ON THE INHIBITION OF CELLULOSE BIOSYNTHESIS BY COUMARIN

H. Esteban HOPP*, Pedro A. ROMERO** and Rafael PONT LEZICA+
Departamento de Biología, Fundación Bariloche, Casilla de Correo 138, 8400 San Carlos de Bariloche, Argentina

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1. Introduction

It has been reported that coumarin inhibits the incorporation of glucose into cellulose fractions of mung bean [1], as well as fiber formation of isolated tobacco leaf protoplasts cultivated in vitro [2]. If the effect of coumarin is specific for the synthesis of cellulose, it could be a very useful compound for the study of cellulose biosynthesis. Particulate preparations of the Chlorophyta *Prototheca zopfii* catalyzed the following reactions [3]:

UDP-Glc + Dol-P → glucolipids → oligosaccharide-linked lipids → glucoprotein $\xrightarrow[\text{GDP-Glc}]{}$ cellulose

We report here that coumarin inhibits the transfer of the oligosaccharide chain from the lipid to the protein acceptor and does not affect the transfer of mannose from lipids to proteins.

2. Materials and methods

Prototheca zopfii strain PR-5 (ATCC 16533) was grown as in [4] and harvested from the log-phase cultures at 50% of maximum growth. Cells were disrupted by sonication (100 W, 30 min, with 5 μm glass powder in a cooling-cell with circulating methano at −30°C) in a buffer containing 0.05 M Tris–HCl, pH 8.0, 5 mM β-mercaptoethanol and 1 mM EDTA. The homogenate was centrifuged 5 min at 1000 × *g* and the resulting supernatant was centrifuged at 100 000 × *g* for 150 min. The resulting pellet was resuspended in 50 mM Tris–HCl, pH 7.5, containing 10 mM β-mercaptoethanol. This was the particulate preparation used in all the experiments.

UDP-[^{14}C]Glc, 268 Ci/mol, UDP-[^{3}H]Glc, 2.42 Ci/mmol, and GDP-[^{14}C]Man, 221 Ci/mol, were a generous gift from the Instituto de Investigaciones Bioquímicas, Fundación Campomar, Buenos Aires. All other chemicals were obtained commercially.

Standard incubations were carried out at 20–25°C for 60 min in total vol. 50 μl. The mixture contained: 5 μmol Tris–HCl, pH 7.5, 1 μmol β-mercaptoethanol, 0.5 μmol $MgCl_2$, 0.4 nmol UDP-[^{14}C]Glc (268 Ci/mol) and 100–400 μg protein. The additions of coumarin, GDP-Glc and GDP-[^{14}C]Man are indicated in each experiment. The reaction was stopped by the addition of 0.1 ml chloroform–methanol (2:1) and the glucolipids and oligosaccharide-linked lipids were extracted as in [5]. The glucoprotein was measured as in [3] and the resulting pellet was extracted to obtain the alkali-soluble and alkali-insoluble fractions [6].

Determination of cellulose in the algal cell walls was made as follows: 45 g packed cells were extracted with acetone and 50% phenol. The resulting pellet was treated with 2% NaOH at 100°C for 5 min 3 times then washed with water and acetone. The remaining alkali-insoluble polysaccharides were submitted to acetolysis [7] for 100 h at 37°C. The cellodextrin

* Present address: Centro de Investigaciones en Ciencias Agronómicas, INTA, CC 25, Castelar, Argentina

** On leave from the Universidad Católica de Valparaíso, Chile. Present address: Instituto de Investigaciones Bioquímicas, Fundación Campomar, Obligado 2490, 1428 Buenos Aires, Argentina

+ Direct correspondence to present address: Department of Biology, University of Kaiserslautern, 6750 Kaiserslautern, FRG

acetates were extracted with boiling 95% ethanol; the extract was filtered while hot and allowed to cool. The resulting crystals were filtered and deacylated with sodium metoxide [8] separated by paper chromatography with isopropanol–acetic acid–water (29:4:9). The disaccharide was eluted, freeze-dried and compared with cellobiose standards by different methods. A sample of cellulose powder for control (Whatman CF1) was treated in the same way as the algal polysaccharides. The corresponding alditols were obtained by reduction with excess of sodium borohydride [3].

3. Results and discussion

It has been reported that particulate preparations from this alga catalyzed the formation of an alkali-insoluble glucan having β-1,4-linkages [3]. Nevertheless, it was not clear whether cellulose is a normal component of *P. zopfii* cell wall, as in other chlorococcales [9]. Glucose, mannose and glucosamine were the only hexoses described as components of cell walls in this organism [10]. In order to elucidate this point, alkali-insoluble cell wall polysaccharides (7.5 g) were submitted to acetolysis, the products were deacetylated with sodium metoxide and the disaccharides isolated. The resulting disaccharide (1.7 g) was compared with cellobiose. Paper chromatography in propanol–ethyl acetate–water (7:1:2) and paper electrophoresis with 0.03 M sodium borate buffer, pH 9.8, showed that the algal disaccharide had the same mobility as cellobiose. Reduction with sodium borohydride and electrophoresis with 0.1 M sodium molybdate, pH 5, gave an alditol undistinguishable from cellobiitol standard. The obtention of cellobiose or a derivative in a good yield (22%) is considered sufficient evidence to show that the polysaccharide in question is cellulose [11]. We must therefore conclude that *P. zopfii* walls contained cellulose as other green algae.

In order to study the effect of coumarin in the growth of the alga, different concentrations of coumarin were added to the culture medium. Algal growth was completely inhibited by 7×10^{-4} M coumarin. A similar concentration affects the normal regeneration of cell wall in tobacco protoplasts [2] and inhibits by 35% the incorporation of glucose into the cellulose fraction of *Vigna angularis* epicotyls [12]. These similarities suggested that coumarin affects the same processes in algae as in higher plants.

To elucidate whether coumarin had any effect on the sequence of reactions leading to cellulose in this alga [3], experiments were performed incubating UDP-[^{14}C]Glc with particulate fractions and different concentrations of coumarin. Radioactivity incorporated into different fractions was measured at the end of the incubation period (fig.1). It is evident that coumarin inhibits the formation of the glucoprotein and the insoluble polymer (cellulose). The glucolipid and the oligosaccharide–lipids formation was not affected by low concentrations of coumarin (0.2 mM). This last product was enhanced by 0.2 mM coumarin, as could be expected if the inhibited step were the transfer of oligosaccharide moiety from the lipid intermediate to the protein acceptor. Higher concentrations of coumarin also inhibited the oligosaccharide–lipid formation.

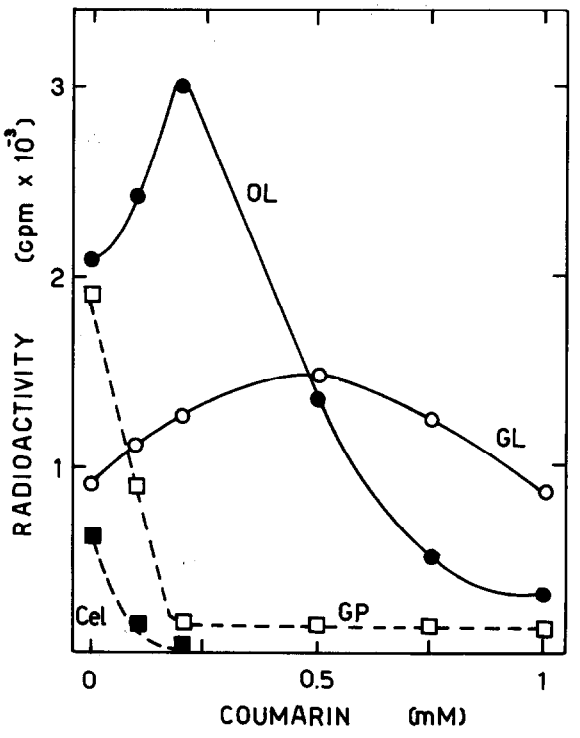

Fig.1. Effect of coumarin concentration on the incorporation of glucose to different fractions. Incubations were carried out as described with the addition of 5 nmol GDP-Glc and coumarin as indicated. GL, glucolipids; OL, oligosaccharide-lipids; GP, glucoprotein; Cel, cellulose.

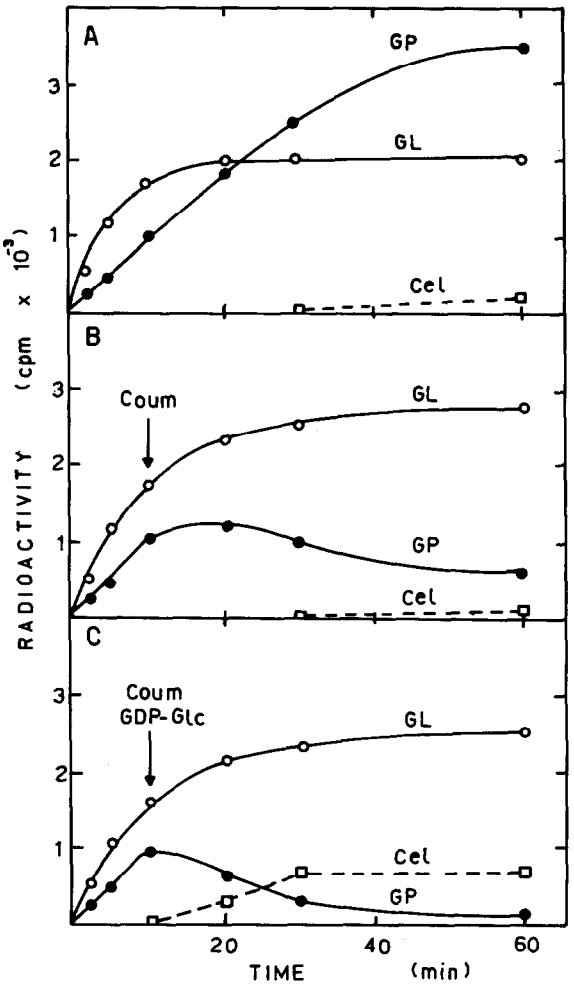

Fig.2. Time-course incorporation of glucose into different fractions. (A) Control with UDP-[^{14}C]Glc as the only precursor. (B) Incubated as in (A) but with the addition of 10 nmol coumarin. (C) Incubated as in (A) but with the addition of 10 nmol coumarin and 5 nmol GDP-Glc. GL, glucolipids; GP, glucoprotein; Cel, cellulose; Coum, coumarin.

Time-course experiments were made in which coumarin (0.2 mM) and GDP-Glc (0.1 mM) were added after 10 min incubation. Results in fig.2A show the incorporation into glucolipids and the glucoprotein when UDP-[^{14}C]Glc was used as precursor in the controls. Very little cellulose was formed in the absence of GDP-Glc. Coumarin (fig.2B) inhibits the formation of the glucoprotein but not the synthesis of glucolipids. When coumarin was added with non-radioactive GDP-Glc (fig.2C), the glucoprotein formation was inhibited as expected, but not the synthesis of the alkali-insoluble polymer (cellulose) that rose until the precursor (glucoprotein) was consumed. These results clearly show that coumarin does not inhibit the formation of glucolipids, oligosaccharide–lipids, or cellulose from GDP-Glc. The only detectable step affected by coumarin is the glucosylation of protein. The incorporation of [^{14}C]glucose into a cytoplasmic β-1,4-glucan and cellulose was inhibited by coumarin in *Phaseolus aureus* hypocotyls [13]. This is in agreement with our results assuming that the cytoplasmic β-1,4-glucan is similar to our glucoprotein.

Coumarin inhibited specifically the incorporation

Table 1
Effect of coumarin on the incorporation of glucose and mannose to different fractions

Fractions	– Coumarin		+ Coumarin	
	[^{14}C]Man (pmol)	[^{3}H]Glc (pmol)	[^{14}C]Man (pmol)	[^{3}H]Glc (pmol)
Glycolipids	5.64	0.38	8.36	0.67
Oligosaccharide–lipids	21.26	0.39	42.18	0.58
Water-soluble polymer	2.85	1.92	3.50	0.02
Alkali-soluble polymer	3.35	0.37	7.29	0.94
Alkali-insoluble polymer	0.00	11.28	0.00	0.10

Incubations were carried out as described with the addition of 2.5 nmol GDP-[^{14}C]Man, 0.25 nmol GDP-Glc, 0.4 nmol UDP-[^{3}H]Glc and 25 nmol coumarin

of glucose into cellulose fractions and no effect was observed in the pectin and hemicellulose fractions [1]. We have shown here that coumarin inhibits the transfer of the oligosaccharide chain from the lipid intermediate to the protein. It was interesting to study the specificity of this inhibition in order to elucidate whether the glycosylation of other proteins, mediated by lipid intermediates, was also blocked by coumarin. Previous observations (unpublished data) showed that [^{14}C]mannose from GDP-[^{14}C]Man could be incorporated into a protein fraction by the lipid intermediate pathway in *P. zopfii*. Membrane preparations were incubated with GDP-[^{14}C]Man, UDP-[^{3}H]Glc and non-radioactive GDP-Glc as precursors (in the same test tube) with or without 0.5 mM coumarin. Incorporation of sugars into different fractions is shown in table 1. As expected, the incorporation of [^{3}H]glucose into the glucoprotein and cellulose was inhibited by coumarin. On the other hand, [^{14}C]mannose was incorporated into the glycolipid, oligosaccharide–lipid and protein fractions (water-soluble and alkali-soluble fractions). Coumarin did not inhibit the incorporation of mannose, but stimulated it. This effect could presumably be due to the availability of the same lipid intermediate not used by the cellulose system.

Evidence obtained up to now suggests that coumarin inhibits specifically cellulose biosynthesis. However, further studies will have to be carried out in order to fully support this assumption.

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