Biosynthesis of cyclic β-(1-3),β-(1-6) glucan in Bradyrhizobium spp.

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Abstract. Inner membranes of Bradyrhizobium japonicum strain USDA 110 produced in vitro soluble and insoluble β-(1-3),β-(1-6) glucans. The reaction proceeded through a 90 kDa inner membrane intermediate protein; used UDP-glucose as sugar donor and required Mg²⁺. Gel chromatography of soluble glucans resolved a cyclic β-(1-3) glucan with a degree of polymerization of eleven from a family of β-(1-3),β-(1-6) glucans with variable degree of polymerization higher than eleven. Bradyrhizobium strains BR4406 and BR8404 isolated from tree legume nodules in Southeast Brazil produce β-(1-3),β-(1-6) glucans very similar to that of B. japonicum. A 100 kDa protein was identified in these strains as intermediates in the synthesis of these glucans. Inner membranes of B. japonicum USDA110, B. japonicum I17, and Bradyrhizobium strains BR4406 and BR8404 incubated with UDP-glucose were unable to synthesize β-(1-2) glucan and lacked the 235 kDa intermediate protein known to be involved in the synthesis of β-(1-2) glucan in Agrobacterium tumefaciens, Rhizobium meliloti and Rhizobium loti.

Key words: Bradyrhizobium japonicum — Bradyrhizobium spp. — Cyclic β-(1-3),β-(1-6) glucan — Laminarin — Glucan — Protein intermediate

Bacterial polysaccharides that may play an important role in plant infection include: extracellular exopolysaccharides (EPS), capsular polysaccharides (CPS), lipopolysaccharides (LPS) and cyclic β-(1-2) glucans. Mutants deficient in the production of EPS in R. meliloti (Leigh et al. 1985), and R. trifolii (Chakravorty et al. 1982) form ineffective empty nodules. In Agrobacterium and Rhizobium, the chromosomal regions chvB, chvA and ndvB, ndvA respectively, are required for the synthesis and secretion of cyclic β-(1-2) glucan (Zorreguieta and Ugalde 1986; Ifi6n de Iannino and Ugalde 1989; Dylan et al. 1986; Stanfield et al. 1988). R. meliloti mutants deficient in the production of cyclic β-(1-2) glucan form ineffective empty nodules (Geremia et al. 1987) and A. tumefaciens β-(1-2) glucan negative mutants are avirulent (Zorreguieta and Ugalde 1986; Puvanesarajah et al. 1985). These results suggest a key role for cyclic β-(1-2) glucan in Rhizobiaceae-plant interaction. B. japonicum does not produce cyclic β-(1-2) glucan (Miller et al. 1990), however a cyclic β-(1-3),β-(1-6) glucan was found in different strains of B. japonicum (Miller et al. 1990). No DNA homology to Agrobacterium chvA or chvB regions was found in B. japonicum (Dylan et al. 1986).

The synthesis of β-(1-3) glucans was studied in plants (Flowers et al. 1968), yeast (Shematek et al. 1980) and alga (Marechal and Goldemberg 1964). Recently in vitro synthesis of β-(1-3),β-(1-6) glucan was communicated in B. japonicum USDA 110 (Cohen and Miller 1991). We describe here that inner membranes of B. japonicum strains USDA 110, I17 and two Bradyrhizobium strains isolated from tree legumes nodules formed β-(1-3),β-(1-6) glucan through an inner membran intermediate protein.

Materials and methods

Bacterial strains and media

Bradyrhizobium japonicum strain USDA 110 was obtained from the United States Department of Agriculture, Beltsville, Maryland, USA. B. japonicum strain I17 was obtained from B. Kamicker (Department of Bacteriology, University of Wisconsin, Madison, USA). Bradyrhizobium strains BR4404 and BR4406 were isolated from Dalbegia nigra and Enterolobium timbouva respectively and
kindly provided by S. M. de Faria (EMBRAPA-UAPNPBS, Km 47, Seropedica, RJ, Brazil). All strains were grown for 4 or 5 days in yeast extract-mannitol medium (AMA) at 28 °C with good aeration on a rotary shaker. AMA contained 10 g mannitol, 1 g yeast extract, 0.2 g MgSO4 7H2O, 0.2 g NaCl, 0.5 g KH2PO4 per litre. Agrobacterium tumefaciens strain A348 was grown in tryptone-yeast extract (TY) media (Zorreguieta and Ugalde 1986).

**Extraction of cell-associated oligosaccharides.** Cells from 100 ml cultures were harvested by centrifugation at 10000 x g for 10 min. Pellets were resuspended with 10% trichloroacetic acid (TCA) for 30 min at room temperature as described previously (Miller et al. 1986). TCA extracts were neutralized with ammonium hydroxide, concentrated and subjected to gel filtration on Bio-Gel P4 columns as described previously (Išion de lannino and Ugalde 1989). Kav was calculated as: Vr - Vc/Vt - Vo, in which Vr is the elution volume, Vc is the void volume and Vt is the total volume.

**Preparation of Bradyrhizobium inner membranes.** B. japonicum cells were harvested after 5 days of culture and capsule removed by shaking in a Omnimixer for 3 min in 30 mM Tris hydrochloride buffer (pH 8.0) with 0.5 M NaCl. Decapsulated cells were washed twice with 30 mM Tris hydrochloride buffer pH 8.0 to remove NaCl. Inner and outer membranes were prepared following the method previously described for gram-negative bacteria (Osborn and Munson 1984). BR8404 and BR4466 strains were grown for 4 days and membrane prepared as described by (Osborn and Munson 1984). PMSF (Phenyl methyl sulfonil fluoride) 2 mM was added previously to French Press shearing.

**In vitro synthesis of glucan.** Inner membranes (200 μg protein) were incubated with UDP-[14C]glucose (90000 cpm; 10.5 GBq/mmol), 10 mM MgCl2, 50 mM Tris hydrochloride buffer (pH 8.2) in a total volume of 50 μl. The reaction was carried out at 20 °C and stopped depending on the experiment, by the following methods:

A) Addition of 300 μl of water, heated at 100 °C for 1 min, centrifuged at 5000 x g for 5 min and supernatant subjected to DEAE-Sephadex chromatography as previously described (Zorreguieta and Ugalde 1986). Membrane pellets were subjected to polyacrylamide gel electrophoresis for 10 min and pellet subjected to polyacrylamide gel electrophoresis as described (Zorreguieta and Ugalde 1989). Pellets were resuspended in 10% TCA and fluorography as described (Zorreguieta and Ugalde 1986). Pellets were resuspended in 1% trichloroacetic acid (TCA) for 30 min and the pellet was subjected to polyacrylamide gel electrophoresis.

**Digestion with laminarinase.** Samples were incubated over night at 37 °C with 20 μg of laminarinase (Sigma EC 3,2,1,6) 5.7 U/mg in 50 mM Na-acetate buffer (pH 5.0).

**Acid hydrolysis and paper chromatography.** Partial and total acid hydrolysis were carried out as previously indicated (Išion de lannino and Ugalde 1989). Hydrolysates were subjected to descending paper chromatography on Whatman No.1 paper (Whatman, Chifton, N.J, USA) with solvent A (butanol-pyridine-water (6 : 4 : 3) (Jeanes et al. 1951), or solvent B (isopropanol-acetic acid-water (27 : 4 : 9)) (Tang and Nordin 1968). Sugars were detected by the alkali silver method (Trevelyan et al. 1950).

**Paper electrophoresis.** Paper electrophoresis was carried out with buffer C (2% sodium phosphate pH 6.0) for 2 h at 15 V/cm (Bourne et al. 1961). Electrophoresis of proteolytic products was carried out at 25 V/cm for 4 h in buffer D (5% formic acid pH 2.0) or buffer E (0.2 M sodium carbonate-bicarbonate pH 10.0).

**Reduction with borohydride.** Oligosaccharides were dissolved in 300 μl of water, 8 mg of NaBH4 were added and incubated overnight in the dark. To remove Na+, an excess of Dowex 50W-X8 resin (Bio Rad Laboratories, Richmond, Calif., USA) was added, and oligosaccharides recovered by washing the resin with water. Samples were dried several times under an air stream, methanol was added and evaporated in order to remove boric acid as methyl borate. Reduced oligosaccharides were subjected to total acid hydrolysis with HCl 1 M 4 h at 100 °C. HCl was eliminated by several evaporation under an air stream and hydrolysate subjected to paper electrophoresis in buffer C.**

**Smith degradation.** Oligosaccharides were oxidized for 3 days with 0.04 M sodium periodate at 4 °C in the dark (final reaction volume 200 μl). The excess of periodate was destroyed with 10 μl ethylene glycol and after 2 h, 8 mg sodium borohydride were added. Samples were kept in the dark for 16 h. Acetic acid (10%) was added to eliminate the NaBH4 excess and chromatographed through Bio-Gel P2 column to remove salts. Desalted samples were ionophorized. Products were subjected to mild acid hydrolysis (HCl 0.1 M at room temperature over night) or to total acid hydrolysis (HCl 1 M 4 h 100 °C). HCl was removed by evaporation under an air stream and hydrolysates subjected to gel chromatography on Bio-Gel P2 columns or to descending paper chromatography as indicated above.

**Trypsin digestions.** They were carried out on inner membrane as described previously (Išion de lannino and Ugalde 1989).

**Proteolysis.** Washed TCA precipitates (15000 cpm) obtained in vitro were incubated with 1 mg of pepsin (Sigma St. Louis, Mo., USA, P-6887) in 2% formic acid in a total volume of 500 μl at room temperature for 48 h. One mg of fresh pepsin was added after 48 h and incubated for 10 days. The reaction was stopped by adding 200 μl methanol, centrifuged 5000 x g 10 min and supernatants subjected to paper chromatography with solvent B. Radioactivity recovered from the origin of the chromatogram was eluted and analyzed for the presence of glycopeptides as described above.