IMMUNOLOGICAL PROPERTIES OF A LECTIN ISOLATED FROM THE PHYTOPATHOGENIC BASIDIOMYCETE *RIGIDOPORUS LIGNOSUS*.

Thierry RICHARD[1], Jean GUILLOT[2] and Bernard BOTTON[1]*

[1] Université Henri Poincaré,
Nancy I, Faculté des Sciences,
Laboratoire de Biologie Forestière associé INRA,
BP 239, 54506 Vandoeuvre-lès-Nancy Cedex, France
[2] Université d'Auvergne, Clermont-Ferrand I,
Faculté de Pharmacie,
Laboratoire de Botanique, Cryptogamie et Microbiologie,
28, Place Henri Dunant, 63000 Clermont-Ferrand, France
* To whom correspondence should be addressed

ABSTRACT: An antiserum raised against the lectin purified from mycelial strands of *Rigidoporus lignosus* (isolate FCA3) was used in Ouchterlony double diffusion to detect lectin in other parts of the colony and in the culture medium, as well as in a number of other African and Asian isolates. The lectin was strongly accumulated in mycelial strands and less concentrated in undifferentiated mycelium. A significant amount was excreted in the culture medium. The lectin in the strands of the FCA3 isolate was serologically identical to those synthesized by the vegetative mycelium, excreted in the culture medium and present in the other isolates. Hemagglutination activity determined with the O group erythrocytes was wery low in a few isolates, and consistent with this, no precipition lines were detected by immunodiffusion, especially in the isolates which hardly ever differentiated mycelial strands. Cross reactions indicated that the lectin of *Rigidoporus lignosus* was related to that of *Boletus edulis* and *Ulex europaeus* I and II, but however not identical with the two latter. No serological relationship was found with 13 other lectins purified from fungi and higher plants, thus confirming the unicity of the lectins in *Rigidoporus lignosus*.

KEY-WORDS: Basidiomycete, Fungi, Lectin, Ouchterlony double diffusion, *Rigidoporus lignosus*

INTRODUCTION

Lectins are diverse multivalent carbohydrate-binding proteins or glycoproteins of non-immune origin that have the property of agglutinating animal and plant cells (Goldstein & Poretz, 1986; Lis & Sharon, 1986). They are widely distributed in animals, plants and fungi and the most extensively studied group of lectins to date is that of the plant family Leguminoseae (Etzler, 1985; Maliarik *et al.*, 1991).

Although many lectins have been isolated and characterized from fungi, the physiological roles of most fungal lectins is still unclear and speculative. In *Conidiobolus lamprauges* and *Neurospora sitophila* the lectin is thought to play an important role in cell wall biosynthesis and mycelial differentiation (Ishikawa *et al.*, 1983; Ishikawa & Oishi, 1989). It has also been postulated that fungal lectins are involved in adhesion and recognition phenomena. Flocculation in *Kluyveromyces bulgaricus* and mating interaction in *Saccharomyces cerevisiae* are mediated by lectins (Al-Mahmood *et al.*, 1988; Terrance *et al.*, 1987), while in lichens, fungal lectins are involved in the relationship between fungi and algae (Lockhart *et al.*, 1978). The lectins of ectomycorrhizal fungi such as *Lactarius deliciosus, L. deterrimus* and *L. salmonicolor* appear to be involved in the early stages of recognition with *Pinus sylvestris, P. abies* and *Abies pectinata*, respectively (Giollant, 1991; Giollant *et al.*, 1993). Adhesion by lectins may also be the initial step in certain infection processes. Thus, the zoospores of *Phytophthora cinnamomi* adhere to root cells of corn by means of lectins (Hinch & Clarke, 1980). In addition, the lectins on the trap surface of many nematode-trapping fungi are important in the attachment of those fungi to nematodes (Tunlid *et al.*, 1992).

Rigidoporus lignosus is a tropical basidiomycete causing great losses in plantations of a number of species, especially *Hevea brasiliensis* (Rubber tree) and *Tectona grandis* (Teak). The fungus differentiates flattened mycelial strands which infest the roots of healthy rubber trees (Nandris *et al.*, 1987). With the aim to control dissemination of the fungus, research has been conducted to gain better knowledge of the mycelial strand differentiation processes. Results have demonstrated the presence in *Rigidoporus lignosus* of a lectin which was composed of four identical submits of 35 kDa, had a high affinity for L-fucose and was synthesized concomitantly with strand formation (Richard, 1995). The lectin was cytosolic in the vegetative cells but was located at the periphery of the strand cells where the affinity sites for the lectin were also distributed (Richard *et al.*, 1994). In addition the affinity sites for the lectin were also present on the root surfaces of *Hevea brasiliensis* (Richard, 1995), suggesting that this lectin may be involved, both in the processes of mycelial strand formation and in host recognition. However a detailed analysis of the accumulation and distribution of this lectin has not been carried out yet.

In the present paper, the lectin extracted from *Rigidoporus lignosus* strands was immunologically compared to the lectins present in the other parts of the colony and in several other strains of this pathogenic fungus. Moreover, the antibodies were used to assess the immunological relationship between the *Rigidoporus lignosus* lectin and a few others isolated from fungi and higher plants.

MATERIALS AND METHODS

Fungi and culture conditions

Ten *Rigidoporus lignosus* strains (also called isolates), isolated from *Hevea brasiliensis* roots were obtained from the Rubber Research Institute in Paris. The details of geographical origin and year of isolation are listed in Table 1. Five strains were isolated from Africa and five from Asia. Purification and characterization of a lectin were carried out from the FCA3 strain coming from Cameroun.

All the strains were conserved as mycelial stock cultures in the dark on 2% malt agar medium at 24°C. For the extraction of the lectins, the fungi were grown in static cultures at 28°C, in 240 ml Erlenmeyer flasks containing 100 ml 2% malt liquid medium.

The surface of the medium was inoculated with a 7 mm-diameter disc of solid medium punched out from the edge of a 7 day-old colony grown on malt agar.

Isolates	Origin	Isolation (year)
FCI9	Ivory Coast (South-West)	1989
FCA3	Cameroun (South-West)	1989
FGA1	Gabon (North-Mitzic)	1989
FGA4	Gabon (North-Mitzic)	1991
FGA7	Gabon (South-Kango)	1991
FID1	Indonesia (North-Sumatra)	1988
FID2	Indonesia (North-Sumatra)	1989
FID3	Indonesia (West-Kalimatan)	1989
FML3	Malaysia (South)	1989
FML4	Malaysia (South)	1989

Table 1. Isolate designation, geographic origin and year of isolation of the studied *Rigidoporus lignosus* isolates

Estimation of lectin activity

To determine lectin content in colonies, all the strains were grown for 3 weeks in liquid medium. Vegetative mycelia and mycelial strands were weighed and extracted separately in PBS (phosphate-buffered saline; 1.5 mM-KH2PO4/10 mM-Na2HPO4, pH 7.4; 3 mM-KCl; 140 mM-NaCl). The resulting homogenate was centrifuged at 40,000 g for 20 minutes to remove insoluble residues. Thereafter, lectin and protein concentrations of the supernatant were determined.

Lectin activity was estimated in microtiter plates by a serial dilution method as already described by Al-Mahmood *et al.* (1988). To 100 µl aliquots obtained by dilution of the lectin solution, an equal volume of a 2% suspension of washed human red blood cells (O group) in PBS was added. Plates were left overnight at 4°C and examined for agglutination. The titre was defined as the reciprocal of the highest lectin dilution that still agglutinated the cells. Specific activity was the titre expressed by 1 mg protein.

Protein concentration was measured according to the method of Bradford (1976) using Bovin Serum Albumin as a standard.

Production of antibodies and Double immunodiffusion assay

Polyclonal antibodies were obtained with the lectin extracted from the mycelial strands of the FCA3 strain according to a method described previoulsy (Botton *et al.*, 1987).

Specificity of the antibodies was tested by Western blot techniques as already described (Richard, 1995). Since the antiserum against the lectin from the FCA3 strain reacted exclusively with the lectin polypeptides on a Western blot of total extracts from other strains and did not react with any polypeptide band when the same extracts were depleted of lectin (result not shown) we assume that the antibodies were monospecific.

Double immunodiffusion was carried out according to Ouchterlony (1959), on the surface of microscope slides (76 x 26 mm) which were covered with 3 ml of 1.2% agarose (Indubiose A37, Reactifs IBF) dissolved in PBS. Lectins and antibodies diffused towards one another at room temperature under saturated atmosphere. Precipitation lines were observed by examining the slides on a black background without any staining. Alternatively, slides weres rinsed overnight with 0.85% NaCl so as to eliminate unprecipitated proteins, then after dehydration, precipitation lines were stained with Coomassie brilliant blue R.

Comparison of the *Rigidoporus lignosus* lectin with lectins from other fungi and higher plants

In order to gain better knowledge of the structure of the lectin isolated from *Rigidoporus lignosus*, cross reactions were performed with antibodies and different lectins isolated and purified from fungi such as *Boletus edulis* (specificity: lactose), *Russula nigricans* (specificity: N-acetyl-β-D-galactosamine) and *Candida albicans* (specificity: L-fucose/N-acetyl-β-D-glucosamine), as well as from higher plants such as *Sophora japonica* (specificity: N-acetyl-β-D-galactosamine), *Canavalia ensiformis* (specificity: α-D-mannose), *Ricinus communis* (specificity: β-D-galactose), *Lens culinaris* (specificity: α-D-mannose/α-D-glucose), *Phaseolus vulgaris* (specificity: N-acetyl-β-D-galactosamine/lactose), *Genista scoparia* (specificity: N-acetyl-β-D-galactosamine/β-D-galactose), *Glycine max* (specificity: N-acetyl-β-D-galactosamine, *Ulex europaeus* I (specificity: α-L-fucose), *Ulex europaeus* II (specificity: N-acetyl-β-D-glucosamine), *Pisum sativum* (specificity: D-mannose), *Laburnum alpinum* (specificity: salicine) and *Cytisus sessilifolius* (specificity: salicine/di-N-acetylchitobiose).

Most of the lectins were provided by Prof. J. Guillot's laboratory of Botany, Cryptogamy and Microbiology, Faculty of Pharmacy, University of Clermont-Ferrand (France). They were purified using protocols previously described (Guillot *et al.*, 1983; Guillot *et al.*, 1991).

RESULTS

Comparison of the lectins from different parts of the colony and excreted in the culture medium

The *Rigidoporus lignosus* lectins of the FCA3 strain were compared by Ouchterlony double diffusion using antiserum raised against the mycelial strand lectin. As shown in Fig. 1, antibodies gave rise to a precipitation line with the strand crude extracts and with those obtained at different purification steps. Similar immunoprecipitations were obtained with crude extracts from vegetative mycelium and with culture filtrates. This suggests that the lectin was produced, not only by mycelial strands, but also by undifferentiated cells and, moreover, partially excreted into the culture medium. The Ouchterlony test gave rise to one continuous precipitation band, indicating that all the lectins were serologically identical to that of the strands.

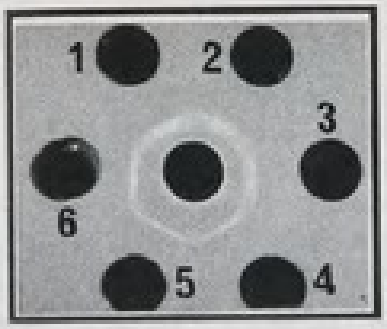

Fig. 1 — Ouchterlony double diffusion patterns of lectins produced by the FCA3 strain of *Rigidoporus lignosus*. Antiserum raised against the mycelial strand lectin was deposited in the central well and challenged with antigens in the outer wells. 1: crude extract of mycelial strands, 2: mycelial strand extract purified by ammonium sulfate (30-80 % saturation), 3: mycelial strand extract purified by subsequent anion-exchange chromatography on DEAE-Sephacel, 4: mycelial strand extract purified by subsequent affinity chromatography on L-fucose-agarose column, 5: crude extract of vegetative mycelium, 6: twice concentrated culture filtrate of *Rigidoporus lignosus*, strains FCA3 (3 week-old-cultures).
Each well contained about 5 µg of purified lectin or antibodies and 25 µg of protein when crude extracts were used. Precipitation lines were observed on a black background without any staining.

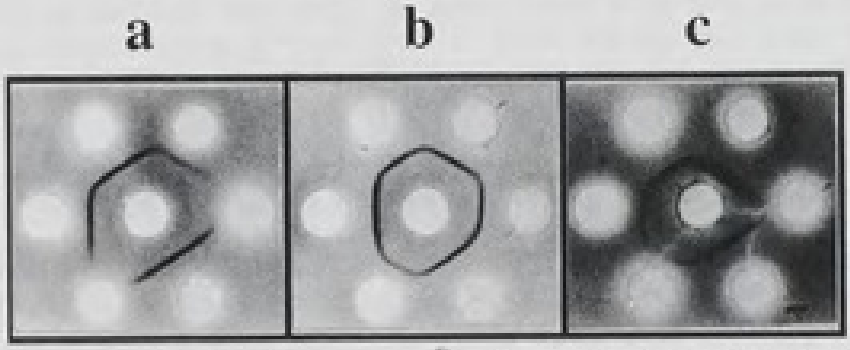

Fig. 2 — Ouchterlony double diffusion patterns of lectins produced by different isolates of *Rigidoporus lignosus*. Antiserum raised against the FCA3 lectin was deposited in the central well and reacted with mycelial crude extracts placed in the outer wells (outer well numbers correspond to those in Fig. 1). a) 1: purified lectin from vegetative mycelium (isolate FCA3), 2: FID1 isolate, 3: FID2 isolate, 4: purified lectin from mycelial strands (isolate FCA3), 5: FGA1 isolate, 6: FML3 isolate, b) 1: purified lectin from mycelium (isolate FCA3), 2: FML4 isolate, 3: FCI9 isolate, 4: purified lectin from mycelial strands (isolate FCA3), 5: RP1 isolate, 6: FGA7 isolate, c) 1: purified lectin from the culture medium (isolate FCA3), 2: FCA3 isolate, 3: Bovine Serum Albumin (control), 4: purified lectin from the culture medium (isolate FCA3), 5: FID3 isolate, 6: FGA4 isolate.
Each well correspond to a crude extract containing 25 µg proteins. Precipitation lines were visualized by Coomassie blue staining.

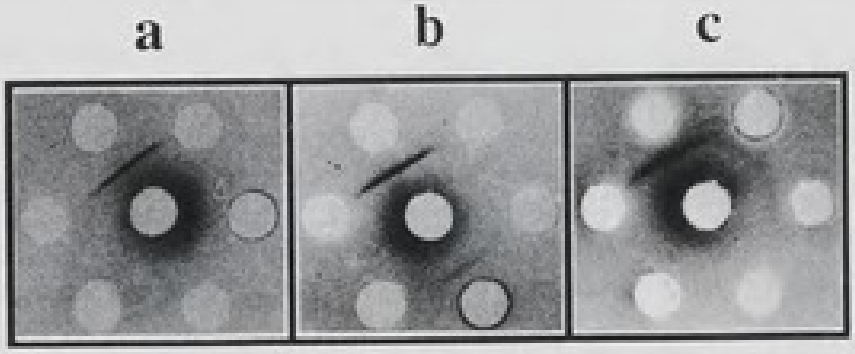

Fig. 4 — Ouchterlony double diffusion patterns of different lectins produced by fungi and higher plants. Antiserum raised against the FCA3 lectin of *Rigidoporus lignosus* was deposited in the central well and reacted with purified lectins of different organisms placed in the outer wells (outer well numbers correspond to those in Fig. 1). a) 1: purified lectin from vegetative mycelium (isolate FCA3: control), 2: *Sophora japonica* lectin, 3: *Canavalia ensiformis* lectin, 4: *Ricinus communis* lectin, 5: *Lens culinaris* lectin, 6: *Phaseolus vulgaris* lectin, b) 1: purified lectin from vegetative mycelium (isolate FCA3: control), 2: *Genista scoparia* lectin, 3: *Russula nigricans* lectin, 4: *Boletus edulis* lectin, 5: *Glycine max* lectin, 6: *Candida albicans* lectin, c) 1: purified lectin from vegetative mycelium (isolate FCA3: control), 2: *Ulex europaeus* II lectin, 3: *Ulex europaeus* I lectin, 4: *Pisum sativum* lectin, 5: *Laburnum alpinum* lectin, 6: *Cytisus sessilifolius* lectin.
Each well contained about 5 µg of lectin. Precipitation lines were visualized by Coomassie blue staining.

Cross reactions with other *Rigidoporus lignosus* strains

Several lectin crude extracts obtained from different isolates of *Rigidoporus lignosus* were challenged with the antiserum in double immunodiffusion assays (Fig. 2). When precipitation lines were obtained they were continuous with each other. Therefore the lectins synthetized by the strains FID1, FML3 (Fig. 2a), FML4, FCI9, RP1, FGA7 (Fig. 2b), FID3 and FGA4 (Fig. 2c) are qualitatively similar to that of the FCA3 isolate, in terms of their immunological properties. However, reactivity of the lectins towards the antibodies was quantitatively different. Indeed, as lectin extraction was carried out from a similar mass of mycelium in each isolate, it can be seen that lectins were much less abundant in FID1 (Fig 2a), RP1 (Fig. 2b), FGA4 (Fig. 2c) and especially in the FID3 isolate (Fig. 2c). No precipitation lines were observed with the isolates FID2 and FGA1 (Fig. 2a).

Estimation of a lectinic activity in different *Rigidoporus lignosus* isolates

Specific activity of the lectins was measured by the hemagglutination test in the isolates mentioned above, except in the RP1 isolate which was not included. As shown in figure 3, the highest productions of lectin were found in decreasing order in the isolates FCA3, FGA4, FCI9, FML4 and FML3. Hemagglutination activity was considerably reduced with the other isolates, especially in the FID1, FID2 and FGA1 isolates, which is in agreement with the Ouchterlony tests, as no precipition bands were detected in the two latter isolates.

The highest lectinic activities were found in mycelial strands and the lowest were usually confined to the vegetative mycelium, an intermediate significant amount of the lectin being excreted into the culture media. However, isolates FID1 and FID2 did not reveal excreted lectins (Fig. 3). Two isolates, FCI9 and FID3 did not differentiate mycelial strands. In the former isolate, lectin was highly concentrated in the mycelium and in the latter isolate, lectin synthesis was strongly reduced (Fig. 2), which is consistent with the faint precipitation band found in the Ouchterlony test (Fig. 2c).

Cross reactions with purified lectins of other organisms

Fifteen purified lectins from higher plants and fungi were challenged with the antibodies in immunodiffusion tests. The FCA3 lectin, isolated from vegetative mycelium was used as a control. Figure 4 shows the results of these studies and reveals that only the lectins of the Basidiomycete *Boletus edulis* (Fig. 4b) and to a lesser extent, the lectins I and II of *Ulex europaeus* (Fig. 4c) gave rise to a weak but discernible reaction upon double immunodiffusion. The position of the *Boletus edulis* lectin on the microscope slide did not allow determining any serological relationship with the FCA3 lectin. On the other hand, the result shown in Fig. 4c reveals that the FCA3 lectin is related, but not identical, to the two *Ulex europaeus* lectins, as evidenced by a substantial spur formation between wells 1 and 2. No precipitation bands were detected with the other lectins.

These results indicate that the FCA3 lectin of *Rigidoporus lignosus* shares some antigenic determinants with the lectins of *Boletus edulis* and *Ulex europaeus*, but reaction with this latter clearly indicates that each lectin is slightly different. The results obtained with the other lectins indicate that their antigenic determinants are different from those of the FCA3 lectin, probably reflecting differences in their primary or/and tertiary structures.

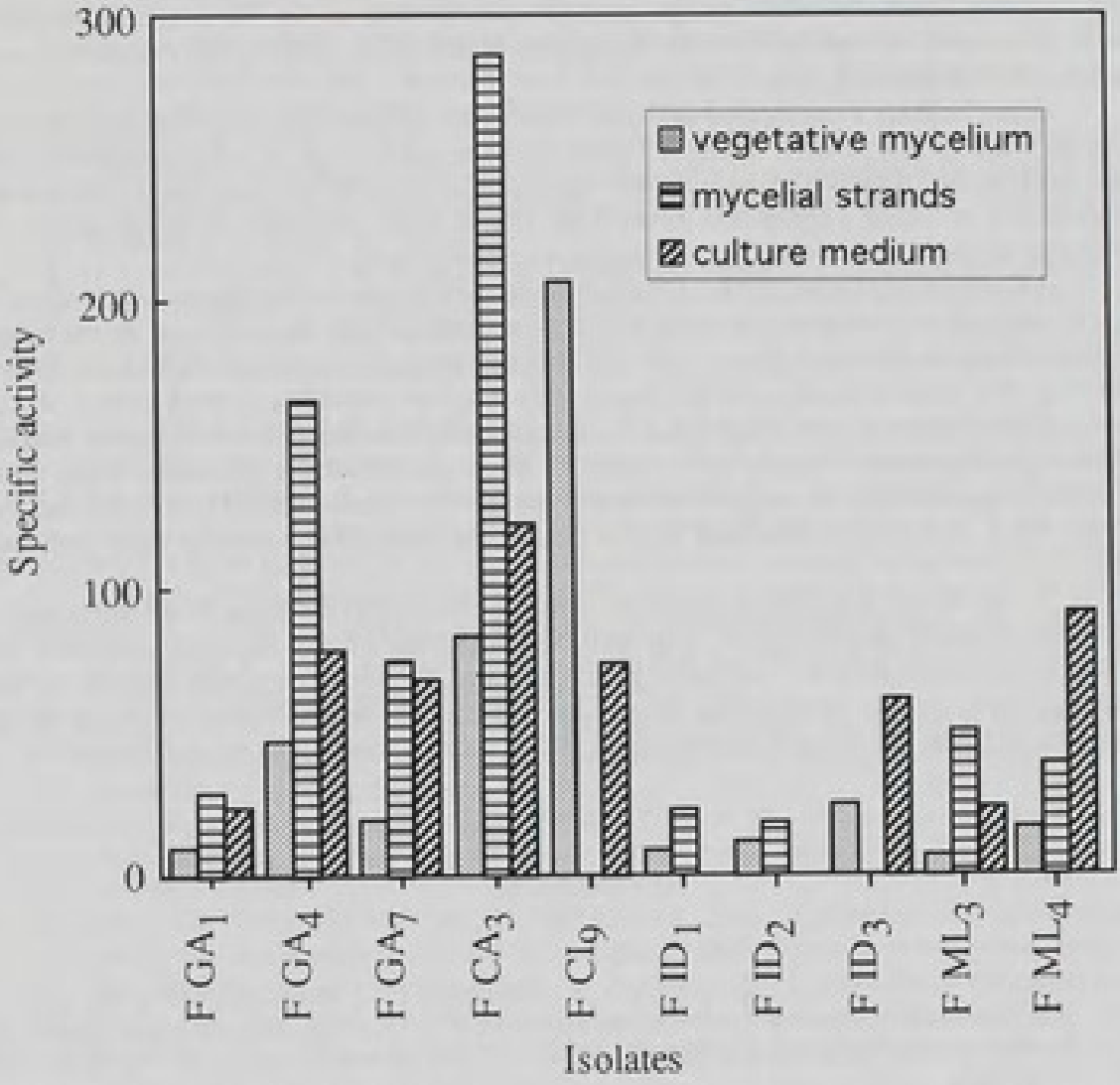

Fig. 3 — Lectinic activity in 10 isolates of *Rigidoporus lignosus*. Hemagglutination tests were carried out separately on vegetative mycelium, mycelial strands and with the culture medium. Age of the cultures: 3 weeks.

CONCLUSION

A lectin was found to be present in *Rigidoporus lignosus* and purified from mycelial strands. The antibodies raised against this lectin allowed an analysis of its accumulation and distribution in fungi and plants. A comparative study carried out by double immunodiffusion assays on ten strains isolated from Asia and Africa revealed that all of them contained a serologically identical lectin. The lectin was found at low concentrations in the vegetative mycelium, while it accumulated much more in mycelial strands. Many lectins have been isolated from fruit bodies of higher fungi and characterized, and there are only a few reports describing lectins in mycelia (Guillot *et al.*, 1991; Kawagishi *et al.*, 1997). The lectin of *Rigidoporus lignosus* is also excreted in the culture medium which

has, so far, rarely been reported, except for a few yeasts such as *Saccharomyces cerevisiae* (Basu *et al.*, 1986), *Candida albicans* (Critchley & Douglas, 1987) and *Kluyveromyces bulgaricus* (Al-Mahmood *et al.*, 1988).

Some isolates investigated in this study did not differentiate mycelial strands and usually produced a low level of lectin. This is consistent with a significant accumulation of lectin found in the strands and with a close correlation between production of lectin and differentiation of these aggregated structures. It has been proposed that the lectin of *Rigidoporus lignosus* be involved in cell aggregation (Richard, 1995).

All the lectins found in the different isolates of *Rigidoporus lignosus* were closely related to each other, obviously sharing the same antigenic determinants and no serological differences were detected. Some slight differences probably exist between the different lectins but they were not detected by the immunological procedures used in this study. Richard (1995) showed that the FCA3 isolate actually produced three different lectins which are glycoproteins slightly different as to their carbohydrate contents. They were respectively accumulated in vegetative mycelium, strands and excreted into the culture medium. Such differences detected within a species, very likely should exist between isolates.

The lectin of *Rigidoporus lignosus* is immunologically different from most of the other lectins already purified from fungi and higher plants. This is in agreement with the amino acid sequence data of the lectin which showed no homology with known lectins (Richard, 1995). Future approaches should be directed to the molecular analysis of the role of this lectin in the fungus, especially in the processes leading to strand formation.

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