preparation of plasma membranes from pea stems. We optimized the aqueous polymer two-phase partition system for isolating plant plasma membranes such that we can prepare 0.12 g of highly purified plasma membranes from 600 g of 8-da old pea stems in one day. Two to three such preparations are then used for digitonin solubilization of GS-II, followed by the product entrapment procedure. This single step gives a ~200 fold purification of GS-II. Further fractionation by preparative isoelectric focusing and by glycerol gradient centrifugation increases the purification to ~350 fold over the specific activity of GS-II in the plasma membrane. In the highly purified preparations only two polypeptides, of 55 and 70 kDa, occur. Immunoblotting shows that the 55 is the same polypeptide that we previously reported, based on partial purification and immunological evidence, is involved in GS-II activity. This is the first time that GS-II has been purified to this extent. The key to this purification was finding that after isolation by product entrapment, GS-II activity can be stabilized by substantially lowering the buffer and detergent concentrations in the media. The detailed data on purification are presented in a manuscript (Dhugga & Ray, 1993), in preparation for submission to J. Biol. Chem., the current draft of which is appended hereto.

Our attempts at directly sequencing the 55 and 70 kDa polypeptides failed, apparently because these polypeptides are N-terminally blocked. We subsequently separated tryptic peptides derived from the 55 and 70 kDa polypeptides on a C-4 HPLC column (equipment that we do not possess, but have kindly been allowed the use of by the

Carnegie Institute of Washington, Stanford). The 55 yielded numerous, sharply resolved tryptic peptides, some of which are now being sequenced. The 70 proved difficult to digest with trypsin, possibly because of its very hydrophobic nature, and has yielded only about five peptide peaks, three of which are sharp and appear suitable for sequencing. At present we are attempting to cleave the 70 first with cyanogen bromide in the hope that more sites will become accessible to trypsin. After we have obtained sequences for some of these peptides we will construct corresponding oligonucleotides and use them to screen cDNA libraries in order to clone the coding sequences for these polypeptides. Dr. Theologis of the Plant Gene Expression Center, Albany, CA has already provided us a pea cDNA library, and Dr. Julie Palmer in Dr. Briggs' laboratory (Carnegie Institute of Washington, Stanford), who is constructing a pea cDNA library in an expression vector (λ-ZAP), has agreed to let us screen it.

Glucan Synthase-I

Because of its poorer stability, localized occurrence in peas (growing tissue only), and localization to Golgi membranes (which are considerably more tedious to isolate in sufficient quantity than plasma membranes), GS-I is less amenable than GS-II to identification or purification. We previously found that after glycerol gradient centrifugation of digitonin-solubilized Golgi membranes 2 polypeptides of 45 and 52 kDa, and a closely-spaced cluster of 4 ranging from 95 to 115 kDa, correlate with GS-I activity. We raised antibodies against the 52 kDa polypeptide, primarily because of its similarity in mol wt to the GS-II 55 kDa polypeptide, and the expectation of some relatedness between different polysaccharide synthases. The antiserum recognized several polypeptide bands in addition to the 52, suggesting that the 52 might be glycosylated. The antiserum furthermore neither inhibited nor adsorbed GS-I activity, which might mean (but does not prove) that the 52 is not a component of GS-I. Since a great deal of work and expense is involved in isolating enough of any of the mentioned polypeptides for raising an antiserum,

we would prefer, as described above for GS-II, to obtain a more purified enzyme preparation that could narrow down the number of polypeptides to be considered, before proceeding with further immunological work. Unfortunately in our hands neither product entrapment nor preparative isoelectric focusing, which are so helpful in purifying GS-II, can yet be made to work for GS-I; this line of attack is awaiting development of alternative method(s) of effective fractionation, which our limited funds and manpower have not yet permitted.

We have, however, made progress with one possible component of the GS-I system, namely the Golgi-localized, 40 kDa doublet that we previously reported is glycosylated by UDP-glc under GS-I assay conditions (Dhugga, Ulvskov, Gallagher & Ray, 1991). The doublet polypeptides may act as primary acceptors of glucose from UDP-glc and then donate this glucose to a separate transferase to be polymerized into (1,4)-β-linked product . We find we can purify the doublet polypeptides to homogeneity by affinity chromatography on UDP-glucuronic acid-agarose; they evidently bind UDP-glucuronic acid as they do UDP-glucose. The purified doublet can be glycosylated by UDP-glc like the membrane-bound one. Availability of purified, enzymatically active doublet will now allow us to perform the label transfer experiment, outlined in the original proposal for this grant, that will test the above-described putative role of the doublet in the GS-I system.

We have obtained 15 amino acid-long sequences for two tryptic fragments of the purified doublet. These sequences do not match anything in the sequence database. Oligonucleotides corresponding to these peptides have been synthesized and will be used to screen a cDNA library. We have also raised an antiserum to the purified doublet, which we plan to use as a second screen (with the expression library mentioned above) to confirm the identity of sequences that encode the doublet polypeptides.

Bibliography

1. **Dhugga KS, Ray PM** (1991) A 55 kilodalton plasma membrane-associated polypeptide is involved in ß-1,3-glucan synthase activity in pea tissue. FEBS Lett **278**: 283-286

2. **Dhugga KS, Ray PM** (1991) Isoelectric focusing of plant plasma membrane proteins: Further evidence that a 55 kD polypeptide is associated with ß-1,3-glucan synthase activity from pea. Plant Physiol **95**: 1302-1305

3. **Dhugga KS, Ulvskov P, Gallagher SR, Ray PM** (1991) Plant polypeptides reversibly glycosylated by UDP-glucose. Possible components of Golgi ß-glucan synthase in pea cells. J Biol Chem **266**: 21977-21984

4. **Dhugga KS, Ray PM** (1991) Purification of (1,3)-β-glucan synthase from pea. Two polypeptides of 55 and 70 kDa copurify with enzyme activity. (in preparation)

DISCLAIMER

SUMMARY

Stanford University
Stanford, CA 94305

Enzymology and Molecular Biology of Cell Wall Biosynthesis

Peter M. Ray, Department of Biological Sciences

Our objective is to identify plant cell wall polysaccharide synthases and clone the corresponding genes, so that the control mechanisms for wall synthesis, mechanisms that could be useful for modifying plant polysaccharide biomass production as an energy source, can be established. Golgi-localized, auxin-regulated β-1,4-glucan synthase (glucan synthase-I or GS-I), which produces the glucan backbone of xyloglucan, and plasma membrane-associated, Ca^{2+}-regulated β-1,3-glucan synthase (GS-II), which synthesizes callose (a wound response polysaccharide), are currently targeted.

We have been able to extensively purify pea GS-II down to 2 polypeptides of 55 and 70 kDa, by a combination of product entrapment (wherein a synthase becomes trapped within polysaccharide micelles that are produced by its synthase reaction and can easily be isolated by centrifugation), followed by isoelectric focussing and density gradient centrifugation. Peptide fragments obtained from each of these polypeptides by digestion with trypsin will be sequenced, and synthetic oligonucleotides corresponding to these sequences will be prepared and used to screen a pea gene sequence library to isolate the coding sequences for these polypeptides.

Except for density gradient centrifugation, the methods that have enabled us to purify GS-II have not worked for GS-I, and since at least 6 different polypeptides correlate with GS-I activity in density gradient fractionation, we are looking for additional methods that might be effective for fractionating Golgi membrane proteins. By affinity chromatography we have been able to purify a Golgi-localized 40 kDa doublet (2 closely related polypeptides) that may be involved in the GS-I system because these polypeptides

reversibly accept glucose units from UDP-glucose (the GS-I substrate) under GS-I assay conditions. The isolated polypeptides will be used in experiments to test the participation of the doublet in polysaccharide synthesis. Tryptic peptides obtained from the doublet have been sequenced, yielding unique sequences not found in the known protein sequence database. The nucleotide equivalents of these sequences will be used, as above, as screens to detect and clone the gene(s) for the doublet polypeptides.

DATE
FILMED
5/2/94
END