Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells

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In homogenates from suspension-cultured soybean cells, 1,3-β-D-glucan synthase activity is increased through preincubation with trypsin or due to action of an endogenous enzyme which presumably is a protease as it is inhibited by soybean trypsin inhibitor. The 1,3-β-D-glucan synthase in untreated membrane preparations is also reversibly stimulated by Ca^{2+}. This Ca^{2+}-dependence is lost on proteolytic activation. Regardless as to whether the enzyme was rendered active by preincubation with trypsin or by the presence of Ca^{2+}, it is inhibited by calmidazolium, trifluoperazine and polymyxin B, suggesting that the activation by Ca^{2+} is not mediated by calmodulin.

Soybean *1,3-β-D-Glucan* *Callose* *Proteolysis* *Ca^{2+}* *Phospholipid interacting inhibitors*

1. INTRODUCTION

The 1,3-β-D-glucan synthase (EC 2.4.1.34) is very active in membrane preparations from a variety of tissues and plants, whereas in the walls of healthy cells its product is present only in minor amounts [1,2]. A function for the enzyme is suggested by the mainly cytological observations that 1,3-β-D-glucan (callose) can be rapidly deposited (sometimes within minutes) at special sites if cells are mechanically injured or attacked by pathogens [1,3]. It appears thus that callose and the speed at which it is formed are important parameters in cell repair and disease resistance mechanisms. Callose is also often transiently deposited when the wall structure undergoes alterations at certain developmental states, for example during sieve pore formation or pollen maturation [1].

All these processes require effective regulation of the 1,3-β-D-glucan synthase activity in plants. We report here data which suggest that this might occur alternatively by action of proteolytic enzymes and/or changes in $[Ca^{2+}]$.

2. MATERIALS AND METHODS

Origin and growth of suspension-cultured soybean cells have been described [4]. The cells were harvested on days 5–6 on a Buchner funnel, on which they were resuspended twice without suction in 100 mM MES/NaOH (pH 6.25) containing 5 mM EGTA; 2 g were homogenized in a ground-glass Potter with 4 ml same buffer but containing 1 mM dithiothreitol, 5 mg/ml bovine serum albumin (fraction V) and 1 mg/ml soybean trypsin inhibitor (omitted where indicated). For membrane preparations, this crude homogenate was further diluted with 6 ml same buffer and centrifuged at 0°C for 3 min at $500 \times g$; membranes were pelleted from the supernatant at $50000 \times g$ for 10 min. The pellet was washed twice with 6 ml 50 mM Tris–HCl (pH 7.0) containing 1 mM dithiothreitol in quartz-distilled water, and suspended in 12 ml of the same buffer. The membrane preparations contained ~0.6 mg protein/ml as determined [5] against a bovine serum albumin standard.

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The 1,3-β-D-glucan synthase assay mixture was combined from [6,7]. For the crude homogenate it consisted of 50 μl 20 mM cellobiose, 16% (w/v) glycerol, 50 mM $MgCl_2$ in water to which 10 μl 0.2% (w/v) digitonin were added. For assay of the particulate membrane preparation 50 μl 20 mM cellobiose, 16% (w/v) glycerol, 10 mM $MgCl_2$ and 4 mM EGTA containing various [$CaCl_2$] in 50 mM Tris–HCl, (pH 7.0) were mixed with 10 μl 0.2% (w/v) digitonin. For assays with >5 μM Ca^{2+} the EGTA was omitted. To these mixtures, 50 μl homogenate or membranes, respectively, were added and the reaction was started with 5 μl 20 mM UDP-[^{14}C]glucose (58 000 cpm) and was terminated after incubation at 25°C at the indicated times by immersion in a boiling water bath.

The assay mixture was quantitatively transferred to strips (3 × 1.5 cm) of Whatman 3MM paper, dried and the paper strips washed twice for 1 h in a mixture of 150 ml ethanol with 350 ml 0.5 M ammonium acetate (pH 3.6). After drying the paper strips were counted in 5 g 2,5-diphenyloxazole/l toluene in a scintillation counter. Each value given represents the means of 2 identical assays minus the zero time control.

For identification of the reaction products assay paper strips were washed after counting in toluene to remove the cocktail constituents and after drying were boiled for 1 h in ~50 ml various solvents, followed by drying and recounting. To show the linkage type in the glucan the strips were wetted by 150 μl solution of 1,3-β-D-glucanase (1 mg/ml of 100 nM citrate/NaOH (pH 5.0)) or α-amylase (1 mg/ml 100 mM Tris–HCl (pH 7.0), 1 mM dithiothreitol) and placed for 48 h in a chamber saturated with water and chloroform vapour. This was followed by washing twice in ethanol/ammonium acetate as for the enzyme assay.

Ca^{2+} was >5 μM as determined with a Radiometer-Selectrode F 2112Ca connected to a Metrohm 605 pH-Meter and calibrated against the Ca^{2+}-standard S3606 (Radiometer), either diluted with quartz-distilled water (for homogenates) or the same but containing 50 mM Tris–HCl, 20 mM cellobiose, 16% (w/v) glycerol, 10 mM $MgCl_2$ (for the particulate preparations). Below 5 μM, the [free Ca^{2+}] was calculated from the Ca^{2+}/EGTA proportion in the buffer used for the assay mixture [8].

Trypsin and α-amylase were from Boehringer (Mannheim) calmidazolium (= substance R 24571) from Janssen Pharmaceutica (Beerse) and trifluoperazine was kindly provided by Röhm-Pharma (Weiterstadt). All other biochemicals were bought from Sigma (St Louis MO). Specific 1,3-β-D-glucan hydrolases were kindly provided by Professor H. Meier (Fribourg).

3. RESULTS

Initial experiments showed that all homogenates prepared, even those from cells washed with buffers not containing EGTA, contained 30–100 μM free Ca^{2+}, which in the light of our later experiments (fig.2) is sufficient to allow high 1,3-β-D-glucan synthase activity. We therefore included 5 mM EGTA in the washing and homogenization buffers. Crude homogenates prepared in this way showed low glucan synthase activity, but could be activated in a time-dependent reaction by preincubation with trypsin (fig.1). In the presence of digitonin higher maximum values were attained. The

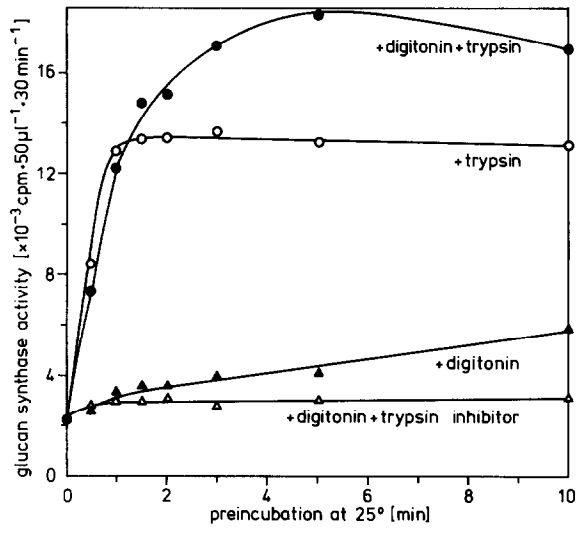

Fig.1. Activation of glucan synthase by trypsin and by endogenous protease. Cell homogenates were prepared without trypsin inhibitor and 500 μl incubated at 25°C with 200 μg trypsin without (○) or with (●) 0.02% (w/v) digitonin. Alternatively, 500 μl homogenate were incubated with 0.02% digitonin without (▲) or with (△) 500 μg soybean trypsin inhibitor. Aliquots were removed at the indicated times and assayed for 30 min in the presence of 50 μg soybean trypsin inhibitor and 0.02% (final conc.) digitonin.

control with digitonin, but without added trypsin, also showed an increase in activity with time. This increase could be almost fully prevented by addition of soybean trypsin inhibitor. The degree of this endogenous activation varied considerably with different batches of cells. In some experiments, where no soybean trypsin inhibitor was added during homogenization and assay, the activity found was so high that the further increase due to preincubation with trypsin or the presence of Ca^{2+} in the assay was small. We therefore added soybean trypsin inhibitor to the homogenization mixture for all further experiments.

The 1,3-β-D-glucan synthase activity in cell homogenates could also be increased ~10-fold by addition of $CaCl_2$ in amounts sufficient to titrate the EGTA present and to create 100–200 μM Ca^{2+}

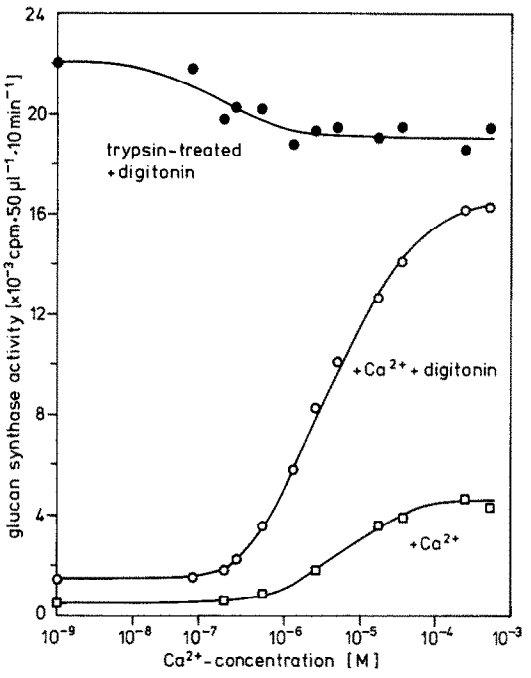

Fig.2. Influence of $[Ca^{2+}]$ on the glucan synthase activity. A particulate fraction was prepared from soybean cells as in section 3 and was used as such (○, □), or was preincubated at 25°C for 2 min with trypsin (100 μg/ml) at 0.02% (w/v) digitonin followed by addition (200 μg/ml) of soybean trypsin inhibitor (●). The assays were performed either without (□) or with 0.02% digitonin (●, ○). In a control experiment in which trypsin and inhibitor were mixed first and then the membranes added and incubated, no significant activation was evident.

(not shown). However, low concentrations of free Ca^{2+} cannot be established or measured with the Ca^{2+}-electrode, using crude cell homogenates. We therefore studied the concentration-dependence with a membrane preparation (fig.2). About 5 μM Ca^{2+} are sufficient for half-saturation, almost maximum activity is found at ~100 μM. A similar effect of Ca^{2+} is evident when the assay did not contain digitonin, although glucan formation occurred at a lower level. No Ca^{2+} appear to be necessary when the activity of 1,3-β-D-glucan synthase was elicited by preincubation of the membranes with trypsin.

The stimulation of the 1,3-β-D-glucan synthase by Ca^{2+} was nullified if EGTA was added in concentrations sufficient to lower the $[Ca^{2+}]$ to <0.1 μM, and this was also observed when the homogenates were preincubated at 25°C in the presence of Ca^{2+} (table 1). When the membrane preparation was assayed as in fig.2 with 150 μM Ca^{2+} or in the presence of 2 mM EGTA, no significant influence was observed upon addition of 0.1 mM ATP or GTP (final conc.). The addition of calmodulin (100 μg/ml) isolated from bovine brain [9] also did not change under the above conditions the activity of the glucan synthase. Trifluoperazine and calmidazolium inhibited glucan formation by the membrane preparation, both when it was rendered active by trypsinization or in the presence of Ca^{2+} in the assay (table 2). Principally, the same was found to be true with polymyxin B and various salts.

Table 1

Influence of Ca^{2+} on the activity of 1,3-β-D-glucan synthase in membrane preparations and its reversion by EGTA.

	Glucan formed [$\times 10^{-3}$ cpm/10 min]	
Membranes + Ca^{2+}	− EGTA	+ EGTA
Not preincubated	9.7	1.0
Incubated 5 min at 25°C	9.6	0.9

Two assay mixtures with membranes were prepared 10-fold scaled-up and supplied with 80 μM Ca^{2+}. Aliquots (100 μl) were assayed by addition of 5 μl radioactive UDP-glucose immediately or after preincubation for 5 min at 25°C either directly or supplied with 2 mM EGTA, which is sufficient to bring $[Ca^{2+}]$ to $<10^{-7}$ M

Table 2

Inhibition by various substances of the 1,3-β-D-glucan synthase, activated by the presence of Ca^{2+} or by preincubation with trypsin

Substance added (final conc.)	Glucan formed in membranes [$\times 10^{-3}$ cpm/10 min]	
	+ Ca^{2+}	Trypsinized
Expt I		
None (DMSO-control)	12.5	20.9
Calmidazolium (100 μM)	0.4 (3)	0.9 (4)
Calmidazolium (20 μM)	9.7 (75)	17.2 (82)
Trifluoperazine (200 μM)	4.2 (33)	7.2 (34)
Expt II		
None (water-control)	14.8	19.0
Polymyxin B (450 μM)	4.4 (30)	10.3 (54)
Polymyxin B (90 μM)	6.2 (42)	13.3 (70)
NaF (45 mM)	2.4 (16)	5.4 (28)
KF (45 mM)	7.0 (47)	9.0 (47)
NaCl (45 mM)	9.2 (62)	13.2 (69)
KCl (45 mM)	11.2 (75)	14.1 (74)

Conditions were as in fig.2. Ca^{2+} was present at 230 μM for the untreated membranes and was about 10^{-9} M (=2 mM EGTA, final conc.) for the trypsinized preparation. For expt I the compounds were added in 2 μl dimethyl sulfoxide (DMSO)/100 μl assay and for expt II in 10 μl water; (% of control)

Tentative identification of the reaction product was based upon its solubility and its resistance against linkage-specific enzymes. About 10% were solubilized from the strips by boiling them in water, representing presumably short chain products or articles which do not sufficiently stick to the filter paper. Boiling with dimethyl sulfoxide removed ~75% and with 1 N NaOH 98% of the glucan. The product was not split by α-amylase, but 90% was solubilized after treatment with an exo-1,3-β-D-glucanase from *Corticium rolfsii*, whereas with an endo-1,3-β-D-glucanase from *Rhizopus arrhizus* 50–60% was rendered soluble. Taken together this is sufficient evidence to tentatively classify the majority of the product as 1,3-β-D-glucan, possibly containing some glucose residues in other linkages [7].

4. DISCUSSION

The data in fig.1,2 clearly show that proteases can render the 1,3-β-D-glucan synthase in the soybean membrane preparation active. The action of the presumed endogenous protease can be counteracted by an inhibitor of protein nature, isolated from the same plant. These findings are reminiscent of reports on the activation of chitin synthase in yeast [10], where it was suggested that such interactions play a role in the regulation of wall biogenesis. With the yeast chitin synthase it remains, however, still unclear whether the proteolytic activation occurs directly at a zymogen or alternatively by removing a protein inhibitor from the enzyme. The picture has even become more occluded by the findings [11] that the vacuolar yeast proteases appear not to be necessary for the regulation of wall formation and tht numerous new proteases of unknown function can be found in yeast. Similar reservations hold also for the question of a possible physiological role of proteases in the regulation of 1,3-β-D-glucan synthase activity in soybean cells.

The observation that Ca^{2+} at low concentrations can stimulate the 1,3-β-D-glucan synthase might explain older reports that these ions are necessary for the callose deposition process [12]. There is now increasing evidence that the [Ca^{2+}] in the cytoplasm of animal and plant cells is held at a rather low level; between 10^{-7}–10^{-8} M [13]. Thus an increase of the integer [Ca^{2+}] due to membrane damage possibly could trigger the glucan synthase directly. This hypothesis requires that the Ca^{2+} of the wall compartment of a live cell has no access to the enzyme. Such an assumption is reasonable as the substrate UDP-glucose presumably comes from the cytoplasmic site, suggesting that the enzyme might be located at the inner membrane surface whereas the product 1,3-β-D-glucan appears to be deposited outside the plasma membrane [1,12].

The data in table 1 suggest that Ca^{2+} must be present throughout the assay period; a short preincubation period is not sufficient. This might indicate that Ca^{2+} are directly effectors of the enzyme and do not act with the help of an auxiliary enzyme system such as phosphorylation or dephosphorylation.

Table 2 shows that the calmodulin-binding sub-

stances calmidazolium [14] and trifluoperazine [13] inhibit the Ca^{2+}-mediated stimulation as well as the reaction enhanced by trypsinization. The above substances bind, however, to calmodulin only in presence of Ca^{2+}. In addition, we had to use relatively high concentrations, and added calmodulin had no effect. These results suggest that the above drugs may exert their action on the glucan synthase not by inhibiting a Ca^{2+}/calmodulin-mediated activation but by non-specific interaction, possibly by binding phospholipids which are associated with the membrane-bound enzyme. This assumption is also sustained by the observed inhibition due to polymyxin B, a cyclic polypeptide with hydrophobic side chains which can influence many membrane functions and inhibit for example the phospholipid-requiring protein kinase C isolated from animal cells [15]. This Ca^{2+}-dependent enzyme can be activated by limited proteolysis and is *not* dependent on calmodulin but nevertheless inhibited by many phospholipid interacting drugs, including the socalled calmodulin-inhibitors trifluoperazine and chlorpromazine [16]. In addition, also Ca^{2+}/calmodulin-dependent enzymes can be non-specifically inhibited by the above drugs. This was discussed in detail for the erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase [17], membrane-bound enzymes which can also be activated by limited proteolysis.

The effects observed with the salts of various monovalent cations (table 2) also appear to be non-specific, and simply resulting from their chaotropic potency.

These results are also of interest in regard to other enzymes involved in cell wall biogenesis. Some indirect evidence was presented that Ca^{2+} might be important for the regulation of cellulose [18] and chitin [19] biosynthesis.

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