AN INTERMEDIATE IN CYCLIC β1-2 GLUCAN BIOSYNTHESIS*

Angeles Zorreguieta, Rodolfo A. Ugalde and Luis F. Leloir

Instituto de Investigaciones Bioquimicas "Fundacion Campomar",
Facultad de Ciencias Exactas y Naturales and CONICET,
Antonio Machado 151, 1405 Buenos Aires, Argentina

Incubation of UDP-[^{14}C]Glc with the inner membranes of Agrobacterium tumefaciens leads to the formation of cyclic β 1-2 glucan and trichloroacetic acid-insoluble compounds. The proteolysis products of the latter show a positive charge in acid and a negative charge in alkaline buffers. The cyclic β1-2 glucan and the trichloroacetic acid insoluble compounds yield the same products on partial acid hydrolysis. Addition of excess non-radioactive UDP-Glc to the reaction mixture nearly stops the formation of radioactive β1-2 glucan and leads to a rapid fall of radioactivity in the trichloroacetic acid precipitate. Alkaline treatment of the insoluble compounds under conditions of β-elimination leads to the partial release of free saccharides (about 30%). It is concluded that β1-2 glucan chains are built up joined to a protein and then released as free cyclic β1-2 glucan.   © 1985 Academic Press, Inc.

Considerable evidence has been presented showing that a polysaccharide

formed by Agrobacteria and Rhizobia is a cyclic β1-2 glucan with a degree of

polymerization which varies from 17 to 24 (1,2) and that it can be formed from

UDP-Glc and enzymes from those organisms (3,4). The enzymatic formation of β1-2

glucan was first detected years ago by Dedonder and Hassid (5).

MATERIALS AND METHODS

The enzyme preparation was the internal membrane fraction (6,7), of Agrobacterium tumefaciens LBA 4001 grown as in (8). Membranes were collected and washed by centrifugation at 100,000 x g for 2 h, and resuspended in Tris-HCl 30 mM pH 8.2.
The incubation mixture contained 60,000 cpm of UDP-[^{14}C]Glc (320 Ci/mol), 50 mM Tris-HCl pH 8.2, 60 mM MgCl$_2$, 20 mM mercaptoethanol and about 0.1 mg of enzyme preparation (total volume: 50 μl). Incubations were at 10°C for the indicated times. The reaction was stopped by heating for 1 min at 100°C and then 300 μl of

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Abbreviation: TCA, trichloroacetic acid.

water were added. After centrifugation, the supernate containing the cyclic glu-
can and the precipitate which contains the intermediate were processed separa-
tely.

Trichloroacetic acid precipitate. The precipitate obtained in the previous step
was suspended in 1 ml of 5% trichloroacetic acid and filtered through a micro-
fibre filter (Whatman GF/C). After washing thoroughly with TCA followed by
methanol, the dry filters were counted with toluene PPO-dimethyl POPOP in a
scintillator. In some cases, the precipitate was washed several times with water
to ensure the removal of all the free glucan. This is referred to as washed TCA
precipitate.

The cyclic glucan fraction was passed through a DEAE-Sephadex column (0.6 x 4
cm). The column was washed with 1.5 ml of water and an aliquot of the eluate was
counted with Bray's solution. It was checked that no radioactive UDP-Glc or low
molecular weight compounds such as free sugars emerged from the column.

Proteolysis: The TCA washed precipitate (50,000 cpm) was incubated at pH 7.5
with about 2 mg of protease type XIV from Streptomyces griseus (Pronase-Sigma)
for 16 h at 37°C. One ml of TCA (5%) was added and the supernate obtained by
centrifugation was washed with ethyl ether.

RESULTS AND DISCUSSION

Previous work on β1-2 glucan formation was carried out with homogena-
tes (3) or permeabilized cells (4). A purer preparation was obtained by dif-
ferential centrifugation of a sonicate. The most active fraction was that
corresponding to the inner membranes, and so it was used in all the experiments.

The formation of a TCA insoluble product. The effect of various changes in the
composition of the reaction mixture is shown in Table 1. The formation of glucan
and TCA insoluble product were approximately parallel. Both were inhibited by
the absence of Mg^{++}, by the presence of UDP or by heating the enzyme. Addition

TABLE 1

Effect of various substances on cyclic β1-2 glucan
and TCA-insoluble compounds formation

Additions	Radioactivity (cpm) in	
	TCA insoluble	Glucan
None	4500	24000
No 2-mercaptoethanol	5400	26000
No Mg^{++}	160	1300
No Mg^{++} + EDTA 5 mM	140	780
UMP 1 mM	4400	17000
UDP 1 mM	1200	2100
Sophorose 1 mM	5900	26000
Glucose 1 mM	5600	27000
Heated enzyme	280	840

Complete system as described in Methods (the incubation lasted 4 min).

of UMP, sophorose, glucose or the absence of 2-mercaptoethanol had hardly any action.

Some properties. The TCA insoluble product could not be solubilized in organic solvents such as chloroform:methanol:water (1:1:0.3) which dissolves many sugar lipids. Heating in 5% TCA for 2 min at 100°C led to no solubilization of radioactivity. Only about 20% was soluble after 5 min in 0.1 N HCl at 100°C.

Proteolysis. Treatment of the washed TCA precipitate with a proteolytic enzyme (pronase) rendered it non precipitable. Electrophoresis (Fig. 1) of the proteolysis products showed that they migrated towards the positive pole in an alkaline buffer (pH 10) and to the negative pole in acid medium (pH 2). Thus, they had the properties of aminoacid containing compounds.

Partial acid hydrolysis. In order to obtain information on the identity of the sugar moiety the proteolysis products were partially hydrolyzed and chromatographed on paper. As shown in Fig. 2, the hydrolysis products behave like a family of homologues. A sample of cyclic β1-2 glucan treated in the same way gave a similar pattern. In other tests the products of acid hydrolysis of the
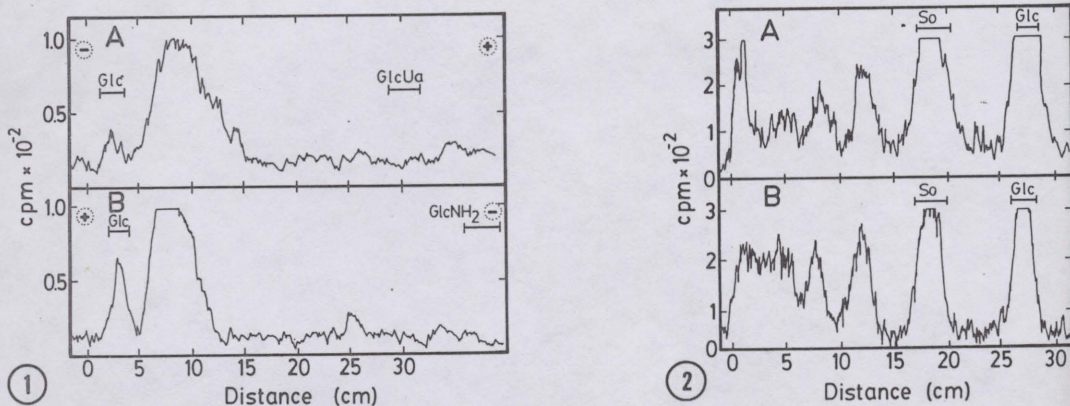
Fig. 1: Electrophoresis of the proteolysis products. An aliquot (4000 cpm) of the products obtained as described in Methods were spotted on paper. Electrophoresis (4 h at 25 V/cm) was carried out: A, in 0.2 M sodium carbonate bicarbonate (pH 10) and B, in 5% formic acid (pH 2). The standards were: glucose, Glc; glucosamine, $GlcNH_2$ and glucuronic acid, GlcUa.

Fig. 2: Paper chromatography of the products of partial acid hydrolysis.
A. The proteolysis products obtained as described in Methods were separated by electrophoresis in 5% formic acid. Positively-charged compounds were hydrolyzed (0,5 N HCl at 100°C for 25 min). The acid was removed "in vacuo" and paper chromatography was carried out with isopropanol:acetic acid:water (29:4:9).
B. A cyclic β1-2 glucan sample was hydrolyzed and run at the same time as well as standards of glucose (Glc) and sophorose (Glc β1-2 Glc, So).

washed TCA precipitate were chromatographed on paper with another solvent (butanol:pyridine:water, 6:4:3). In all the experiments, the mobilities of the smaller products was the same as those of standards of glucose and sophorose (Glc β1-2 Glc). Thus, it may be concluded that the TCA precipitate contains a β 1-2 linked glucan.

Alkaline treatment. It is generally accepted that sugars linked to hydroxyaminoacids are split by β-elimination by a rather mild alkaline treatment. If the carboxyl group of the hydroxyaminoacid is free or becomes free by hydrolysis, then the sugar moiety cannot be released as easily (9). When the washed TCA precipitate was treated with 0.2 N NaOH, 0.5 M sodium borohydride at 37°C for 72 h, about 40% of the sugar was still precipitable with TCA, 24% was charged positively (electrophoresis in 5% formic acid) and 36% was neutral. In other experiments, the charged substance was observed to have a negative charge when electrophoresis was carried out in alkaline buffer (pH 10). A more drastic treatment which splits asparagine-linked sugars (2 N NaOH, 2 M sodium borohydride at 80°C for 16 h) led to the disappearance of all the TCA precipitable material and 80% of the sugar became neutral. These results do not allow a clear conclusion on the nature of the aminoacid involved, although they exclude a linkage of the type of hydroxylysine-sugar which is very stable to alkali. Other methods will have to be used in order to clarify the nature of the protein-sugar linkage.

Chase experiment. The result of adding non-radioactive UDP-Glc to the reaction mixture is shown in Fig. 3. It may be observed that after non-labeled UDP-Glc addition the radioactivity in the TCA precipitate decreased very rapidly and thereafter very slowly. Incorporation of radioactivity in the glucan became very slow after UDP-Glc addition. In the experiment of Fig. 3, the radioactivity that disappears from the TCA precipitate should appear in the glucan, but this is not clearly visible because the scales differ by a factor of ten. These curves had the aspect of intermediate and product of a reaction.

The information obtained on the TCA insoluble compound formed during cyclic β1-2 glucan synthesis points strongly in favour of a reaction inter-
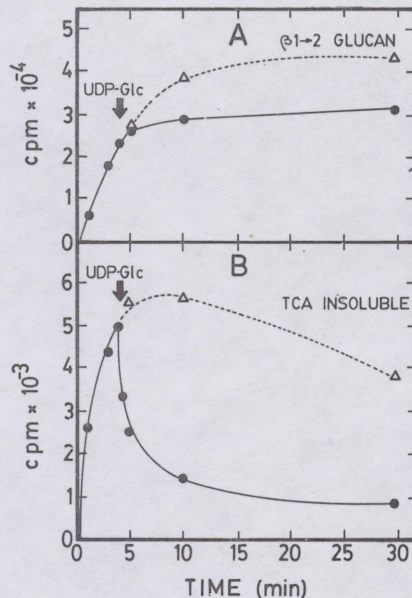
Fig. 3: Chase experiment. Incubation mixture as described in Methods. Solid lines: radioactivity after adding 1 mM of non-radioactive UDP-Glc at the arrow point. Dotted line: control with no addition.
A. Radioactivity in the cyclic β1-2 glucan.
B. Radioactivity in TCA precipitate.

mediate. It contains β1-2 glucan chains and the chase experiments were quite clear. The protein nature of the rest of the molecule seems certain as judged by the effect of a protease. Furthermore, the products of protease action had the properties of a glycopeptide, but the aminoacid combined to the saccharide was not identified.

It seems that the β1-2 glucan grows joined to a protein and that when it reaches 17 to 24 units in length, a transfer reaction occurs that leads to cyclization and release.

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