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Glycogen, Amylase and α-Glucosidase as Possible Components in the Glucose Release System of the Cyanobiont of *Peltigera horizontalis*. Partial Purification and Characterization

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Abstract

Glycogen, amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) and α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) from cultured symbiotic *Nostoc* were partially purified and characterized; phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1) activity was not detected. The role of these enzymes and of glycogen in glucose release by the cyanobiont is discussed.

Keywords: amylase, α-glucosidase, glycogen, *Nostoc*, symbiotic cyanobacteria

Abbreviations: BSA = bovine serum albumin; DEAE = diethylaminoethyl; EDTA = (ethylenedinitrilo)-tetraacetic acid; MOPS = 3-(N-morpholino)propanesulfonic acid; TCA = trichloroacetic acid

1. Introduction

Glucose has been recognized as the carbohydrate moving from cyanobacterium to fungus in *Peltigera polydactyla* (Drew and Smith, 1967; Hill and

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Smith, 1972) and this seems to be valid also for other lichens containing blue-green photobionts (Richardson et al., 1986). Evidence for a glucan as a precursor of released glucose came from tracer studies with intact thalli (Hill, 1972, 1976) and with the cultured cyanobiont (Meindl and Loos, 1990). The insoluble glucan fraction could be degraded by addition of amylolytic enzymes (Hill, 1976; Meindl and Loos, 1990) and also by a cell-free lichen homogenate (Hill, 1976). In this work quantitative data are presented for a glucan, an amylase and an α-glucosidase in the isolated cyanobacterial partner of *P. horizontalis*. The possible involvement of each in glucose release is discussed.

2. Materials and Methods

Plant material

Nostoc sp. isolated from *P. horizontalis* (Meindl and Loos, 1990) was grown autotrophically in medium BG 11 (Stanier et al., 1971) in which $NaNO_3$ was replaced by NaCl (1 mM). Other culture conditions were as given by Fischer et al. (1989). Cells were harvested by centrifugation 7–13 days after inoculation at the start of the stationary phase when a cell density (wet weight) of 13–42 g/l had been reached corresponding to 0.35–1.12 g/l on a dry weight basis.

Preparation of Nostoc *glycogen*

Nostoc filaments were washed and suspended in 12 mM MOPS/KOH buffer pH 6.8 containing 4 mM EDTA to give a density of 0.25 g wet weight/ml. After cell breakage in a French press the resulting homogenate was centrifuged for 25 min at 48,000 g and the supernatant which contained most of the glycogen was slowly mixed with one quarter of its volume of a solution of 25% (by weight) trichloroacetic acid; after further stirring for 10 min, the precipitated proteins were removed by centrifugation (10 min, 48,000 g), the supernatant was neutralized with solid $NaHCO_3$ and dialyzed against several changes of water (Weber and Wöber, 1975). The dialyzed material was subjected to ultracentrifugation (1 hr, 106,000 g and the pellet containing most of the glycogen was dissolved in 12 mM MOPS/KOH buffer pH 6.8 and applied to a DEAE cellulose column previously equilibrated with the same buffer. The bulk of glycogen was eluted by 1.2 M NaCl in 12 mM MOPS (pH 6.8), dialyzed against water and concentrated under reduced pressure for analyses. All procedures except harvesting/washing of the cell and DEAE cellulose chromatography were carried out at 0–4°C.

Purification of amylase

Nostoc filaments (20–50 g wet weight) were washed once with 0.75 M potassium phosphate buffer pH 7.0, resuspended with the help of a glass potter in fresh phosphate buffer at a density of 0.5 g wet weight/ml and were passed twice through a French press. The homogenate was centrifuged at 48,000 g for 10 min and the supernatant was further centrifuged at 175,000 g for 1 hr. An orange-yellow band at the top of the centrifuge tube was discarded and also the pellet consisting largely of phycobilisomes and membranes. All steps up to here were carried out at room temperature and in the presence of strong phosphate buffer to obtain an effective removal of the phycobilisomes (Gantt et al., 1979); further procedures were carried out at 0–4°C. The supernatant was dialyzed against 20 mM imidazole/HCl buffer pH 6.8 containing 1 mM $CaCl_2$, made 40% (v/v) with respect to ethanol and clarified by centrifugation. Amylase was precipitated with glycogen essentially according to Schramm and Loyter (1966) with a ratio of 5 mg glycogen (from oyster, Sigma Type II) per unit of amylase activity (see below) being sufficient for optimum yield. The precipitate was spun down, resuspended with a glass potter in 20 mM imidazole/HCl pH 6.8 and was incubated for 3 hr at 30°C to solubilize most ($\geq$ 90%) of the amylase activity. Insoluble material was removed by ultracentrifugation (1 hr, 175,000 g) and the enzyme preparation was concentrated by ultrafiltration (Amicon, Centricon C 10) before application to columns.

Purification of α-glucosidase

Cell breakage in strong phosphate buffer and removal of insoluble material by centrifugation was as described below. Dialysis of the soluble cell fraction was carried out against 20 mM imadazole/HCl pH 6.8, the temperature was 0–4°C as in all following steps. The dialysate was concentrated by treating the filled dialysis tubing with solid polyethylene glycol 20,000 (Serva) and was clarified by centrifugation before application to columns.

Enzymatic tests

Activity of amylase was assayed with the dinitrosalicylic acid reagent essentially according to Bernfeld (1955), but with 20 mM imidazole/HCl pH 6.8 as incubation buffer containing 1 mM CaCl. The assays were made 0.25 mM with respect to maltose from the beginning to avoid the effects of nonlinearity in the calibration curve. Samples were incubated routinely for 15 min at 30°C. Enzyme activity was expressed in units as defined by Schramm and Loyter (1966). Activity of α-glucosidase was measured at 30°C in 20 mM imidazole/HCl pH 6.8 with 1 mM p-nitrophenyl-α-D-glucoside as substrate in a total

volume of 0.5 ml. After 1 hr, 1 ml of 0.2 M sodium borate buffer pH 9.8 was added and the absorption of free p-nitrophenol was read at 405 nm. Checks were made to assure linearity of activity with time and with enzyme concentration. Phosphorylase was assayed using a coupled optical test based on NADP reduction (Steup, 1990).

Determination of protein, carbohydrate and glycogen

Protein was measured according to Lowry et al. (1951) and for samples of low (1–12 μg) content after Smith et al. (1985) with BSA as standard throughout. Carbohydrate was determined with the phenolsulfuric acid method of Dubois et al. (1956) using D-glucose as standard. For determination of glycogen the polysaccharide was digested with amyloglucosidase and the resulting glucose was assayed with the hexokinase/glucose-6-phosphate system (Keppler and Decker, 1974). Controls were run without amyloglucosidase treatment.

Hydrolysis of glycogen and analysis by gas liquid chromatography

Hydrolysis was carried out for 2 hr in 1 M trifluoroacetic acid at 120°C. After removal of acid under reduced pressure, the monosaccharides were converted to their respective alditolacetates and separated by gas liquid chromatography as described previously (Brunner and Loos, 1985).

3. Results and Discussion

Glycogen-like material in Nostoc*; partial purification*

The polysaccharide found in the cultured symbiotic *Nostoc* was of high molecular weight and could be degraded by amyloglucosidase. Therefore, and because of the general occurrence of glycogen in cyanobacteria (Fredrick, 1951; Weber and Wöber, 1975) it will be called glycogen here. Its content per cell dry weight was highest in young cultures (17%) and declined already during the logarithmic phase of growth to come down finally to around 5% (Fig. 1); another experiment gave a similar result. These values and the kinetics are comparable to those of free-living *Anabaena* which have been discussed in relation to nitrogenase activity (Ernst et al., 1984). In nonheterocysteous cyanobacteria and in heterotrophic bacteria, however, highest cellular levels of glycogen are attained in the stationary phase (Allen and Smith, 1969; Weber and Wöber, 1975; van Liere, 1979).

For purification of glycogen it proved to be essential to include EDTA in the homogenization buffer to prevent formation (even at 0–4°C) of smaller degradation products by a Ca^{2+}-dependent endogenous amylase. After removal of

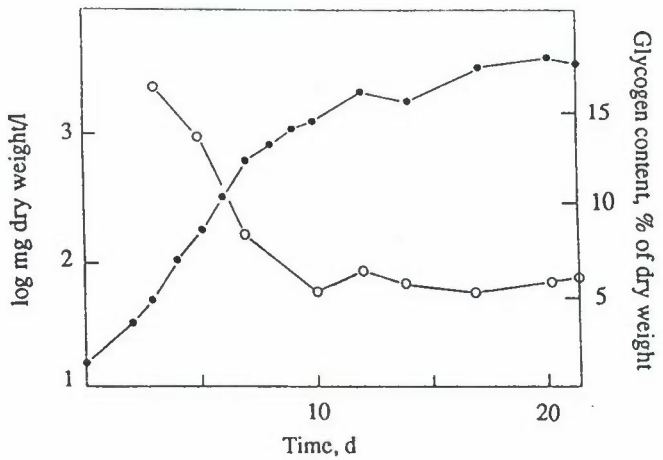

Figure 1. Kinetics of growth (•——•) and glycogen content (o——o) of cultured *Nostoc* of *P. horizontalis*. For determination of glycogen a cell homogenate (see *Material and Methods: Preparation of Nostoc glycogen)* was treated for 5 min at 100°C and 400 µl were assayed. Dry weight was determined after washing the cells twice with water and drying for 2 hr at 105°C.

protein by TCA precipitation, ultracentrifugation was used to concentrate and purify glycogen (Table 1). Obviously, the glycogen occurs in the form of particles as the α-granules of *Nostoc muscorum* (Chao and Bowen, 1970). In a final chromatography step the glycogen was eluted by NaCl from DEAE cellulose and thus seems to contain charged groups and/or to be associated with protein. The preparation obtained in this way consisted largely of glycogen (Table 1) and, after hydrolysis and gas liquid chromatography, showed a sugar composition of 94.4% glucose, 4.2% arabinose and 1.2% fucose.

Table 1. Purification of glycogen from 23.0 g wet weight cultured *Nostoc* of *P. horizontalis*. Details of purification steps are given in *Materials and Methods*.

Fraction	Glycogen [mg]	Carbohydrate [mg]	Glycogen/ carbohydrate	Yield [%]
Homogenate	59.3	563	0.11	100
Dialyzed supernatant of TCA precipitation	19.7	91.7	0.21	33.2
Pellet after ultracentrifugation	18.2	41.7	0.44	30.8
Eluate from DEAE cellulose column	8.75	9.99	0.88	14.8

Table 2. Purification of amylase from 54.7 g wet weight of cultured symbiotic *Nostoc*. Anion exchange chromatography was done on a Mono Q HR 5/5 column (Pharmacia) at a flow rate of 1 ml/min applying a linear gradient of NaCl from 0–0.4 M in 20 mM imidazole/HCl containing 1 mM $CaCl_2$; total volume of the gradient was 20 ml and fractions of 1 ml were collected. For gel filtration a superose 12 HR 10/30 column of 25 ml volume (Pharmacia) was operated at a flow rate of 0.4 ml/min with 50 mM imidazole/HCl containing 1 mM $CaCl_2$; samples of 1 ml were collected.

Fraction	Protein content (mg)	Specific activity (U/mg)	Yield (%)	Purification
Homogenate (dialyzed soluble fraction)	339	0.358	100	1
Glycogen-precipitated material	16.1	3.44	45.8	9.62
Anion exchange chromatography, fractions 8 + 9	0.775	38.7	24.8	108
Gel filtration chromatography, fraction 13	0.145	89.5	10.7	247

Table 3. Dependence of amylase activity from cultured symbiotic *Nostoc* on the concentration of free Ca^{2+}. The assays contained protein from a 255-fold purified enzyme preparation (67.8 μg/ml), 20 mM imidazole/HCl pH 6.80, 1 mM ethylene glycol bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and different amounts of $CaCl_2$; other conditions see *Materials and Methods*. The concentrations of free Ca^{2+} were measured with a Ca-sensitive minielectrode. The control had an absolute activity of 5.19 units/ml. n.d. = not detected.

Concentration of free Ca^{2+} [μM]	Activity [%]
n.d.	0
0.14	43
2.80	84
11.90	109
Control (1 mM $CaCl_2$, no EGTA)	100

Partial purification and characterization of amylase and α-glucosidase

Amylase could routinely be enriched 250–300-fold from the soluble cell fraction by co-precipitation with glycogen followed by two column steps (Table 2). Gel filtration on a calibrated column (last purification step) indicated an apparent molecular weight of 42 kD. The pH optimum of amylase activity was at pH 6.4 with half-maximal activities at pH 5.0 and pH 7.8; the temperature dependence displayed a broad optimum over the range 30–54°C and a sharp drop in activity at temperatures > 54°C (data not shown). To see whether the enzyme cleaved its substrate in an exo- or an endo-fashion, the kinetics of release of reducing groups and of maltose were followed. In the beginning

Table 4. Purification of α-glucosidase from 37.0 g wet weight of cultured symbiotic *Nostoc*. A hydroxyapatite column (bed volume 16 ml) was eluted with a linear gradient from 0.02 M imidazole/HCl pH 6.8 to 1.1 M K-PO_4 pH 5.8 (total volume 90 ml, fractions size 3.5 ml). The phenylsepharose column (5 ml bed volume) was equilibrated with 0.7 M K-PO_4 pH 6.8, loaded with the pooled active fractions from the previous column step, washed with two bed volumes of equilibration buffer and eluted with a linear gradient from 0.2 M K-PO_4 pH 7.0 to 0.02 M imidazole/HCl pH 7.8 (total volume 60 ml, fraction size 2 ml).

Fraction	Protein content (mg)	Specific activity (A_{405}/h·mg)	Yield (%)	Purification
Homogenate (dialyzed soluble fraction)	150	0.17	100	1
Hydroxyapatite chromatography, fractions 11–17	25	0.65	61	3.8
Phenylsepharose chromatgraphy, fractions 26–30	0.91	4.36	15	26

Table 5. Activity of a 26-fold purified α-glucosidase preparation with various p-nitrophenyl-glycosides (1 mM) as substrate. Activity of 100% corresponded to a rate of 0.58 μMol/h·mg protein.

Substrate	Activity [%]
p-Nitrophenyl α-D-glucoside	100
p-Nitrophenyl β-D-glucoside	3
p-Nitrophenyl α-D-galactoside	< 1
p-Nitrophenyl β-D-galactoside	< 1
p-Nitrophenyl α-D-mannoside	2

of enzymatic digestion of starch only a small part of the liberated, reducing products consisted of maltose; the percentage of this sugar increased only after prolonged incubation (Fig. 2). This pattern of starch break-down is typical for an endo-enzyme. Thin-layer-chromatographic analyses revealed mainly oligosaccharides as early reaction products and thus confirmed this conclusion. The enzyme had a strict requirement for Ca^{2+} with maximal activity observed already at micromolar concentrations (Table 3). This and the kinetics of reaction products are typical for α-amylases. Perhaps the cellular concentration of free Ca^{2+} is regulating the activity of the amylase in the intact thallus and, in consequence, the release of glucose.

The activity of amylase in the soluble cell fraction ranged from 0.09–0.20 μmol maltose equivalents/h·g wet weight. This is very comparable to the rate of glucose excretion from whole cells (0.3–0.5 μmol/h·g wet weight;

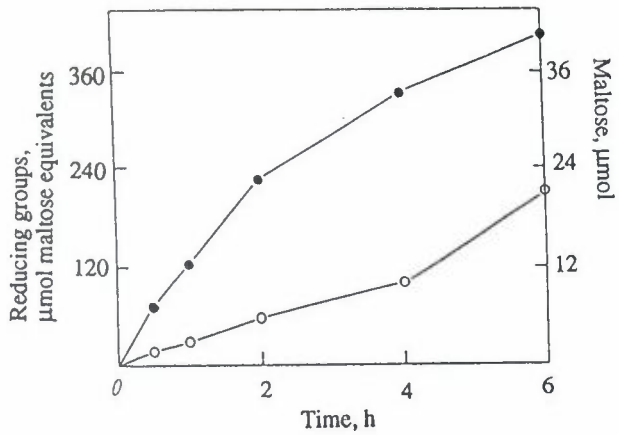

Figure 2. Kinetics of formation of reducing groups (•——•) and of maltose (o——o) by a 37-fold purified amylase preparation from cultured symbiotic *Nostoc*. The reaction mixture contained in a total volume 36 ml 225 μg protein corresponding to an activity of 3.75 units. After different times, aliquots were withdrawn and assayed for reducing groups with the dinitrosalicylic acid reagent and for maltose enzymatically; for details see *Materials and Methods*.

Meindl and Loos, 1990) and thereby underlines the possible physiological relevance of amylase.

α-Glucosidase could be purified from the *Peltigera* cyanobiont 10–30-fold by two column steps (Table 4). For the purified preparation, a molecular weight of 180 kD was indicated by gel filtration (data not shown). The enzyme cleaved with high preference the α-glucosidic bond when different p-nitrophenyl glycosides were tested as substrate (Table 5). The properties of di- and oligosaccharides as substrate are under investigation.

Apparent lack of glycogen phosphorylase activity

Despite several attempts, no activity of phosphorylase could be detected in cell homogenates, neither with exogenous substrates (oyster glycogen, soluble starch) nor with the purified endogenous glycogen. Controls with purchased phosphorylase showed no inhibition of activity by *Nostoc* homogenates. Thus, glycogen phosphorylase appears to be absent in cultured *Nostoc* sp.; it has been demonstrated, however, in free-living *Anacystis* (Lehmann and Wöber, 1978).

4. Conclusions

This work supports the view that a glucan (glycogen), amylase and α-glucosidase are essential components in the path of glucose formation in cyanobacterial photobionts (Hill, 1976) whereas phosphorylase seems not to be involved.

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