

Extraction, chemical composition, use in induced protection and cross-reactive antigens between exopolisaccharides from *Tremella fuciformis* Berk and *Xanthomonas campestris* pv. *citri* (Hasse) Dye

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Abstract — Exopolysaccharides (PS) are the major components on the surface of bacteria and also produced by fungi. These molecules are important in human health, in order to control diabetes as well as protect plants against attacks of foliage diseases. The objective of the present work was to study the partial chemical structure of the carbohydrate, use in control disease in plants and cross-serological relationship (cross-reactive antigens between isolates from fungi (*Tremella fuciformis* (Tf) and bacteria (*Xanthomonas campestris* pv. *citri* (Xcc)). Tf was developed in culture medium containing sorghum seeds during 20 days, and Xcc in the PDA (potato dextrose agar) medium for an 8 days period. The polysaccharide was removed from the culture medium, precipitated with ethanol, and quantified total sugar. By TLC was observed that 2 isolates presented galactose, glucose, mannose, arabinose and xylose in different proportions. Fucose and ribose was not found in the PS from Xcc but present in Tf. In ELISA, antiserum to Xcc revealed an antigenic homologous reaction with the same bacteria and heterologous with Tf. Barley plants pretreated with PS from Tf and later challenged with conidia from *B.sorokiniana*, demonstrated protection against the pathogen. Results suggested that PS from Tf presented induction of protection. Both PS (antigens) present an identical epitope demonstrated by reaction in Elisa test. The antibody against Xcc was specific for an epitope and bounded to another antigen due to having similar chemical properties.

Keywords— Polysaccharides, fungi, bacterial, cross-reactive antigen.

I. INTRODUCTION

Natural biopolymers (polysaccharides - PS) have a potential application in food, cosmetics, pharmaceuticals and oil industries. The macromolecules are water soluble which may be ionic or non-ionic in nature, and which increase the viscosity of the medium in conjunction with different physical and chemical agents. Because of their wide diversity in physical structure and properties, they can change the rheological properties and texture of the products in which they are incorporated into [1]. In recent years, there has been a major emphasis on the search for novel microbial polysaccharides, and a wide variety of microbial strains are reported to produce polysaccharides with varied compositions and useful properties [2].

Tremella fuciformis (family *Tremellaceae*, order *Tremellales*, class *Basidiomycetes*) is probably one of the most beautiful fungi growing in subtropical and tropical areas, or even temperate zones. It was first found in Brazil but has developed to an artificially cultivated species in Taiwan, China and some other countries in Asia [3]. This mushroom is called snow fungus, white jelly mushroom, and have been used as food and folk medicines for centuries in Asian countries [4, 5].

At the moment in Brazil, *Tremella fuciformis* (Tf) was grown in medium with sorghum for obtained PS. The polysaccharide was given by gavage for rats when induced by streptozotocin in order to develop type 1 diabetes mellitus (DM1) for a period of 30 days. Results indicate that PS is beneficial in control of DM1 when the level from blood glucose is up to 130mg/dL accomplished

by reducing cholesterol, triglyceride, GPT, urea and increasing HDL cholesterol [6, 7].

Another important application from PS was with promoted induction of protection against disease in plants. Bach et al. [8] demonstrated that PS from *Tf* in three concentrations, stimulated the ability of plants to compensate the damaging effects of the pathogens (*Bipolaris sorokiniana*) on barley plant (Variety BRS Brau) metabolism. The same protection was observed with xanthan gum from *Xanthomonas campestris* pv. *citri* (*Xcc*), *Xanthomonas campestris* pv. *campestris* and commercial xanthan gum against the same disease on barley and wheat plants [9, 10].

About chemical structure of the PS obtained from dried *Tf*, commercially available in China, and extracted by boiling water, determined that it consists of a linear backbone of (1→3) α -D-mannan with side chains composed of glucuronic acid, xylose and fucose [11, 12]. *Xanthomonas* produced xanthan gum and is an important industrial biopolymer as example food. According to several authors, the PS are antigenic macromolecules and may be related to pathogenicity [13 - 15].

The objective from the present work was to study the partial chemical structure of the carbohydrate and investigate the use under greenhouse conditions as an inducer of protection in barley plants (cultivar Embrapa BRS Elis) against pathogen. Another method for identification PS was study the cross-serological relationship (cross-reactive antigens between isolates from fungi (*Tremella fuciformis* *Tf*) and bacterial (*Xanthomonas campestris* pv. *citri* *Xcc*). This could aid in the possible explanation for its use in control of diseases both for the use in plants such as for humans beings.

II. MATERIALS AND METHODS

Preparation of polysaccharide from *Tremella fuciformis*

Tremella fuciformis received from Brasmicel, were cultured in potato dextrose agar (PDA) for 8 days, and then transferred to plastic bag containing sorghum seeds. The bags were incubated during 45 days for micelial growth in chamber with controlled temperature ($27 \pm 1^\circ\text{C}$) and dark. For production of polysaccharides (PS), a solid medium was made and added 100g of sorghum seeds (Embrapa seed variety 308) that was first cooked in water and after crushed in a blender in 400mL of water and boiled again. The mixture was filtered through sieve, gauze, cotton cloth, completely to 500mL water and supplemented with 0.5g of agar. After boiled, the solution was transferred to bottles and sterilized [8].

Sorghum seeds with mycelium were inoculated to bottle with solid medium and incubated for 10, 20, 30 days in chamber with controlled temperature ($25 \pm 1^\circ\text{C}$) and dark.

After the period, PS was removed and solution was reduced to half-volume by vacuum evaporation. This reduced volume was, then, treated with cold ethanol (70%) to polysaccharide precipitation. To facilitate the precipitation (ppt), the solution was kept at 4°C for an additional 24 h and then centrifuged at 4000g for 10 min at 18°C , and the precipitates were collected, washed twice with alcohol and then solubilized in water. After, the gum was submitted to dialysis in bags with 10.000 daltons against buffer phosphate pH=7 (0.05mol/L) for retired phenols. Tests were performed to quantify beta glucan and total sugar. Beta-glucan was determined by Lever method [16] involving the beta-glucanase enzyme (Sigma). For standard in test, was used laminarin that said one unit of enzyme can be liberated $1 \mu\text{M}$ of glucose/min at 37°C [17]. For total sugar the method used was Anthrone [17-19].

The PS was loaded onto a Sepharose CL_4B column (2.4 cm x 100 cm, Pharmacia) and eluted with the same buffer at a flow rate of 2ml/min. The carbohydrate moiety in the PS was monitored by absorbance at 480 nm. A pool fractions from 10, 12 and 14mL was quantify by Anthrone.

Preparation of Suspension of pathogen

The pathogen used was *B.sorokiniana* obtained from infected barley leaves (Fundação Guarapuava- Agraria, Paraná) and grown on plates using potato dextrose agar (PDA). After 10 days of incubation, conidia, removed by brushing the surface of the agar, were suspended in 10 ml of sterile water and filtered through gauze. The concentration of conidia was adjusted to 10^5 conidia per ml and Tween 20 (poly-oxyethylene sorbitan monolauret, Sigma Chemical Co) was added to give a final concentration of 0.05% [20, 21].

Preparation of barley and treatments

Barley plants (Embrapa Elis – from Foundation Agraria, state of Paraná), were grown in clay pots (15cm diameter, ten seeds) contained red soil fertilized (NPK 10:10:10 and micronutrients) in a greenhouse under a 12h photoperiod (approximately $190\text{IE}/\text{m}^2/\text{s}$) for approximately 3 weeks when plants reached the tillering stage (stage 5) [22]. Groups of 10 plants was used in each treatment and replicated three times. The data were submitted to variance analysis. Plants were arranged in a complete randomized block design and the combination of challenger and protector in each treatment. Plants were maintained at room temperature and a 12h photoperiod (7.35Wm⁻² of fluorescent light) throughout each treatment unless indicate otherwise [20]. Approximately 10ml of the conidia suspension, PS of *Tf*, or water were used in each treatment. Treatments were: (a) healthy:

plants were sprayed with water; (b) Inducer: plants were sprayed with PS of *Tf* (conc 2mmol of sugar); (c) pathogen inoculated: plants were pulverized with the conidial suspension of the pathogen; (d) 24 hour Inducer-pathogen: first treated with PS and 24 hours later inoculated with the conidial suspension; (e) 48 hour Inducer-pathogen: as in group d but inoculated with the conidial suspension 48 hours after inducer; (f) 72 hour Inducer-pathogen: as in group d but inoculated with the conidial suspension 72 hours after inducer. During the first 24 hours after inoculation with the pathogen, all plants were kept in the dark at room temperature in a humid chamber (80% relative humidity). After that, plants were transferred to the greenhouse. Protection level was evaluated 7 days after inoculation with the pathogen, based on the number of infected leaves in ten plants [20, 21].

Preparation of polysaccharide and antisera for *Xanthomonas campestris* pv. *citri*

Bacterial cells were grown for 8 days on potato dextrose agar (PDA) in Petri dishes at 27° C in the dark. The PS was removed from surface with water. One hundred mL of solution were centrifuged (7,000g- 20min), supernatants were reduced to half-volume by vacuum evaporation at 40°C and precipitated with 75% ethanol. The precipitates were dried under a N₂ stream and dissolved in distillate water. Beta glucan and total sugar was measured as described for *Tremella*.

Antisera for *Xanthomonas campestris* pv. *citri* pathotype A were produced in New Zealand White rabbits (approx. 2kg) in Biological Institute, São Paulo, Brazil in year 2000. Rabbits were immunized with one single 1.0 mL intra-lymph node injection of 900ug of glucose equivalents emulsified with an equal volume of Freund complete adjuvant (Difco). Sera were collected from the marginal ear veins 20 days after the injection [23].

Immunoglobulin (Ig) was precipitated from rabbit sera with 100% sat. ammonium sulfate, repeated twice, and dissolved in 0.85% NaCl solution followed by dialysis against the same solution. Enzyme conjugation was performed by adding 0.5mL of Ig (1 mg/mL) to 0.09 mL of alkaline phosphatase (Boehringer) followed by dialysis against 2mM sodium phosphate buffer pH 7.4 plus 0.85% NaCl and 0.8mL of 0.25% glutaraldehyde for 1h at 4°C. Ig and conjugates were diluted 1:2 and antigens (PS) were used in the concentration of 1mmol of glucose equivalents/mL. Normal sera were prepared with the same method as negative control.

Treatment of antigens with sodium periodate

The method was based in Bach and Guzzo [13] when 1mmol of glucose/mL of each PS, as measured by the

anthrone test [19] and treated with 0.05mol sodium periodate. Another group of equally treated samples were then reduced with sodium borohydride (Sigma) and subjected to hydrolysis with 0.03mol acetic acid for 1h (Hydrolyzed sample). Control samples were treated with ethylene glycol prior to the periodate treatment. Periodate-treated, hydrolyzed and control samples were used as antigen for serological tests by ELISA test.

Elisa Test

Antigens (PS) from *Xcc* and *Tf* were tested by double antibody sandwich-ELISA [24] and the experimental conditions for the serological reactions and immunoglobulins was based in Bach and Alba [23] and, Bach and Guzzo [13]. ELISA values were obtained by measuring absorbance at 405nm. The concentration of PS from *Tf* and *Xcc* used in work was equivalent to 1mmol of glucose.

Chromatography Thin layer (TLC)

The sugar was analyzed by thin layer chromatography (TLC) carried out on Merck silica gel 60 F254 plates (20 cm x 20 cm). Aliquots of standards solutions of glucose, galactose, mannose, xylose, arabinose, ribose and fucose (10mg/mL of each from Sigma) were applied as spots at the origin on a plate and developed with butanol: acetic acid: water (4:1:1 by vol.) in a pre-saturated chromatography chamber. The thin layer chromatography plates were dried at 60°C and the sugars were visualized at 254nm UV light and after detected by spraying chromatograms with 5% ethanolic sulphuric and heating in the oven at 100°C until clear sugars spots appeared [25, 26]. The CPATLAS program was used to determine the area of spots and relative front from each standard of developed chromatoplate. 10mmol from PS samples were treated with 100uL of beta glucanase (10UI) and 200uL of alfa glucanase (10UI) from Sigma in presence of citrate buffer (50mM, pH=5) and after 10min at 37C, was submitted to boiling for one hour at 100C. Those was separated in TLC.

III. RESULTS

Extraction of polysaccharide and concentration of beta glucan

When isolates of *Tf* and *Xcc* grown in medium, was observed as loose slime secreted by the microorganisms. After precipitation and solubilized in water we have a gum solution. Table 1 compared concentration of sugars present in PS from *Xcc* and from *Tf*. *Tf* polysaccharide increased until day 20, being after it decreased, most likely due to the increased oxidation, or enzyme presence. Present sugars were Alfa-glucose (millimol) and Beta-glucan.

Table.1: Concentration of beta-glucan linked-(1,3)(1,6) and total sugars from preparations of samples from *Tremella fuciformis* (Tf) and *Xanthomonas campestris* pv. *citri* (Xcc).

Polysaccharide from	mg Beta-glucan/mL	mmol total sugar
Tf (10days)	85.2 +/- 0.586a*	12.5d
Tf (20days)	153.6 +/- 0.625c	28.0a
Tf (30days)	130.5 +/- 0.615b	27.3c
Xcc	120.8 +/- 0.666d	21.4b

*Different letters on columns indicate statistically significant differences among groups ($p < 0.01$; ANOVA + Student's test).

In parallel studies with suspension of Tf was observed presence of spores. The appearance in 10 days was ovoid like yeasts. In 20 and 30 days the appearance was type yeast elongated. In work we used for studies Tf from 20 days because PS presented white color and from 30 days PS was transformed a brown color.

Gel Filtration of PS

The PS from Tf 20 days and from Xcc was monitored by a gel filtration in a Sepharose CL_4B column, by which one polysaccharide was eluted as shown in Fig. 1. Peaks from tubes 0-4 included external volum. Pool fractions (tubes 5,6,7) from Tf presented 5.12mmol of glucose and from Xcc only 2.04mmol of glucose.

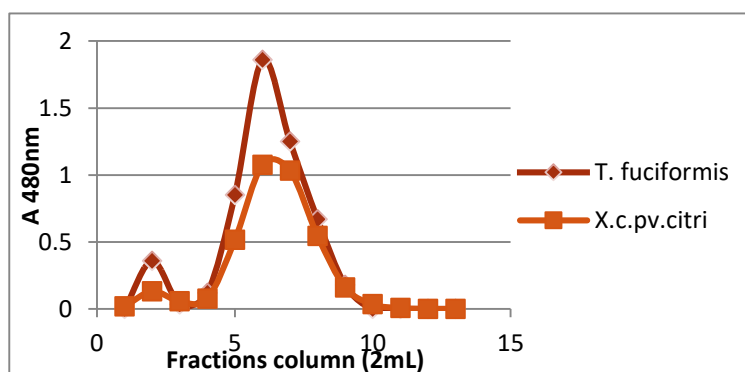


Fig.1: Elution profile of polysaccharides produced from *Tremella fuciformis* (20days) and *Xanthomonas campestris* pv. *citri* in Sepharose CL-4B chromatography.

Thin Layer Chromatography

By TLC was possible to observe that isolates from Xcc and Tf contain galactose, glucose, mannose, arabinose and xylose in different proportions but similar. Fucose and ribose was not found in the PS from Xcc (Table 2).

Table.2: Composition of the PS from *Tremella* and *Xanthomonas* obtained by TLC.

Isolates	galactose Rf=0.16	Glucose Rf=0.18	mannose Rf=0.20	arabinose RF=0.21	fucose RF=0.27	xylose RF=0.28	Ribose RF=0.31
<i>T. fuciformis</i> 20days	3.54*	132.5	121.6	3.85	1.56	5.48	2.85
<i>X. c. pv. citri</i>	3.85	147.8	125.4	3.15	x	6.48	X

* area of spots in CPATLAS.

Elisa test

Results obtained through ELISA from the combination of PS from Xcc and the same antisera have specific reaction. In contrast, negative reactions occurred with normal serum when used in the test. Similarly, antigen from Tf reacted specifically in heterologous antigen-antibody

combinations and give reaction in Elisa at 0.82. The results can indicated that reaction was similar as observed with antigen from Xcc or that was equivalent to 100% of interaction. To confirm that the serological reaction depends from the polysaccharides antigen, samples could be treated with sodium periodate. Homologous and

heterologous serological reactions were completely abolished by treating the PS antigens with periodate for antisera against *Xcc*. The carbohydrate contents in all periodate treated samples decreased in comparison to the

non-treated samples. Reactions in Elisa test were not recovered in homologous and heterologous reactions (Table 3).

Table.3: Effect of periodate treatment on antigenicity of PS from *Xanthomonas* and *Tremella* and contents of carbohydrate antigenic fractions.

Antigens	Treatments	Antibody PS from <i>Xcc</i> *	Concentration of sugar after treatment of periodate	
			control	treated
<i>Xcc</i>	Control	0.85	1mmol	1mmol
	Treated	0.00	1mmol	0.28mmol
	Hydrolyzed	0.00	1mmol	x
<i>Tf</i> 20days	control	0.82	1mmol	1mmol
	treated	0.00	1mmol	0.18mmol
	hydrolyzed	0.00	1mmol	x

* Absorbance (A405) Elisa Test

Induction of protection

Barley plants when treated with PS from *Tf* presented protection ranged from 70 to 85% as compared with infected leaves (Table 4).

Table.4: Percentage of protection in barley leaves against *B.sorokiniana* by an PS from *Tremella fuciformis* at 2mmol of sugar.

Treatments	% protection*
tremella Control	x
tremella 24h	70b
tremella 48h	80b
tremella 72h	85b
healthy	x
infected	0a

See Materials and Methods for a description of each treatment. * mean percentage of protection from total of 10 plants per treatment and three repetitions. ** a different letter in collums indicates a statistically significantly difference from the infected plants (P<0.05)

IV. DISCUSSION

Polysaccharides have the highest capacity for carrying biological information and differ greatly in their chemical composition, molecular weight, conformation, glycosidic linkage, and degree of branching, etc [27]. Weintraub [28] working with immunology said that the surface polysaccharide confers protection against the disease. The immunological properties of PS from bacterial can be used in vaccines, for study cross-reactive antigens, serogrouping or serotyping systems and others. PS from *Xcc* and *Tf* were used and demonstrated protection in barley plants against infection by *Bipolaris sorokiniana*

[8-10, 13]. So the objective was to evaluate the presence of crossed antigens.

The chemical composition and efficiency of extraction processes of polysaccharides in fruiting bodies from *T. fuciformis* were not completely clear because there are different methods. In Brazil, it is difficult to find fruiting bodies, and work in labor is possible when grown in medium. Many solid medium were used, but the best was made with sorghum seeds (variety 308-Embrapa).

Polysaccharides from *Tf* in this work, after precipitation with alcohol, presented white color that can be transformed in brown color in some days because enzyme

peroxidase was presented and it acted in compound. After washing, precipitate and dialysis, the compound became white colored that can be kept in freeze for several years [7, 8]. With PS from *Xcc*, the color after precipitation, was white.

The concentration of beta glucan from *Tf* demonstrated that increased until day 20, being after it decreased, most likely due to the increased oxidation and correlated with the color from precipitated after 20 days. About the content of sugars *Tf* present more beta glucan and total sugars when compared with *Xcc* (Table 1). The results with production of polysaccharides with spores its important and the appearance are according with those observed by Chen & Hou [29]; Cho et al. [30] and Seviour et al.[31]. Chen & Hou [29] said that spores were broadly ellipsoid with 7-9 x 6-7um, smooth and hyaline. According to Cho et al.[30] and Seviour et al.[31], exopolysaccharide production from *Tf* revealed that the morphological form grows in mainly three different yeast-like forms: ovoid, elongated, and double yeast forms. Cho et al.[29] said that it is noteworthy to mention that the increased population of elongated yeast probably contributed to an increased PS production. So, in results (Table 1) we have formation of elongated form in *Tf* in 20 days that coincide with a higher glucose concentration.

Gel permeation chromatography of the polysaccharides on a Sepharose CL-4B column yielded two major peaks: a narrower peak in fractions (tubes 0-4) and another broad peak present in tubes 5-6 corresponded from 10 to 12mL. In this separation can be see that have the same peaks for *Xcc* and *Tf* that correspond to 5.12mmol of glucose for *Tf* and 2.04mmol for *Xcc*.

By TLC was observed that PS from *Xcc* and *Tf* presented in polymers the sugars as: galactose, glucose, mannose, arabinose and xylose. The results with *Tf* in part are in accord with several authors that estimated ratio of mannose, fucose, xylose, and glucuronic acid is 9:1:4:3 making glucuronic acid accounted for 17.6% of the polysaccharides [32-34]. Khondkar [34] was isolated PS from liquid cultures of nine *Tremella* species grown in Malt-yeast extract media for 6 days at 27°C, and the results demonstrated that the polysaccharides in aqueous solution consisted of the following monomeric sugars: fucose, ribose, xylose, arabinose, mannose, galactose, glucose and glucuronic acid. The backbones of the polysaccharide structures consisted of α -(1→3)-links while the side chains were β -linked. In results (Table 2) from this work have not observed glucuronic acid.

Bach and Guzzo [13] worked with PS from *Xanthomonas campestris* pv. *citri* and *Xanthomonas campestris* pv. *manihotis* and observed the sugar compositions in isolates that suggesting that antigenic determinants may depend

on the sequence of sugars, linkages, branching and stereochemistry of PS. Lozano and Sequeira [35] said that PS coats the outer membrane and seems to play a role in the specific recognition mechanism between the bacterial or fungal cell and the host cell walls. The PS can also prevent the interaction to host cell wall.

For determinated serological relationships among the preparations of PS, preliminary assays showed that Ouchterlony double-diffusion technique detect a weak cross-reactive antigens between *Xcc* and *Tf*. This indicates that these substances occur in low concentration, both in bacterial and fungi. For more contributions was made by sensitive serological techniques like ELISA.

Table 3 demonstrated that have a clear indication that cross-reactive antigens must be involved in this case. This assumption is corroborated by the evidence that antiserum to *Xcc* reacted with PS from *Xcc* (homologous reaction) and also with PS from *Tf* (heterologous reaction). To confirm that serological reaction depends from the polysaccharides antigen, samples was treated with sodium periodate. Sodium periodate oxidation eliminated all serological reactions suggesting that, perhaps, periodate-susceptible 1,2; 1,4; 1,3; 1,6 linked non-terminal residues or non-reducing terminal units that could be present in antigenic sites. It can be assumed that all antigenic determinants did not recover serological activity after reduction with sodium borohydride and mild acidic hydrolysis, suggesting that perhaps the periodate degradation led to splitting of the polysaccharide chain. Apart from this, it can be concluded that PS have antigenic determinants of *Xanthomonas* and *Tremella* and with an identical epitope. The antibody against *Xcc* was specific for an epitope and bounded to another antigen due to having similar chemical properties.

The results presented in Table 4 indicate that protection was conferred to barley plants when the inducer (PS) was applied in the plant, and after 72h from the treatments the protection was higher. This same effect was observed by Bach et al [20], Castro and Bach [21] in work with other elicitors.

V. CONCLUSION

The use of polysaccharides in agriculture aimed at disease control on plants has been important as a alternative control in biotechnology. The results suggest that PS from *Tremella* can be induce protection against disease in barley plants. In study from PS in the *Xanthomonas-Tremella*, interaction there is a "key" cross-reactive antigens. The hypothesis is that the molecular structures of polysaccharides should be similar and have the same common properties and both can be used for the same aim: the plant protection against diseases or as a medicinal usage.

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