Soluble uridine diphospho-D-glucose: mycosporin glucosyltransferase from spores of *Ascochyta fabae* Speg.

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**Abstract.** The enzyme properties of a soluble uridine 5'-diphosphate (UDP) glucose: mycosporin-2 glucosyltransferase from spores of *Ascochyta fabae* Speg. (Fungi imperfecti) were studied. The optimal conditions for the glucose transfer from UDP-glucose to the mycosporin-2 (the amide form being the best acceptor) were determined; for maximal activity the glucosyltransferase requires a pH of about 8.5 and the presence of divalent cations (Mn\(^{2+}\) being more efficient than Ca\(^{2+}\) or Mg\(^{2+}\)). The reaction was not reversible in presence of large amounts of UDP.

**Key words:** *Ascochyta* – Fungi imperfecti – Glucosyltransferase – Mycosporin.

**Introduction**

*Ascochyta fabae* Speg. is a phytopathogenic Deuteromycete parasite of the broad bean and has been studied for its production of ascochitine, an antibiotic drug which is responsible for considerable damages to leguminoses (Oku and Nakanishi 1963). This fungus, like many other fungi, synthesizes a hydrophilic, low-molecular-weight compound – belonging to the mycosporin group (Arpin and Bouillant 1981) – which has a strong UV absorbance at 310 nm and is characterized by the fact that it is produced, in presence of light, during reproduction and accumulates mainly in spores as glucoside derivatives (Pittet et al. 1983). Furthermore, in *A. fabae*, the amide (IV) and acid (III) forms (respectively 95% and 5%) account for 5% of the spore dry weight (Pittet et al. 1983). In this paper we have studied the subcellular distribution and the kinetic properties of the enzyme responsible for the glucosylation of the aglycone mycosporin.

**Material and methods**

**Microorganism and culture methods.** *Ascochyta fabae* Speg. was maintained by subcultures on slopes of Brewer's medium agar (1959) in complete darkness. Roux bottles, containing a liquid Brewer's culture medium with ammonium tartrate as nitrogen source, were inoculated with 2-ram-diameter inocula from the stationary culture. The incubation temperature was 20 °C and the lighting conditions were: 2.5 W m\(^{-2}\) during 12 h per day.

**Chemicals.** Uridine 5'-diphosphate (UDP)[\(^{14}\)C]glucose was purchased from the Radiochemical Centre, Amersham, UK. The aglycones of mycosporins were isolated from *Trichothecium roseum* (Pers.) Link ex Gray and purified according to the general procedure of Leach (1965) modified by Bouillant et al. (1981).

**Subcellular fractionation.** Sporulated cultures (18 d old) were shaken to free the spores which were separated from the mycelium by filtration on a glass filter No. 2 (Corning, Avon, France). The spores were spun down by a centrifugation at 1000 g for 10 min. The spores, washed and resuspended in lots of 10 g in 50 ml of ice-cold 0.25 M sucrose buffered with 50 mM 2-amino-2[(hydroxymethyl)-1,3-propanediol (Tris)-HCl pH 7.3 and 1 mM β-mercaptoethanol, were disrupted in a MSK Braun mill (Melsungen, FRG) with 0.11-mm-diameter glass beads at maximum speed for 1 min. All subsequent operations were done at approx. 4 °C unless otherwise specified. The crude homogenate thus obtained is referred to as the cell-free extract and was used as an enzyme source for glucosyl transfer. The homogenate was cleared of cell debris and nuclei by centrifuging for...
were obtained, the upper layer containing the glucosylated mycosporin. The standard incubation medium was composed of 100 μl of the enzyme fraction in 0.25 M sucrose buffered with 50 mM Tris-HCl (pH 7.3); and 1 mM β-mercaptoethanol, 5 μl of 20 mM MnCl₂, 5 μl of 20 mM MgCl₂, 5 μl of 3% Triton X-100, 5 μl of the amide form of mycosporin-2 (IV) (5 mM); and 0.4 nmol of UDP-[14C]glucose (11.8 MBq μmol⁻¹). The reaction, carried out at 25°C, was stopped by addition of 2 ml of chloroform-methanol (2:1, v/v) and 0.37 ml of water according to Folch et al. (1957). The mixture was vortex-mixed and centrifuged; two phases (211, v/v) were obtained, the upper layer containing the glucosylated mycosporin.

Marker enzymes. The following marker enzymes were used to assess the purity of the fractions: cytochrome oxidase (EC 1.9.3.1), NADPH-cytochrome-c reductase (EC 1.6.2.4) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) for the inner mitochondrial membrane, microsomes and the cell sap, respectively. The NADPH-cytochrome-c reductase was assayed spectrophotometrically by following the reduction of added cytochrome c at 540 nm, in presence of KCN according to Sottocasa et al. (1967). The glucose-6-phosphate-dehydrogenase activity was tested by measuring the reduction of NADP⁺ at 340 nm according to Lohr and Walker (1965), and the cytochrome oxidase was assayed according to Colbeau et al. (1971), in presence of 0.02% Triton X-100. The protein content was estimated according to Hartree (1972).

Chromatography. Analysis of mycosporin glycosylation products was carried out by three types of chromatography. (1) Ion-exchange chromatography: the upper phase was chromatographed on a (5 cm long, 1 cm diameter) column packed with Dowex 50 W X-8, 100-200 mesh in the H⁺ form (Serva, Heidelberg, FRG); the column was eluted with distilled water with a flow rate of 0.2 ml min⁻¹. Elution was followed by measuring the absorbance at 310 nm of 5 ml fractions. (2) High-performance liquid chromatography (HPLC): the HPLC of mycosporins was carried out at room temperature on a 30-cm long, 0.4-cm-diameter, microbondapak C-18, reverse-phase column (10 μm; Waters Associates, Milford, Mass. USA) equilibrated and eluted with distilled water. The flow rate was: 1 ml min⁻¹ and the detection was monitored at 280 and 310 nm (Waters, model 440 UV detector). (3) Thin-layer chromatography (TLC): the mycosporins or their hydrolysis products (HCl 2 N, 2 h at 100°C, in a sealed ampoule) were lyophilized and subjected to TLC on cellulose developed in phenol:water (4:1, v/v) (solvent A) or in butanol:pyridine:HCl 0.1 N (5:3:2, by vol.) (solvent B) in the presence of reference samples. The mycosporins were located after spraying the chromatogram with bis-diazobenzidin according to Sherma and Hood (1965). The sugars were located by using aniline oxalate as staining reagent (Partridge 1948).

Determination of radioactivity. Hydromethanolic and aqueous samples (0.5 ml) were counted in presence of 7.5 ml of ACS scintillator (Amersham) in a Packard (Tri-carb 300) liquid scintillation spectrometer. Radioactive zones on thin-layer chromatograms were detected with a radiochromatogram scanner Packard (Model 7201), scraped off and counted in the same liquid scintillation system. Radioactivity was calculated from quench curves established in the same counting conditions.

Results

Mycosporin-2 glucosylation. The homogenate of spores from Ascochyta fabae was incubated as described under Material and methods in the presence of UDP-[14C]glucose and the lower and the upper phases obtained by the Folch’s extraction procedure (Folch et al. 1957) were analysed. The lower organic layer contained a radioactive lipid with the chromatographic properties of a sterol glucoside (results not shown). The upper hydromethanolic layer was chromatographed on a Dowex cationic-exchange column; unreacted UDP-[14C]glucose was followed by a radioactive fraction which displayed a strong UV absorption at 310 nm with the typical mycosporin-2 spectrum. Figure 1 shows the HPLC elution profile of this fraction. The major peak A (85% of the total radioactivity) co-migrated with mycosporin-2 amide form (IV). Two minor products (C and B) which absorbed at 310 nm were identified as mycosporin-2 acid form (III) and its glucoside derivative (Pittet et al. 1983). Thin-layer chromatography (TLC) of peak A on cellulose gave a single radioactive spot corresponding to the glucoside of the mycosporin-2 amide form (IV) (Fig. 2).

The radioactivity released by acid hydrolysis co-migrated with glucose on TLC both in solvents A and B (Fig. 3).