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Cellulase production by Rhizobium

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Summary The production of cellulase by *Rhizobium* species was studied. *Rhizobium trifolii* cellulase was induced by a variety of polysaccharides, including celluloses and hemicelluloses. Cellobiose and myo-inositol also allowed enzyme expression but mannitol prevented it at concentrations higher than 0.25%. Both soluble and insoluble plant root substances moderately stimulated cellulase production by *Rhizobium trifolii.*

Most substances tested did not induce the production of cellulases by the "slow-growing, cowpea type" rhizobia strain CIAT 79. Effective inducers were carboxymethylcellulose, gluconate and myo-inositol.

Cellulase production was very low under all conditions tested. In most cases the enzyme activity was loosely bound to the capsular material. The enzyme in fast-growers is an 1,4-β-D-glucan-4-glucanohydrolase (endo-glucanase EC 3.2.1.4) with specificity for high molecular weight polysaccharides.

There was no correlation between infectiveness of *Rhizobium trifolii* strains and cellulase production. One strain, which lacks the nodulation plasmid, produced cellulase at the same rate as its parental infective strain.

Introduction

The infection of legume roots by Rhizobium is a complex process showing a high degree of specificity. Many strains of Rhizobium can nodulate only certain species of leguminous plants. The expression of this specificity occurs prior to the initiation of the infection thread[7]. The biochemical mechanism by which the infection thread is initiated is unknown. Nutman[14] proposed that rhizobia induced the redirection of root hair cell wall growth from the growing tip to the infection site. This would cause an invagination of the cell wall forming the tubular structure of the infection thread.

Ljunggren and Fahraeus[9] proposed that rhizobia induced the production of polygalacturonases by the plant. These enzymes would "soften" the root hair cell wall allowing *Rhizobium* to penetrate and trigger infection thread formation. Efforts to implicate the production of polygalacturonases in the infection process have been unsuccessful[8,11,17] as well as successful[15,16,19]. Confirmation of the polygalacturonase hypothesis is difficult perhaps because of the very low levels of enzymatic activity found.

The involvement of polysaccharide degrading enzymes in the infection

Table 1. Sources and characteristics of Rhizobium strains used in the research

Species	Strain	Host	Infectiveness	Source
Rhizobium trifolii	BAL	*Trifolium repens*	+	F. B. Dazzo.
Rhizobium trifolii	BART A	*Trifolium repens*	–	G. Fahraeus
Rhizobium trifolii	0403	*Trifolium repens*	+	G. Fahraeus
Rhizobium trifolii	0435	*Trifolium repens*	+	A. N. McGregor
Rhizobium trifolii	0435-2	*Trifolium repens*	–	A. N. McGregor
Rhizobium trifolii	521	*Trifolium repens*	+	W. Zurkowski.
Rhizobium trifolii	521 nod^{-8}	*Trifolium repens*	–	W. Zurkowski.
Rhizobium sp. "cowpea type"	CIAT 79	Wide range*	+	P. H. Graham

* Infectivity tested on *Macroptilium atropurpureum.*

process is strongly suggested by electron microscope data[2,3,13]. Rhizobia appear to degrade the plant cell wall. In response the plant deposits a layer of new wall material at the infection site. The continuous process of deposition results in the formation of the infection thread[2].

It has been shown that rhizobia produce cellulases and hemicellulases[12] as well as polygalacturonases[6].

This work reports further study of cellulase production by Rhizobium *in vitro* and the possible involvement of cellulase in the infection process.

Materials and methods

The strains of Rhizobium used in the study, their sources and legume hosts are listed in Table 1. The Rhizobium strains were kept on yeast extract mannitol agar (YEMA) slants and/or in lyophilized form. The infectivity of the strains was verified by nodulation of host plants growing in partially enclosed glass assemblies[20]. *Trifolium repens* seedlings were used as host for *Rhizobium trifolii* strains, *Macroptillium atropurpureum* seedlings for "cowpea type" Rhizobium CIAT 79.

Cellulase activity

Two methods were used for assaying enzymatic activity. The first was measuring the decrease in viscosity of a solution of CMC (0.2% w/v) as described by Martinez-Molina *et al.*[12] using Cannon-Fenske viscometers.

The second method used was measurement of the increase in reducing power at 30°C of a reaction mixture consisting of 1 ml of CMC solution (0.2% w/v) in PCA (0.1 *M* K_2HPO_4 and 0.1 *M* citric acid mixed to pH 5.2) buffer and 1 ml of enzyme extract. Reducing sugars were assayed by the method of Somogyi[18].

For the viscosimetric assay one enzyme unit was defined as the amount of enzyme that reduces the viscosity of CMC solution (0.2% w/v) by 50% in 10 hours or has a slope (plot of the reciprocal specific viscosity against reaction time, hereafter called $1/\eta_s$) of 1.883×10^{-2} h^{-1}. For the reducing sugar assay one enzyme unit is the amount of enzyme that produces an increase in reducing power in a CMC solution equivalent to a μmole of glucose/min.

Enzymatic activity was corrected for increase of the bacterial mass as a result of growth during the induction experiment. Bacterial growth was estimated by measuring the bacterial dry weight. The dry weight of bacterial cultures was determined by filtration of samples (polycarbonate filters, 0.4 μm, Nucleopore Corp., Pleasanton, CA). The filters were washed twice

with filtered deionized water and dried overnight at 70°C. The weight of the bacteria was calculated as the difference in weight of the filter before and after filtration.

Glucose was measured by the glucose oxidase method (Sigma Chem. Co., St. Louis, MO.).

Growth conditions

To test for the induction of cellulase production the bacterial strains were grown in Bergersen's medium[1] in which the carbon source was replaced by the carbohydrate to be tested. Culture flasks containing 100 ml of medium were incubated in an orbital shaker at 28°C and 120 rpm.

Inocula for experiments were prepared by resuspending the bacterial cells from YEMA Petri plate cultures in sterile deionized water. The bacterial population was counted using a Petroff-Hausser chamber and the amount of bacterial suspension to be used calculated to obtain a initial population of 1.5×10^7 cells/ml in the culture.

Enzyme extraction

After 8 days of incubation, 100 ml of culture was centrifuged at 3,500 × g for 1 hour at 4°C. Each pellet was resuspended in 10 ml of PCA buffer and sonicated for 1 min at 60 W (Model W140, Heat Systems-Ultrasonics Inc. Plainview, NJ). The sonicated suspension was centrifuged at 15,300 × g for 15 min at 4°C. The supernatant (hereafter called enzyme extract) was tested for cellulase activity by one of the methods described above.

Plant root extracts preparation

Seeds of clover (*Trifolium repens* var. Louisiana Nolin) were surface sterilized with $HgCl_2$ and germinated aseptically[20]. The seedlings were kept at room temperature under fluorescent lights for 10 days. The roots were cut with a razor blade and macerated in PCA buffer in a mortar over ice. The macerate was centrifuged at 15,300 × g for 15 min at 4°C. The supernatant (hereafter called root solubles) contained 32 μg/ml of protein (average) [assayed by the method of Lowry *et al.*[10]] and 71 μg/ml of total carbohydrates (average) [assayed by the phenol sulfuric acid method[4]].

Purification of enzyme activity

Cells from 6.35 liters of broth culture (Bergersen's medium with myo-inositol as the C source) of *Rhizobium trifolii* strain BAL were collected by centrifugation at 3,500 × *g* for 1 h at 4°C and resuspended in 120 ml of glycine-urea buffer (hereafter called GU buffer; solution A: 0.2 *M* K_2HPO_4, 1% w/v glycine, 1 *M* urea. Solution B: 0.1 *M* citric acid, 1% glycine, 1 *M* urea. Solution A was mixed with solution B until a pH of 7.5 was obtained). Chloramphenicol and dithiothreitol were added to a concentration of 100 μg/ml and 0.15 μg/ml, respectively. The cell suspension was sonicated twice for 1 min at 60 W and centrifuged at 15,300 × *g* for 15 min at 4°C.

The protein of the supernatant was fractionated by addition of crystalline $(NH_4)_2SO_4$ in 20% saturation steps up to 80% saturation, at 4°C under constant stirring. The precipitates were redissolved and dialyzed (using Spectra/Por 6 membranes, Spectrum Medical Ind., Los Angeles, CA) against GU buffer with 0.05 μg/ml of $CaCl_2.2H_2O$ for 5 hours at 4°C. Fractions precipitated at 20%–60% saturation contained cellulase activity and were pooled. The pooled material was placed on a column (10.5 × 5 cm) of DEAE cellulose (DE 52, Whatman, Clifton, NJ) stabilized with GU buffer. The column was eluted with a linear gradient of NaCl (from 0 to 1 *M*) and pH (from 7.5 to 4.8) at a flow rate of 0.96 ml/min. Fractions of 4.6 ml were collected and assayed for cellulase activity by viscosimetry.

Results and discussion

Rhizobium strains showed differential cellulase production and growth according to the medium used. Cellulase was produced by *R. trifolii* strain BAL in the presence of a variety of carbohydrates

such as cellobiose, CMC, xylan, locust bean gum, gum arabic and the sugar alcohol myo inositol. *Rhizobium* sp. strain CIAT 79 seemed to be more restricted and only showed cellulase production with cellobiose, CMC, gluconate and myo inositol. Enzymatic activity was detected in preparations of both strains by the viscosimetric and the reducing sugar assays but *R. trifolii* strain BAL cellulase caused a large decrease in viscosity with a relatively small increase in reducing power of the CMC solution while *Rhizobium* sp. strain CIAT 79 cellulase had the reverse effect. Mannitol inhibits the cellulase expression above a concentration of 0.25% while myo inositol stimulates the production of cellulase throughout the range of concentrations tested (0.25 to 2.0%) but without significant difference about a concentration of 0.5% for *R. trifolii* strain BAL and of 1.5% for *Rhizobium* sp. strain CIAT 79.

Effect of root extracts

Root extracts stimulate the production of cellulases by *R. trifolii* strain BAL (Table 2). Root solids showed a striking stimulation of enzyme production as measured by the release of reducing groups. However the same stimulation was not observed in the results of the viscosimetric assay. In *R. trifolii* strain BAL cultures with root solids, cells tended to adhere to the root pieces and did not grow as well as in other treatments. Addition of root extracts showed no effect on *R. trifolii* strain BART A growth or cellulase production as measured by viscosimetry, but the release of reducing groups was strongly stimulated by root solids and, to a lesser extent, by agar. The difference in results obtained with these two assays may indicate that different enzymes are induced by these substances.

Enzyme purification

Most of the cellulase produced by *R. trifolii* strain BAL seems to be loosely associated with the capsular material. Although there is a small detectable activity in the culture supernatant (ranging from 0.05 to 0.20 viscosimetric EU/ml) it is very difficult to separate from the extracellular polysaccharide. The main proportion of the total enzyme produced remains associated with the cells after centrifugation. The enzyme is not tightly bound to the cell surface however, since a few hours of shaking a cell suspension in buffer will release the cellulase into solution as does sonication.

The type of extraction solution used influences enzyme recovery. The highest recovery was obtained with deionized water. PCA and GU buffers extract around 60% of the amount extracted with water.

Table 2. Effect of root extracts on cellulase production by *Rhizobium trifolii* strains BAL and BART A

Culture amendment	Enzymatic activity measured by the release of reducing groups assay		Enzymatic activity measured by the viscosimetric method	
	Strain BAL*	Strain BART A*	Strain BAL**	Strain BART A**
Root solubles	183	173	1.1	1.7
Root solids	279	1156	0.3	1.2
Agar	67	453	0.4	1.2
No amendment	96	222	0.4	1.4

* Enzymatic activity expressed as μEU/ml of enzyme extract.

** Enzymatic activity expressed as viscosimetric EU/ml of enzyme extract.

Note: Each datum from the reducing groups assay is the average of 4 replications. Each datum from the viscosimetric assay is the average of 2 replications. The basal medium used was Bergersen's with myo-inositol as C source (2% w/v) (the pH was adjusted to 7.0 with a solution of NaH_2PO_4, 20% w/v) which received after autoclaving 5 ml per 100 ml of medium of filter sterilized root solubles, or autoclaved root solids suspension, or sterile PCA buffer or agar (0.2% w/v). No enzymatic activity was detected in uninoculated medium amended with root solubles or root solids.

Tris-HCl buffer (pH 8.0) releases only 20% of this amount. Enzyme extracts made up with water maintain enzymatic activity for several weeks when stored at 4°C and for 2–3 months when sterilized by filtration (polycarbonate filters, 0.4 μm, Gelman Ind., Ann Arbor, MI) and stored at 4°C.

After ammonium sulfate precipitation, the fractions with 20–60% saturation show cellulase activity after dialysis. GU buffer was essential for maintaining enzymatic activity. Without glycine and urea the precipitate did not dissolve completely and usually part of it reprecipitated after a few hours. A small amount of $CaCl_2.2H_2O$ (0.05–0.1 μg/ml) helped to solubilize the protein but calcium or magnesium did not affect the activity of Rhizobium cellulase (data not shown). Short dialysis time (4–5 hours, 10 hours maximum) is required; lengthy extensive dialysis resulted in complete loss of cellulase activity. This may be due to binding of the enzyme to the cellulosic dialysis membranes or to denaturation. Several procedures for recovery of enzyme from the surface of the dialysis membranes were tested without success.

Fig. 1 shows the elution pattern of proteins from a DEAE cellulose column. Cellulase activity appeared in fractions eluting immediately before the main protein peak. Elution should be performed with a gradient of salt and pH; a salt gradient alone does not release the proteins from the column.

Enzyme characterization

Incubation of *Rhizobium trifolii* strain BAL enzyme extract with

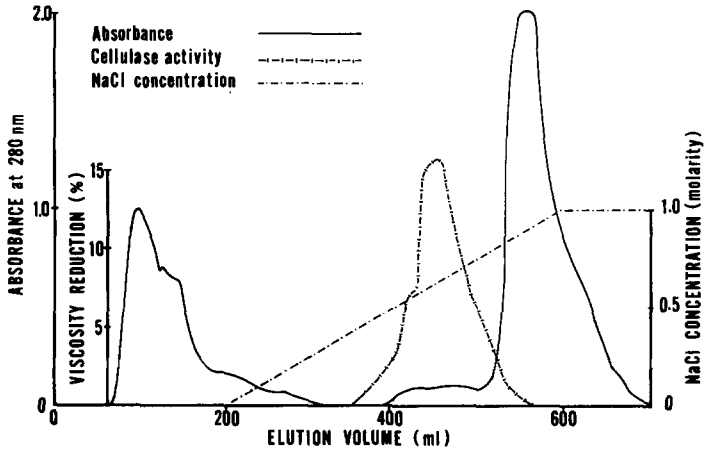

Fig. 1. Elution pattern of *Rhizobium trifolii* strain BAL cellulase after DEAE cellulose column chromatography. Cellulase activity is expressed as percentage of viscosity reduction of a CMC solution. The pH gradient follows the same pattern as the salt gradient.

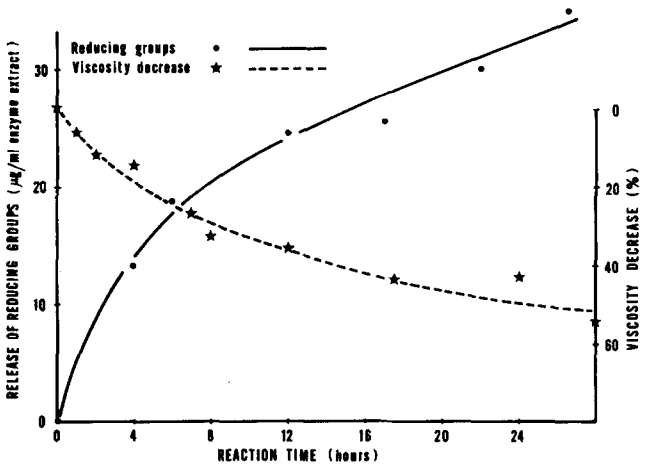

Fig. 2. Comparison of plots of reduction of viscosity and release of reducing groups by *Rhizobium trifolii* enzyme extracts. Each viscosity reduction value is the average of 2 replications. Each value of release of reducing groups is the average of 4 replications.

CMC resulted in a slow release of reducing groups with a relatively larger decrease in viscosity (Fig. 2). This indicates that the enzyme is an 1,4-β-D-glucan-4-glucanohydrolase (endo-1,4-β-D-glucanase). CMC seems to be degraded to oligosaccharides.

The relatively low activities observed and the difficulty of concentrating and purifying the enzyme may indicate that CMC is not the best substrate for the enzyme. Enzymatic activities on xylan, locust bean gum and gum arabic have been detected but at even lower levels than cellulase.

Rhizobium cellulase is resistant to heat. Overnight incubation at 30°C did not affect or, occasionally, increased enzymatic activity.

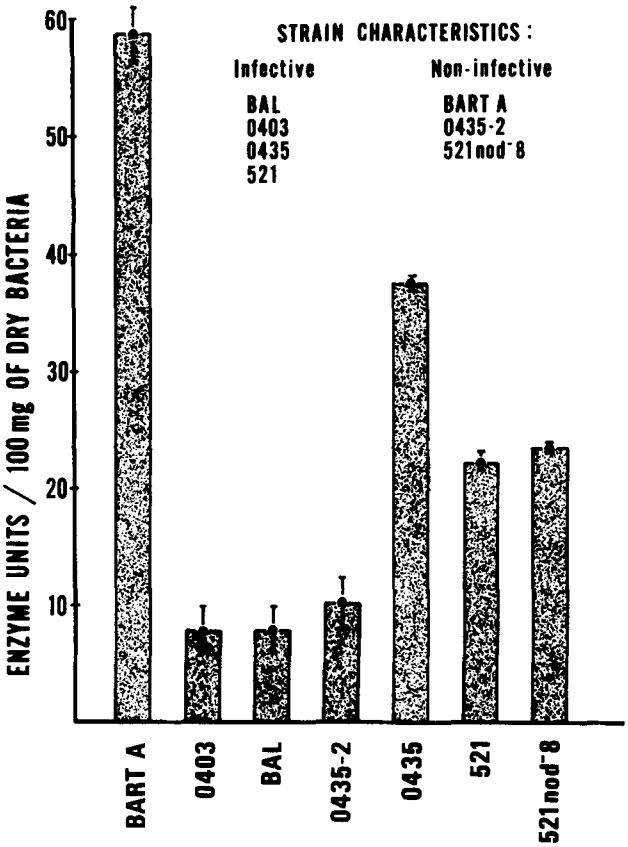

Fig. 3. Production of cellulase by different strains of *Rhizobium trifolii*. Enzymatic activity was measured by the viscosimetric assay. Each value is the average of 4 replications.

Heating at 60°C for 30 min destroyed 70% of the activity while 15 min of boiling destroyed 96% of the cellulase.

Production of cellulase by different Rhizobium strains

Fig. 3 shows the level of cellulase production by strains of *Rhizobium trifolii* with different infectiveness. There were differences between pairs of infective and non-infective strains. BART A, a non-infective derivative, produced 6 times more cellulase than the infective parental strain 0403. Both of these strains have three plasmids, but BART A has a deletion in the nodulation plasmid (sym plasmid) (R. Taylor, M.Sc. Thesis, University of Florida, Gainesville, 1981). In contrast, infective strain 0435 produced almost 4 times the amount of cellulase produced by strain 0435-2, its non-infective derivative.

The strain 521nod^{-8} is a derivative of 521, cured of the sym plasmid (190×10^6 daltons[21]). The two strains did not show any difference in cellulase production in the cultural conditions used.

The results obtained indicate that the gene(s) coding for the *Rhizobium trifolii* endo-glucanase is not in the sym plasmid. Hooykaas *et al.*[5] showed that the transfer of the sym plasmid of *Rhizobium trifolii* strain LPR 5001 (180×10^6 daltons) to a Ti plasmid cured derivative of *Agrobacterium tumefaciens* makes the transconjugants capable of inducing nodule formation in clover roots. It seems, therefore, that the sym plasmid carries all the genes needed for nodulation.

Other polysaccharide-degrading enzymes, induced only in proximity to the root hair cell wall, may have a role in plant infection. It is also possible that Rhizobium does not produce the complete enzyme set required for infection but induces the plant to produce the enzymes that it lacks[9]. There are many proteins in the Rhizobium enzyme extracts, in addition to cellulase, which have a high affinity for plant cell wall materials.

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