Multiplicty of soluble glucan-synthase activity in spinach leaves: Enzyme pattern and intracellular location

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Abstract. Buffer-extractable proteins from leaves of Spinacia oleracea L. were separated by non-denaturing polyacrylamide gel electrophoresis. Gels were stained for adenosine diphosphoglucose (ADPglucose)-dependent glucan-synthase (GS) activity (EC 2.4.1.21). Three major forms of activity were observed. No staining was detectable when ADPglucose was replaced by an equimolar concentration of either uridine, guanosine or cytosine diphosphoglucose. Two of the three GS forms exhibited both primed and citrate-stimulated unprimed activity whereas one enzyme form was strictly dependent upon the presence of an exogenous glucan. For intracellular localization, mesophyll protoplasts and intact chloroplasts were isolated and their enzyme pattern was compared with that of the leaf extract. Intactness and purity of the chloroplast preparations were ascertained by polarographic measurement of the ferricyanide- or CO2-dependent oxygen evolution, by determination of marker enzyme activities, and by electrophoretic evaluation of the content of chloroplast- and cytosol-specific glucan-phosphorylase forms (EC 2.4.1.1). The three GS forms were present in mesophyll protoplasts. Intact chloroplasts possessed both primer-independent enzyme forms but lacked the primer-dependent one. The latter form was enriched in supernatant fractions of leaf homogenates when the intact chloroplasts had been pelleted by centrifugation. Thus, in spinach-leaf mesophyll cells soluble ADPglucose-dependent GS is located both inside and outside the chloroplast.

Key words: Chloroplast (glucan synthese) – Enzyme (multiple forms) – Glucan synthase (intracellular location) Spinacia (glucan synthese) – Starch synthase

Introduction

In higher-plant leaves the plastid is generally assumed to be the only starch-containing organelle. In contrast, enzymes acting on α-1,4/1,6-linked glucans possess a more complex intracellular distribution. Glucan-hydrolyzing enzymes (such as endoamylase, debranching enzyme, α-glucosidase), glucan phosphorylase, and D-glucose all have been localized both inside and outside the chloroplast (Steup 1988; Beck and Ziegler 1989; Beers and Duke 1990).

Chloroplast and extra-chloroplast proportions of several of these enzyme activities have been attributed to distinct compartment-specific enzyme forms (Steup 1988, 1990). Owing to the compartmentation of the plant cell, transitory starch granules are accessible only to those enzyme forms/activities which reside inside the chloroplast and, therefore, all the extra-chloroplast enzymes are not immediately involved in the metabolism of transitory starch. If any of these non-chloroplast enzymes exerts a metabolic function in vivo, an oligo- or polysaccharide pool has to be postulated which is situated at the same subcellular site. Recently, a heteroglycan has been isolated from spinach and pea leaves (and from pea cotyledons as well). This high-molecular-weight polysaccharide contains glucose residues and interacts strongly with the cytosol-specific phosphorylase isozyme (Type I; see Steup 1990). Interaction with the chloroplast-specific phosphorylase form (Type II) was minor (Yang and Steup 1990). As revealed by indirect immunofluorescence, the heteroglycan is located in the cytosol of parenchyma cells and, presumably, functions in vivo as the carbohydrate substrate of the cytosolic phosphorylase form (Steup et al. 1991). Thus, it appears that in the cytosol of higher-plant cells glucan polymerization and depolymerization reactions take place which differ largely from those involved in chloroplast starch metabolism.

These results prompted us to reinvestigate the intracellular location of other ‘starch’-metabolizing enzymes. Until now, soluble glucan synthase (GS; EC 2.4.1.21) was considered to be located exclusively inside...
the plastid. Localization has been based upon measurements of the enzyme activity following leaf tissue or protoplast fractionation (Mares et al. 1978; Okita et al. 1979; Echeverria and Boyer 1986; Robinson and Preiss 1987). In these experiments GS activity was usually determined by monitoring $^{14}$C-glucosyl transfer from ADPglucose to a primer. In principle, any concomitant glucan depolymerization, as catalyzed by amylolytic activities, can interfere with the glucan synthesis and result in an underestimation of GS activity (Dang and Boyer 1988). This holds especially true for samples containing a high level of amylase but a relatively low amount of GS activity. Because the bulk of leaf glucan hydrolyses resides outside the chloroplast, the various subcellular fractions of leaves or protoplasts contain different amounts of amylolytic activities and, therefore, the reliability of the GS assay may vary. Interference is expected to be low if chloroplast extracts are assayed but it may be significant for non-chloroplast cell fractions.

In order to avoid these complications we have taken an approach which had been successfully applied in intracellular localization studies on glucan phosphorylase (Steup and Latzko 1979). Buffer-soluble GS activity from spinach leaves was resolved into distinct enzyme forms by non-denaturing polyacrylamide gel electrophoresis. The pattern observed with the leaf extract was then compared with that of mesophyll protoplasts and of intact chloroplasts. By this procedure, even minor components of the total GS activity can be localized.

The results obtained provide evidence that soluble GS possesses a dual intracellular location.

### Material and methods

**Plant material.** Spinach (*Spinacia oleracea* L. cv. ‘Estivato’ or ‘Wiremona’, purchased from Nebeling, Münster, FRG) plants were grown hydroponically as described elsewhere (Steup and Latzko 1979). Buffer-soluble GS activity from spinach leaves was resolved into distinct enzyme forms by non-denaturing polyacrylamide gel electrophoresis. The pattern observed with the leaf extract was then compared with that of mesophyll protoplasts and of intact chloroplasts. By this procedure, even minor components of the total GS activity can be localized.

**Leaf crude extract.** Approximately 8 g of deveined leaves were frozen in liquid nitrogen and were then ground with a mortar. The leaf material was suspended in 20 ml grinding buffer (60 mmol Tris $\cdot$ $1^{-1}$, adjusted to pH 7.3 with orthophosphoric acid, and containing 5 mmol dithioerythritol (DTE) $\cdot$ $1^{-1}$, 2 mmol EDTA $\cdot$ $1^{-1}$, plus 0.4 mmol phenylmethylsulfonyl fluoride (PMSF) $\cdot$ $1^{-1}$), and was further ground with an Ultra-Turrax homogenizer. The homogenate was filtered through four layers of a nylon net and was centrifuged (10 min at 26 000 $\times g$). Proteins of the centrifuged leaf homogenate (see above). Precipitated proteins were added ammonium sulfate (70% saturation) to the supernatant of the centrifuged leaf homogenate (see above). Precipitated proteins were collected by centrifugation (10 min at 26 000 $\times g$), dissolved in 20 ml grinding medium (see above) using a glass homogenizer. The homogenate was centrifuged (10 min at 26 000 $\times g$). Proteins of the supernatant were concentrated by ammonium-sulfate precipitation (70% saturation) and were then passed through a Sephadex G-25 column (see above). In some experiments the concentration step was omitted. The chloroplast-deprived cell fraction was obtained by adding ammonium sulfate (70% saturation) to the supernatant of the centrifuged leaf homogenate (see above). Precipitated proteins were collected by centrifugation (10 min at 26 000 $\times g$), dissolved in 20 ml grinding buffer and were centrifuged again (as above). The supernatant was passed through a nitrocellulose filter and through Sephadex as described for the leaf extract.

**Polyacrylamide gel electrophoresis.** For non-denaturing discontinuous electrophoresis the system described by Jolley and Allen (1964) was used. Electrophoresis was performed using a Mini-Protein II Dual Slab Cell (1.5 mm spacer; Bio-Rad, Munich, FRG). The total monomer concentration of the separation gel was 8.5% (w/v). Except where stated, 1 to 1.35 mg protein $\cdot$ cm$^{-2}$ gel surface was applied. Samples contained 15% (w/v) sucrose. Following electrophoresis (2 h 25 min at 250 V, 11.5 to 7.5 mA per slab gel; $2^{3}$ C) gels were stained for starch synthase or, alternatively, glucan phosphorylase activity.

**Non-denaturing continuous electrophoresis was performed as described elsewhere (Steup 1990).**

**Enzyme-activity staining.** For both primed and unprimed starch-synthase-activity staining, gels were equilibrated (45 min at room temperature under continuous agitation) in a mixture containing 50 mmol Tricine-NaOH $\cdot$ $1^{-1}$, 2 mmol EDTA $\cdot$ $1^{-1}$, 25 mmol potassium acetate $\cdot$ $1^{-1}$, and 5 mmol DTE $\cdot$ $1^{-1}$ (pH 8.5; solution A). Gels were then incubated overnight at room temperature in either solution B (0.1% [w/v] soluble starch, 1 mmol ADPglucose $\cdot$ $1^{-1}$, dissolved in solution A; primed synthase activity) or in solution C (0.5 mol sodium citrate $\cdot$ $1^{-1}$, 0.025% [w/v] BSA, 1 mmol ADPglucose $\cdot$ $1^{-1}$, dissolved in solution A; unprimed activity). For substrate-depletion tests, ADPglucose was omitted. In some experiments, ADPglucose was replaced by an equimolar concentration of either UDPglucose, GDPglucose or CDPglucose.

**Mesophyll protoplasts.** Spinach-leaf mesophyll protoplasts were prepared essentially as described by Edwards et al. (1978) using approx. 20 g of leaf material. Isolated protoplasts were suspended in 3 ml grinding buffer and were broken with a glass homogenizer. Following centrifugation (as above) samples were processed as described before.

**Cell fractionation.** For localization studies, both isolated intact chloroplasts and a chloroplast-deprived cell fraction were prepared. Spinach leaves (50 g) were harvested during the first 2 h of the light period and were homogenized in 200 ml chilled isolation medium (330 mmol sorbitol $\cdot$ $1^{-1}$, 50 mmol Hepes $\cdot$ $1^{-1}$, adjusted to pH 6.8 with NaOH, and containing 1 mmol $\cdot$ MgCl$_2$ $\cdot$ $1^{-1}$, 1 mmol MnCl$_2$ $\cdot$ $1^{-1}$, 2 mmol EDTA $\cdot$ $1^{-1}$, and 0.1% (w/v) bovine serum albumin (BSA)). The homogenate was filtered through two layers consisting of Miracloth (Calbiochem, San Diego, Cal., USA) and nylon net (80 mm pore size) and the filtrate was centrifuged for 5 min at 1100 $\times g$. The supernatant was collected and used for the preparation of the non-chloroplast cell fraction (see below). For chloroplast purification, a modified version of the procedure of Stitt and Heldt (1981) was applied. The pelleted chloroplasts, resuspended in 10 ml resuspension medium (as isolation medium, but pH 7.6), were passed through a sevenfold volume of 38% (v/v) Percoll (Pharmacia; 15 min at 4000 $\times g$). Prior to use, Percoll had been dialyzed overnight against charcoal-containing water. Following centrifugation through Percoll, chloroplasts were suspended in 10 ml resuspension medium and were collected by centrifugation (90 s at 3000 $\times g$). For determination of the intactness of the chloroplasts an aliquot of the preparation was withdrawn and kept in an ice bath for approx. 2 h. For electrophoresis or enzyme-activity assays, the pelleted chloroplasts were immediately broken in approx. 6 ml grinding medium (see above) using a glass homogenizer. The homogenate was centrifuged (10 min at 26 000 $\times g$). Proteins of the supernatant were concentrated by ammonium-sulfate precipitation (70% saturation) and were then passed through a Sephadex G-25 column (see above). In some experiments the concentration step was omitted. The chloroplast-deprived cell fraction was obtained by adding ammonium sulfate (70% saturation) to the supernatant of the centrifuged leaf homogenate (see above). Precipitated proteins were collected by centrifugation (10 min at 26 000 $\times g$), dissolved in 20 ml grinding buffer and were centrifuged again (as above). The supernatant was passed through a nitrocellulose filter and through Sephadex as described for the leaf extract.