BIOCELL
2003, 27(2): 173-179

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Effect of sugars on the association between cowpea vicilin (7S storage proteins) and fungal cells

T.L. Rose*, V.M. Gomes*, M. Da Cunha**, K.V.S. Fernandes*** and J. Xavier-Filho***

* Laboratório de Fisiologia e Bioquimica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brasil.
** Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brasil.
*** Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brasil

Key words: vicilin, cowpea seeds, *Saccharomyces cerevisiae*, *Fusarium oxysporum*

ABSTRACT: Vicilins (7S storage proteins) found in various legume seeds have been previously shown to interfere with the germination of spores or conidia of phytopathogenic fungi and inhibit yeast growth and glucose stimulated acidification of the medium by yeast cells. In the present work vicilins from cowpea (*Vigna unguiculata*) seeds were added to the growth medium of *Saccharomyces cerevisiae* cells and *Fusarium oxysporum* conidia. *Helix pomatia* lectin, wheat germ agglutinin and *Ulex europaeus* lectin were used to identify differences in the binding of the vicilins to the surface of cells of *S. cerevisiae* and *F. oxysporum* treated with this protein. After the growth period, the material in suspension (yeast cells) was centrifuged and the final pellet was also treated with different sugar (glucose, sucrose, glucosamine, N-acetyl-glucosamine) concentrations and 0.1 M HCl for extraction of vicilins associated to chitinous structures present in yeast cells. Our results showed that vicilin sub-units were present in the different sugar extracts of yeast cells pre-treated with the vicilins and these proteins were eluted by 0.5 M solutions of sugars in the following order of efficiency of elution: N-acetyl-glucosamine, sucrose/glucose and glucosamine.

Introduction

Vicilins are seed storage proteins of the 7S globulin class, which are present in seeds of leguminous and other plants. These proteins are characterized as oligomers of 150-170 kDa formed by three similar subunits of about 40-70 kDa with no disulfide linkages. All vicilins purified from legume seeds are highly heterogeneous and consist of many different subunits. This heterogeneity is due to the expression of multigene families whose individual genes are very closely related and also to post-transcriptional processing and glycosylation (Derbyshire *et al*., 1976; Casey *et al*., 1986).

We have recently shown that vicilins from cowpea (*Vigna unguiculata*) and other legume seeds strongly associate with chitin, chitosan and fully acetylated chitin (Sales *et al*., 1996). Recent results have also shown that vicilins interfere with the germination of spores or conidia of phytopathogenic fungi and inhibit yeast growth and glucose stimulated acidification of the medium by yeast cells (Gomes *et al.,* 1997; Gomes *et al*., 1998a). Chung *et al*. (1997) also showed that vicilin-related basic proteins isolated from cotton seeds inhibit the growth of various filamentous fungi. The same way others different types of antimicrobial proteins have been

Address correspondence to: Dr. V.M. Gomes. Laboratório de Fisiologia e Bioquimica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego, 2000, 28015-620 Campos dos Goytacazes, RJ, BRASIL. Fax: (55-227) 261520; E-mail: valmg@uenf.br
Received on April 29, 2002. Accepted on August 26, 2002

also purified from plants seeds (Wang and Bunkers, 2000).

A mechanism by which vicilins exert their effects on fungi growth was recently proposed by us. This mechanism is based on the ability of vicilins to bind to the cell walls and the plasma membranes of fungi (Gomes *et al.,* 1998b). A similar mechanism of action is attributed to proteins containing the so called chitin binding domains (specific chitinases, hevein and hevein-like proteins and peptides from *Amaranthus caudatus* seeds) which are involved in defense mechanisms of plants against pathogens (Raikhel *et al*., 1993; Broekaert *et al*., 1992). The aim of the present work was to investigate the binding of vicilins to fungal cells and whether this binding could be reversed by different sugars like glucose, sucrose and N-acetyl-D-glucosamine.

Materials and Methods

Seeds

Cowpea (*Vigna unguiculata)* seeds of the cultivar pitiúba were supplied by the Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, Brasil where they were developed. Seeds of the cowpea line IT81D-1045, which are resistant to the bruchid beetle *Callosobruchus maculatus,* were obtained from IITA, Ibadan, Nigeria through the Centro Nacional de Pesquisa Arroz-Feijão (EMBRAPA), Goiânia, Goiás, Brasil. This line was bred at IITA from the bruchid-resistant cultivar TVu 2027.

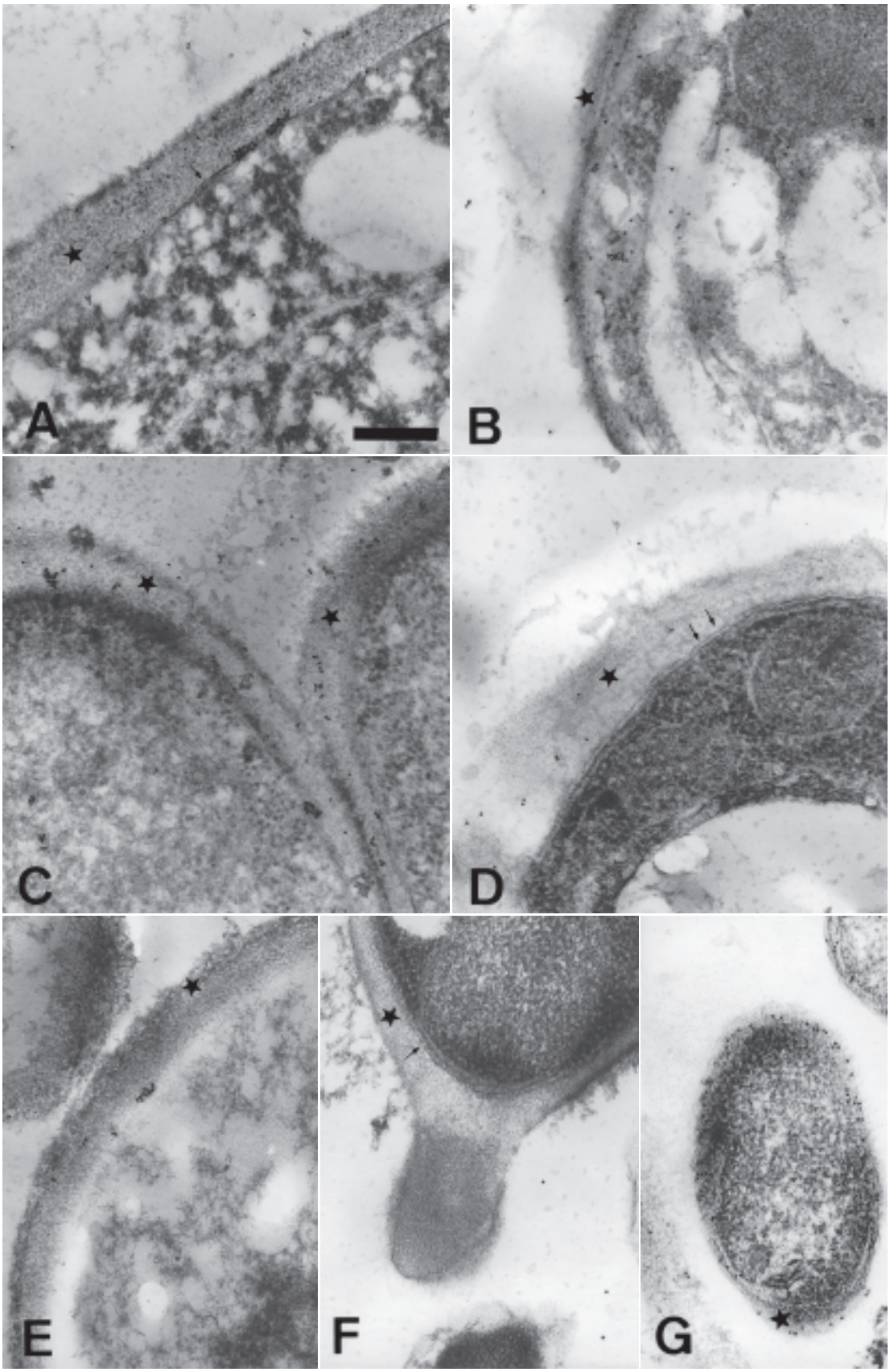
Preparation of vicilins

Vicilin type proteins from *V. unguiculata* were prepared following the protocol developed by Macedo *et al.* (1995) with some modifications. We extracted the finely ground seed meal (1:10 meal to buffer ratio) with 100 mM Tris-HCl buffer, pH 8.0, for 30 min at room temperature. After centrifugation for 30 min at 8,000 x g and 4°C, the proteins in the supernatant were fractionated by ammonium sulphate precipitation and the 70 to 90% saturation fraction was dialyzed against water and freeze dried. The vicilins from cowpea seeds were further purified by chromatography on a Sephacryl S-200 column (3 x 40 cm), equilibrated and eluted with the same buffer used for extraction. The vicilin-rich fractions were recovered and submitted to an ion-exchange chromatography step on a DEAE-Sepharose column (2 x 12 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, and eluted with a NaCl gradient (0 to 1 M) in the same buffer. Vicilin-rich fractions were recovered and submitted to chromatography on a Sephacryl S-400 column (2.5 x 60 cm) in 100 mM Tris-HCl, 0.25 M NaCl, pH 8.0. Vicilins were recovered by dialysis and freeze drying. The degree of purification of the vicilins obtained by the above procedure was ascertained during the purification process itself since only the most quantitatively significant and symmetrical protein peaks were collected. Purified vicilins showed similar patterns to those obtained previously by us (Macedo *et al*., 1995) and by others (Derbyshire *et al*., 1976; Sammour *et al*., 1984).

Fungi

Fungal isolates utilised were: *Fusarium oxysporum,* kindly supplied by CNPAF/EMBRAPA, Goiania, Goiás, Brazil, and *Saccharomyces cerevisiae* (1038) obtained from Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Brazil. The fungi were maintained on Agar Sabouraud. For the preparation of yeast cell cultures, inoculum was transferred to Petri dishes containing Agar Sabouraud and allowed to grow at 28°C for 2 days; after this period cells were transferred to sterile 0.15 M saline solution (10 ml). Yeast cells were quantified in a Neubauer chamber for further calculation of appropriate dilutions. For the preparation of conidia of *F. oxysporum*, fungal cultures were transferred to Petri dishes containing Agar Sabouraud for 12 days (Leah *et al*., 1991); after this period, sterile distilled water (10 ml) was added to the dishes and these were gently agitated for 1 min for spore liberation with the help of a Drigalski spatula. Spores were quantified in a Neubauer chamber for appropriate dilutions.

FIGURE 1. Localization of glycoconjugates using lectin-gold complexes in cells of *F. oxysporum* conidia (A, C and E) and *S. cerevisiae* (B, D and F) after lectin binding assays (gold particles. 10 nm). A and B -control fungi sections treated with WGA lectin without previous treatment with vicilins. C and D - Fungi sections treated with WGA lectin with previous treatment with vicilins. E and F- Fungi sections treated with *Helix pomatia* lectin with previous treatment with vicilins. G, Cell walls of *F. oxysporum* conidia submitted to vicilin treatments labelled by anti-vicilin serum. Cell wall (stars); cell plasma membrane (arrows); Bar represents 0.3 μm for all figures.


A
B
C
D
E
F
G

Incubation of fungi in the presence of vicilins

For the growth of fungi in the presence of vicilins, yeast cells and fungal conidia (2 x 10^4 ml^{-1} in Sabouraud broth) were incubated in 10 ml Kitasato flasks followed by the addition of cowpea (pitiúba and IT81D-1045) vicilins (0.8 mg/ml). The flasks were then maintained at room temperature (25ºC). The fungi were grown for the following 72 h. A general control without addition of protein was also utilized.

Lectin-binding assay

S. cerevisiae cells and *F. oxysporum* conidia, grown for 72 h in Sabouraud broth in the presence of vicilins, were washed with 0.1 M phosphate, pH 7.3 and submitted to fixation for 2 h at room temperature in a solution containing 0.1% glutaraldehyde, 2.0% formaldehyde in the same buffer. The materials were rinsed three times with 0.1 M phosphate buffer, pH 7.3, dehydrated in solutions of increasing concentrations of methanol (30% - 90%) and processed for LR Gold embedding. For labelling binding assay of the *Helix pomatia*, wheat germ (WGA) and *Ulex europaeus* (UEA I) lectins (Sigma Chemical Co.), ultrathin sections were treated following the protocol described by Benhemau and Quellete (1986) with some modifications. PBS/BSA (10 mM phosphate and 0.15 M NaCl pH 7.5, containing 1% bovine serum albumin) was used for all rinsing steps and for dilution of the reagents. Sections were labelled by immersing grids in drops (30 µl) of solutions in the following sequence: (1) 50 mM ammonium chloride in PBS, 30 min; (2) PBS/BSA, 30 min; (3) lectin solution (1:10) in PBS/BSA, over night, 4°C; (4) five changes of PBS/BSA, 10 min each; (5) five changes of buffer, 10 min each; (6) five changes of deionized water, 10 min each. Sections were then air-dried onto a Formvar film, stained with uranyl acetate and lead citrate and observed in a ZEISS 900 transmission electron microscope. Control sections were made by labelling of yeast cells grown for 72 h in Sabouraud broth not treated with vicilins.

Immunogold labelling of vicilins in fungi structures

For immunocytochemical analysis, fungi were first submitted to fixation for 2 h at room temperature in a solution containing 0.1% glutaraldehyde, 2.0% formaldehyde in 0.1 M phosphate buffer, pH 7.3, rinsed three times with 0.1 M phosphate buffer, pH 7.3, dehydrated in solutions of increasing concentrations of methanol (30% - 90%) and processed for LR Gold embedding. For immunogold labelling, ultrathin sections were treated following the protocol described by Gomes *et al*. (1998b).

Extraction of vicilins associated to yeast cells by different sugars

After a 72 h growth period, the yeast cells in suspension and corresponding to each treatment with vicilins were centrifuged at 3,000 x g in an Eppendorf microcentrifuge for 2 min. The supernatant was discarded and the pellet was exhaustively washed with 0.1 M Tris-HCl, pH 8.0, until no more protein could be measured by the Bradford (1976) method. Each pellet was treated with 1 ml of different sugars (N-acetyl-D-glucosamine, glucose, sucrose and glucosamine) at different concentrations (0.05, 0.1, 0.2, 0.4, 0.5 M). The final precipitates were then treated with 1 ml of 0.1 N HCl. All resulting supernatants were concentrated in a speed vac apparatus. The dried samples were redissolved in 0.1 ml of 0.1M Tris-HCl pH 8.0 and utilized for SDS-PAGE and western blotting.

Quantitative analysis of proteins associated to yeast cells and extracted by different sugars

The quantitative analysis of the proteins extracted by different sugars was carried out by the method of Bradford employing bovine serum albumin as standard (Bradford, 1976). After extraction of the pellets with 1 ml of the different sugars (N-acetyl-D-glucosamine, glucose, sucrose and glucosamine) in different concentrations (0.1, 0.5 M) and 0.1 N HCl, the resulting supernatants were collected and utilized for the Bradford assay. The reaction was performed by incubating 0.04 ml of supernatant with 1 ml of the Bradford reagent and the absorbance was measured at 595 nm.

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate electrophoresis (SDS-PAGE) was carried out according to the denaturing method of Laemmli (1970). Protein markers employed were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa). The transfer of proteins to nitrocellulose membranes was carried out as described by Towbin *et al.* (1979). Blots were developed using a chemiluminescence detection Kit (Amersham ECL reagent) accord-

ing to the manufacturer's instructions. Antisera against purified vicilins (from cowpea seeds) were prepared by immunization of white rabbits (Macedo *et al*., 1995). Purified antisera were obtained by affinity chromatography of the crude immune sera in a column of protein A covalently bound to Sepharose CL-4B.

Results and Discussion

Several carbohydrate-binding proteins with antifungal activity have been isolated from many plant species, especially from their seeds (Leah *et al*., 1991; Chrispeels and Raikhel, 1991). The present study was initiated in order to show whether vicilins (7S storage proteins), with chitin-binding properties, isolated from cowpea seeds can bind to different sugars like glucose, sucrose, N-acetylglucosamine, glucosamine and triacetyl-chitotriose. The association of proteins of the chitin-binding family is not restricted to chitin, since they also bind to various complex glycoconjugates containing N-acetylglucosamine (GlcNAc) or N-acetyl-D-neuraminic acid (NeuNAc) (Raikhel *et al*., 1993). Vicilins probably show affinity towards other glyconjugates as well. In order to reinforce this idea we utilized lectins (*Helix pomatia* lectin, wheat germ agglutinin and *Ulex europaeus* lectin) with different sugar specificities to identify N-acetylgalactosamine, N-acetylglucosamine and L-fucose sugar residues on the surface of *S. cerevisiae* cells and *F. oxysporum* conidia after treatment with vicilins. Lectins have prooved to be useful tools for identifying and locating glyconjugate components of microbiall cells (Recobet *et al.,* 1996). Through immunogold labelling performed on sections of fungi (*F. oxysporum* and *S. cerevisiae*) structures, we have previously suggested the association of vicilins with fungi cell walls and/or plasma membranes (Gomes *et al*., 1998b). Figure 1 shows the results of lectin binding assays with cells of *S. cerevisiae* and *F. oxysporum* conidia after localization of glycoconjugated reaction. N-acetylgalactosamine and N-acetylglucosamine binding lectins are able to bind to cell wall and/or cell plasma membrane of *F. oxysporum* and no qualitative differences between the samples (treated or not treated with vicilins) were seen (Fig. 1A, 1C and 1E). These results suggest that not only the chitin monomer N-acetyl glucosamine is involved in the binding of vicilins to the cell wall of fungi. Glycoconjugates containing N-acetylgalactosamine residues may also be involved in the binding. However only N-acetylglucosamine binding lectin is able to bind to cell wall and/or plasma membrane of *S. cerevisiae* (Fig. 1B, 1D and 1F). Control fungi sections treated with *Helix pomatia* lectin without previous treatment with vicilins (data not shown) showed identical labbeling to fungi sections with previous treatment with vicilins (Fig. 1E and 1F). Cell walls of *F. oxysporum* conidia submitted to vicilin treatments were labelled by anti-vicilin serum as show in figure 1G. Apparently bound vicilins do not interfere with lectins association; furthermore, no binding occurs with the L-fucose binding lectin in both fungi (Data not shown). After a 72 h growth period, control cells or cells grown in the presence of cowpea vicilins from pitiúba and IT81D-1045 seeds were washed with buffer (Tris-HCl 0.1 M, pH 8.0) and also submitted to a final washing with different sugars and subsequently by 0.1 N HCl. Proteins extracted by all treatments were analyzed by western blotting using an antiserum raised against cowpea vicilins. All vicilins showed the same pattern of sequential sub-unit elution by 0.5 M sugars (glucose, N-acetylglucosamine, glucosamine and sucrose) and 0.1 M HCl indicating that some vicilin subunits are extracted by all sugars and others are more strongly bound to yeast cells since they are extracted only after subsequent acidic treatment as was already shown by Gomes *et al*. (1998a) (Fig. 2). Western blotting of vicilins eluted by increasing

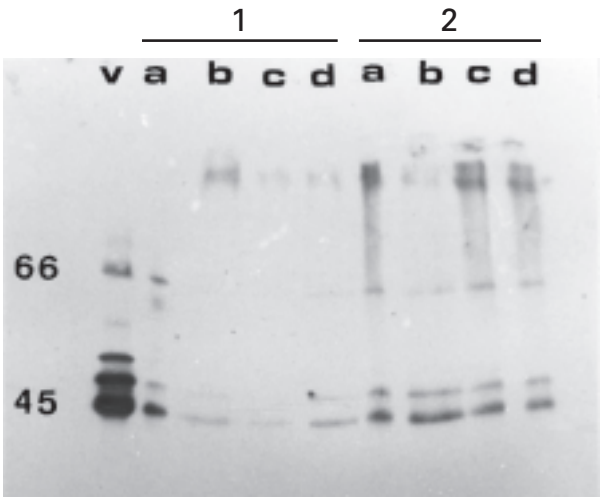


FIGURE 2. Western blotting of vicilins extracted from yeast structures with different sugars (0.5 M) (PANEL 1) and then subsequently with 0.1 N HCl (PANEL 2) using an anti-IgG IT81D-1045 vicilin. (V) IT81D-1045 vicilin standard. (a) N-acetylglucosamine, (b) glucose, (c) glucosamine, (d) sucrose. The numbers on the left refer to molecular mass standards.

sugar concentrations (Fig. 3) shows the same banding pattern of vicilins eluted by 0.5 M sugar solutions shown in figure 2. Glucose (Fig. 3A) and N-acetylglucosamine (Fig. 3B) as well as sucrose and glucosamine (data not shown) are able to extract vicilins in concentrations as low as 0.1 M. Based on previous findings that vicilins associate with chitin and chitin containing structures (Firmino *et al*., 1996; Yunes *et al*., 1998), we conclude that all sugars tested (N-acetylglucosamine, glucose, sucrose and glucosamine) are able to extract vicilin subunits when employed at appropriate concentrations.

In order to compare the ability of different sugars in extracting vicilins from yeast cells we measured the protein content of the resulting supernatants (see Methods). As seen in figure 4A, vicilins from pitiúba seeds were better eluted by 0.5 M solutions of sugars in the following elution efficiency order: N-acetylglucosamine, sucrose/glucose and glucosamine. In the case of vicilins from IT81D-1045 (Fig. 4B) a more subtle difference among sugars was observed even though the same order of elution efficiency was noted. However, a distinct behavior was shown when comparing the two cowpea vicilins. Similar measurements of extracted proteins were also made by employing 0.1 M sugar solutions with no detectable differences in elution efficiencies (data not shown).

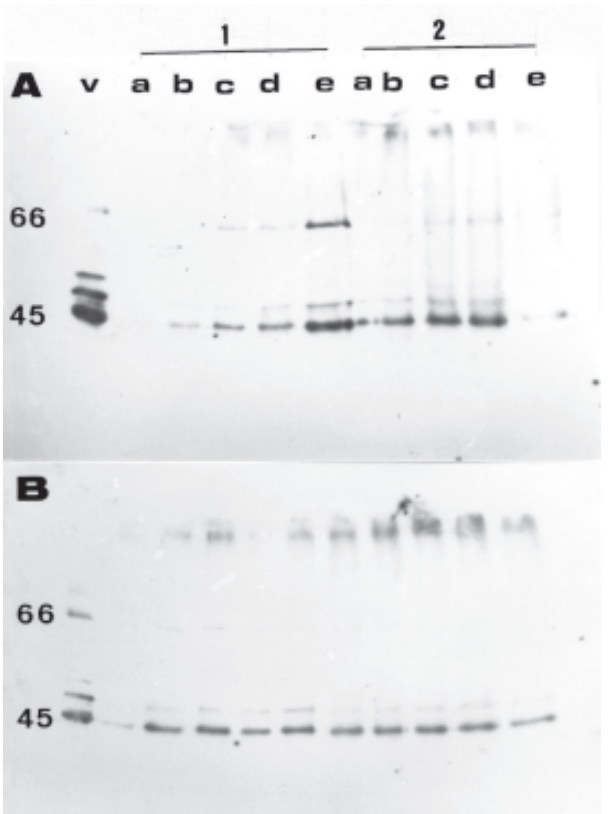


FIGURE 3. Western blotting of vicilins extracted from yeast structures with different sugar concentrations (PANEL 1) and then subsequently with 0.1 N HCl (PANEL 2) using an anti-IgG IT81D-1045 vicilin standard. (A) glucose, (B) N-acetylglucosamine. (V) IT81D-1045 vicilin, (a) 0.05 M, (b) 0.1 M , (c) 0.2 M, (d) 0.4 M, (e) 0.5 M. The numbers on the left refer to molecular mass standard.

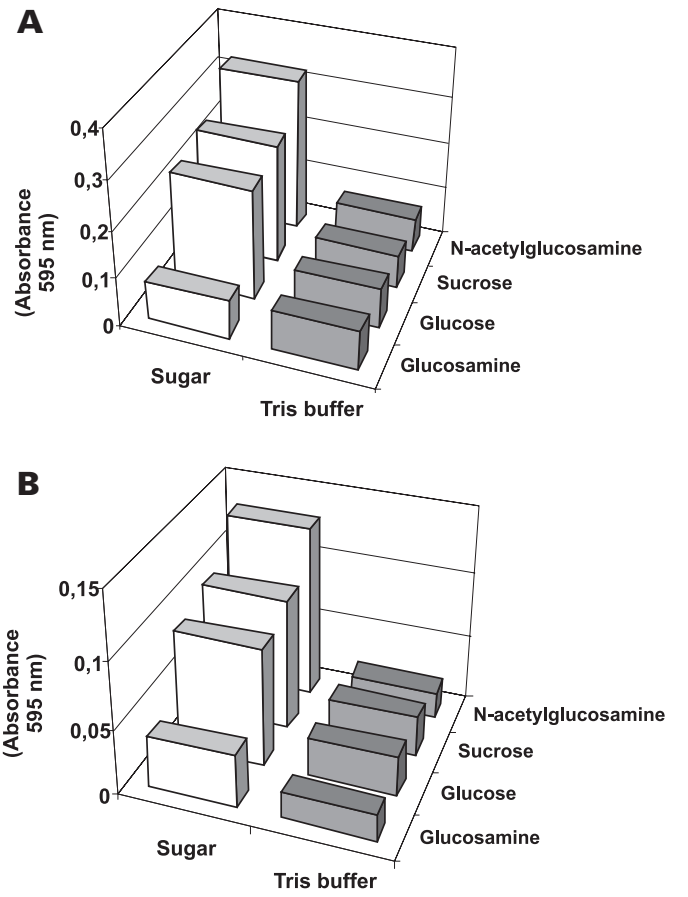


FIGURE 4. Vicilins extracted from yeast structures with different sugars (0.5 M). (A) Pitiúba vicilin, (B) IT81D-1045 vicilin. Experiments were run in triplicate and the standard errors (coefficient of variation not more than 20%) are omitted for clarity.

We are systematically investigating the association of vicilins with chitin components of fungi (Gomes *et al.,* 1997), chitin derivatives (Yunes *et al*., 1998) and chitinous structures of the midgut of insects (Firmino *et al.,* 1996). The findings reported in this paper and other results on the association of vicilins with chitin structures suggest that vicilins associate with organisms containing chitin or various others complex glycoconjugates, and that different sugars are able to reverse this association.

Acknowledgements

This work is part of the doctoral dissertation of VMG presented to the Escola Paulista de Medicina, UNIFESP and of the undergraduate monography of TLR presented to UENF. We acknowledge the financial support of the Brazilian agencies CAPES, CNPq, PRONEX and FINEP and of the Universidade Estadual do Norte Fluminense supporting body FENORTE.

References

Benhemau N, Quellete GB (1986). *Ultrastructural localization of glycoconjugates in the fungus Ascocalxy abietina, the scleroderris canker agent of conifers, using lectin-gold complexes*. J Histochem Cytochem 34: 855-867.

Bradford MM (1976). *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*. Anal Biochem 72: 248-254.

Broekaert WF, Marrien W, Terras FRG, De Bolle MFC, Proost P, Van Damme J, Dillen L, Claeys M, Ress SB, Vanderleyden J, Cammue BPA (1992). *Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin binding proteins*. Biochem 31: 4308-4314.

Casey R, Domoney C, Ellis N (1986). *Legume storage proteins*. Oxford Surv. Plant Mol Cell Biol 3: 1-95.

Chrispeels MJ, Raikhel NV (1991). *Lectins, lectin genes, and their role in plant defence*. Plant Cell 3: 1-9.

Chung RP–T, Neumann GM, Polya GM (1997). *Purification and characterization of basic proteins with in vitro antifungal activity from seeds of cotton. Gossypium hirsutum.* Plant Sci 127: 1-16.

Derbyshire E, Wright DJ, Boulter D (1976). *Legumin & vicilin, storage proteins of legume seeds*. Phytochem 15: 3-24.

Firmino F, Fernandes KVS, Sales MP, Gomes VM, Miranda MRA, Domingues SJS, Xavier-Filho J (1996). *Cowpea (Vigna unguiculata) vicilins associate with putative chitinous structures in midgut and feces of the bruchid beetles Callosobruchus maculatus and Zabrotes subfasciatus*. Braz J Med Biol Res 29: 749-756.

Gomes VM, Mosqueda M-I, Blanco-Labra A, Sales MP, Fernandes KVS, Cordeiro RA, Xavier-Filho J (1997). *Vicilin storage proteins from Vigna unguiculata (legume) seeds inhibit fungal growth*. J Agric Food Chem 45: 4110-4115.

Gomes VM, Okorokov LA, Rose TL, Fernandes KVS, Xavier-Filho J (1998a). *Legume vicilins (7S storage globulins) inhibit yeast growth and glucose stimulated acidification of the medium by yeast cells*. Biochim Biophys Acta 1379: 207-216.

Gomes VM, Da Cunha M, Miguens FC, Fernandes KVS, Rose TL, Xavier-Filho J (1998b). *Ultrastructure and immunolabelling of Vigna unguiculata vicilins (7S storage proteins) associated to fungi cells*. Plant Sci 138: 81-89.

Laemmli UK (1970). *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature 227: 680-685.

Leah R, Tommerup H, Svendsen I, Mundy J (1991). *Biochemical and molecular characterization of three barley seed proteins with antifungal properties*. J Biol Chem 266: 1564-1573.

Macedo MLR, Fernandes KVS, Sales MP, Xavier-Filho J (1995). *Purification and some properties of storage proteins (vicilins) from cowpea (Vigna unguiculata) seeds which are susceptible and resistant to the beetle Callosobruchus maculatus.* Braz J Med Biol Res 28: 183-190.

Raikhel NV, Lee HI, Broekaert WF (1993). *Structure and function of chitin-binding proteins*. Annu Rev Plant Physiol Plant Mol Biol 44: 591-615.

Recobert G, Steinberg C, Alabouvette C (1996). *Quantitative variability of sugar resudues at the surface of Fusarium oxysporum conidia as detected by lectin typing*. Lett App Microbiol 22: 233-236.

Sales MP, Gomes VM, Fernandes KVS, Xavier-Filho J (1996). *Chitin binding proteins from cowpea (Vigna unguiculata) seeds*. Braz J Med Biol Res 29: 319-326.

Sammour RH, Gatehouse JA, Gilroy J, Boulter D (1984). *The homology of the major storage protein of jack bean (Canavalia ensiformis) to pea vicilin and its separation from a-mannosidase*. Planta 161: 61-70.

Towbin H, Staehelin NT, Gordon J (1979). *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedures and some applications*. Proc Natl Acad Sci USA 176: 4350-4354.

Wang X, Bunkers GJ (2000). *Potent heterologous antifungal proteins from Cheeseweed (Malva parviflora)*. Biochem Biophys Res Commun 279: 669–673.

Yunes A, Andrade MT, Sales MP, Morais RA, Fernandes KVS, Gomes VM, Xavier-Filho J (1998). *Legume seed vicilins (7S storage proteins) interfere with the development of the cowpea weevil [Callosobruchus maculatus (F.)]*. J Sci Food Agric 76: 111-116.