The chemical bonds between mycotoxins and cell wall components of *Saccharomyces cerevisiae* have been identified

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Abstract

Several studies carried out in vivo during the last decade on animals indicate that addition of yeast cell walls to contaminated feeds alleviate the harmful effects of mycotoxins. The term of mycotoxin binder has been used to characterize the effect of yeast cell wall, but nothing was known on the chemical interactions between the binder and mycotoxins.
We showed that β-D-glucans are the yeast component responsible for the complexation of ZEN, and that the reticular organization of β-D-glucans and the distribution between β-(1,3)-D-glucans and β-(1,6)-D-glucans play a major role in the efficacy. Using ^{1}H-NMR, X-ray diffraction and UV spectral techniques, in association with equilibrium studies with bound and free toxins separated by a dialysis membrane and balanced according to various environmental conditions, we showed that weak hydrogen and van der Waals bonds are involved in the chemical complex formation between ZEN and β-D-glucans. Thus, the chemical interaction is more of "adsorption type" than "binding type". Molecular modelling was performed to validate the concept on other mycotoxins such as aflatoxin B1, deoxynivalenol and patulin. The potential energy of the formed complexes was calculated and used to estimate their stability.
Keywords: *Mycotoxins; binders, yeast cell wall; β-D-glucans; animal production.*

Introduction

Mycotoxins are secondary metabolites produced by various moulds of which *Aspergillus*, *Penicillium* and *Fusarium* are the most common genera as indicated in Table 1. Fungal contamination of plants can occur in the field on contaminated seeds or during growth, or at transport and storage in certain environmental conditions. The level of mycotoxin contamination in fields varies according to the plants and depends of climatic conditions, which is explained by large differences between years (Table 2). Detectable levels of nearly 300 potentially toxic metabolites secreted by moulds contaminate between 25 and 40% of cereals and cereal derivatives worldwide, as well as fruits, spices, coffee and many other ingredients (Figure 1). Ingestion of contaminated feeds and foods can have harmful effects in animals and humans. Some ingested mycotoxins can also be recovered

into edible animal products such as milk or animal tissues, under their native form or after being metabolized.

Despite recent crises (bovine spongiform encephalitis, listeriosis, salmonellosis, dioxin contamination, etc.) in animal production, consumers are still largely unaware of the risks associated with the natural presence of toxins or their metabolites in food and feed consumed by humans and animals (Guerre *et al.*, 2000). Since these toxins are of natural origin, consumers generally believe that they do not carry risks. In fact, mycotoxins can be hepatotoxic, haematotoxic, nephrotoxic, immunotoxic, neurotoxic, mutagenic, genotoxic, reprotoxic, teratogenic and carcinogenic. Furthermore, multicontaminations often occur and result in synergy, increasing the dangerousness of individual mycotoxins. They can cause numerous disorders and diseases, which sometimes prove to be fatal in animals (*Pfohl-Leszkowicz, 2000*) or humans *(CAST, 2003; Krishnamachari et al., 1977)*, which is a clear indication of the potential danger associated with mycotoxins. This risk has existed from the origin of organised agricultural practice. For example, the Old Testament makes reference to ergotism (*Schoental, 1984*) and indirectly attributes the decline of the Etruscan civilisation to the T-2 toxin and/or zearalenone (*Schoental, 1991*). The first modern mycotoxicosis due to aflatoxins was reported in Great Britain, in the 1960s, after the death of 100,000 turkeys due to liver necrosis and biliary hyperplasia. Fortunately, this kind of acute toxicosis, which is easy to detect, remains rare. However, chronic toxicoses due to ingestion of low amounts of contaminated feeds over long periods are more common, and also more difficult to detect.

An effective control of mycotoxin contamination of feed requires an integrated strategy "from the field to the fork". Mould growth and mycotoxin production must be controlled at field level by using adapted agricultural practices (resistant plant varieties, crop rotation, elimination of crop residues on soil, tillage, appropriate pesticides/insecticides, avoiding watering of aerial plant fraction, avoiding physical damage and over-contamination during harvest, eliminating damaged grains) and during storage (no humidity, ventilation, possible preservatives). Physical, chemical or biological decontamination procedures can be advised if mycotoxins have been detected after storage. Despite all these measures, it remains impossible to totally eliminate fungi and mycotoxins (CAST, 2003). Strategies based on sorting *(Jemmali, 1990; Pasikatan and Dowell, 2001)* and/or destruction of contaminated foods (*Karlovsky, 1999; Scott, 1998*) are not realistic, given their cost. Furthermore, dilution of contaminated feed to decrease the level of contamination until it falls below the authorised levels is forbidden in Europe since July 2003.

I- THE MYCOTOXIN BINDERS

Due to the lack of practical solutions to totally preclude mycotoxin contamination in feeds, binders have been proposed to sequestrate the toxins and prevent them from being absorbed in the animal's digestive tract, thereby limiting their effect on animals and thus their transfer to edible animal products. However, they have not yet been allowed by the European Food Safety Authority.

Inorganic materials such as bentonites and aluminosilicates, or clays in general, were early used for their adsorptive properties to reduce the toxic effect of aflatoxins *(Galvano et al., 2001; Grant and Phillips, 1998; Huwig et al., 2001; Lemke et al., 2001; Patterson and Young, 1993; Ramos and Hernandez, 1997)*, but they have limited efficacy against other mycotoxins. Furthermore, they need to be incorporated at high levels and can have side effects on some dietary nutrients, thus reducing the nutritional value of animal diets. They may also contain dioxins and heavy metals, which is a huge limit for their use as feed additive. Finally, their excretion in manure can alter the soil quality on which they are spread out. This is why several studies have been carried out on other types of binders during the last decade. Among them, organic compounds such as yeast cell walls *(Dawson et al., 2001; Devegowda et al., 1998a; Devegowda et al., 1998b; Freimund et al., 2003; Newman, 2000), Lactobacilli, Bifidobacteria* or *Propionibacteria (Ahokas et al., 1998; El Nezami, 1998; Pierides et al., 2000; Yoon and Baeck, 1999*) have been proposed as an alternative solution to bind several mycotoxins without impairing nutrient bioavailability or inducing detrimental environmental effects. All these studies have tested the efficacy of yeast cell wall through animal performance or through more specific biomarkers in blood, urine or milk of animals fed with naturally contaminated feed or feed supplemented with pure mycotoxins and supplied or not with the binder (*Devegowda et al., 1998a; Dvorska, 2003; Dvorska and Surai, 2001; Dvorska et al., 2003; Newman, 2000; Smith et al., 2001; Smith et al., 2002; Surai et al., 2002; Swamy et al., 2002a; Swamy et al., 2002b; Whitlow et al., 2000; Whitlow and Hagler Jr, 2002; Whitlow and Hagler, 1999*). However, only few studies have investigated the mechanisms underlying the *in vitro* formation of chemical complexes between toxins and yeast cell wall components *(Dawson et al., 2001; Diaz et al., 2002)*. Recent studies carried out on aluminosilicate clays, consisted in drawing the adsorption curves and modeling them to determine physical factors such as adsorption capacity, number of sites involved in the binding process, strength of the interaction between binder and mycotoxin, as well as certain thermodynamic parameters *(Grant and Phillips, 1998; Lemke et al., 2001). Freimund et al., (2003)* applied these results to organic binders and made some speculations on the nature of the complexes formed between yeast cell walls and certain mycotoxins, suggesting that possible hydrophobic interactions could occur between ZEN and yeast cell wall components. Nothing more was done to finely analyze, at the molecular level, the

chemical bonds that established between mycotoxins and yeast cell wall components during the binding process.

II- STUDY OF THE CHEMICAL BONDS BETWEEN MYCOTOXIN AND YEAST CELL WALL COMPONENTS

2.1 THE CHEMICAL ORGANIZATION OF YEAST CELL WALLS

The cell wall fraction of *Saccharomyces cerevisiae* is mainly composed of polysaccharides (80 to 90%), which makes up 15 to 30% of the dry weight of the whole cell. The mechanical resistance of the cell wall is due to an inner layer composed of ◎-D-glucan chains (50 to 60% of the wall's dry weight), which consist in a complex network of ◎-(1,3)-D-glucans with a high degree of polymerisation (2 kDa for soluble forms; up to 35 kDa for insoluble forms) (Lee, 2002), branched with linear side chains of ◎-(1,6)-D-glucans with a low degree of polymerisation (Figure 2). The 3D-structure of ◎-D-glucans is made of either random coils or more organised conformations made of several single helix chains, which are associated in a more or less densely-packed triple helix and stabilised by inter- or intra-hydrogen bounds (*Kogan, 2000*). This inner layer is firmly bound to the plasma membrane through linear chitin chains made of around ninety N-acetylglucosamine units. Chitin plays a significant role in the insolubility of the overall structure and the packing of ◎-D-glucans, which both influence the plasticity of the cell wall. Chitin molecules make up 2 to 4% of the polysaccharides in the cell wall, and up to 10% in some mutant laboratory strains. The outer layer of the yeast cell wall is made of mannoproteins, which play a major role in the exchanges with the outside medium and environment of yeasts. Mannans (over 150 units of D-mannose) are linked to ◎-D-glucans via a GPI anchor where the proteins are located at the external part of cell walls (Lipke and Ovalle, 1998). This structure is highly dynamic and can vary according to the yeast strain, since about 1200 genes drive the synthesis of these cell wall components. Culture conditions including pH, temperature, oxygenation rate, nature of the medium, and concentration or nature of the carbon source strongly modulate the quantity and structural properties of ◎-D-glucans, mannans and chitin in cell walls. Moreover, the cell cycle stage also interacts with the cell wall composition. For example, budding induces strong changes in the distribution of the structural components of the cell wall such as chitin.

2.2 OUR STRATEGY TO ANALYZE THE CHEMICAL BONDS BETWEEN MYCOTOXINS AND YEAST CELL WALL COMPONENTS AT THE MOLECULAR LEVEL

The present study was aimed (i) to identify the cell wall components responsible for the complexation of mycotoxins and (ii) to characterise the chemical bonds involved in the chemical interactions between yeast cell wall components and

mycotoxins. Most of the study was carried out on zearalenone (ZEN), which was used here as a mycotoxin model for the following reasons: it has a good balance between polar and hydrophobic groups, it harbours a benzene and a non-benzene cycle, and has an average molecular size (molecular weight = 318). The results obtained on ZEN were checked on 3 other mycotoxins: aflatoxin B1 (AFB1), deoxynivalenol (DON) and patulin (PAT).

Our research program was technically based on the following steps: (*i*) set up an *in vitro* method to quantify the capacity of binders to bind mycotoxins through some physical constants calculated from modelling of binding curves. This method has been applied to the whole cell wall isolated from various yeast strains, to each component of the fractionated cell wall, and to pure ◎-D-glucans used as models of cell wall components; (*ii*) use spectroscopic techniques (NMR, UV spectroscopy, X-ray diffraction) to assess the chemical interactions between the binder and mycotoxins at the macromolecular level; (*iii*) perform *in silico* analysis (molecular mechanics) of both the toxin and the binder molecules, separately, with the aim of constructing 3D-models of the molecular complex resulting from the two attached molecules.The most stable complexes were assessed by the lowest calculated potential energy used as an indicator of the entropy of the pair-molecule system. The nature of the chemical bonds within the complexes was also tested in the models.

Material and methods

2.3.1 PRODUCTION OF YEAST BIOMASS

Four strains of *S cerevisiae,* the wild type *wt292*, the *fks1* mutant type, the *mnn*9 mutant type and the industrial strain *sc1026* (Alltech Inc. KY, USA) were cultured in flasks containing a YPD medium (1% w/v yeast extract, 2% w/v bacteriological peptone and 2% w/v glucose) at + 30°C, shaken at 200 rpm. Cultures were stopped when the concentration reached the concentration of 2×10^7 cells/mL.

2.3.2 ISOLATION AND CHEMICAL ANALYSIS OF YEAST CELL WALLS

Cells were disrupted with glass beads and then isolated according to *Dallies et al., (1998*). Mannans and glucans were quantified by mannose and glucose analysis after 2N-H_2SO_4 hydrolysis at + 100°C for 4 h. An enzymatic method was used to determine the chitin content (*Popolo et al., 1997*).

2.3.3 ALKALI FRACTIONATION OF YEAST CELL WALL COMPONENTS

Cell walls were fractionated by alkali treatment with 1 M-NaOH and 0.5% of $NaBH_4$ for 24 h at + 37°C under agitation (*Catley, 1988; Fleet, 1991)*. Supernatant and pellet fractions were separated by centrifugation (10,000 *g* for 5 min). Supernatants

were dialyzed (1:100, v/v) on cellulose-ester membranes (MWCO: 6 to 8,000) with 0.02 M Tris/HCl buffer (pH 7.4) for 16 h at + 4°C with magnetic stirring. ◎-D-glucans were separated from mannans by liquid chromatography using a concanavalin A sepharose column and a mixture of 0.02 M Tris/HCl buffer (pH 7.4) and 0.5 M NaCl as eluant. The pellets were washed thoroughly with 1 mL of 75% ethanol/10 mM Hepes buffer (pH 7.1), then suspended in 2 mL of 0.1 M Tris/HCl buffer (pH 8.5), and finally stored at - 20°C until use.

2.3.4 GLUCOSE AND MANNOSE ANALYSES

Analyses were performed in triplicate on a Dionex Bio-LC system (Sunnyvale, CA) with a pulsed amperometric detector equipped with a gold electrode. Separation of carbohydrates was performed by liquid chromatography on a CarboPac PA1 anion-exchange column (4 × 250 mm) equipped with a guard column. Elution was performed at a flow rate of 1 mL/min at + 20-22°C with 18 mM NaOH.

2.3.5 ANALYSIS AND QUANTIFICATION OF MYCOTOXINs

Mycotoxins were analyzed by isocratic HPLC on a HP-1090 Series II HPLC (Hewlett-Packard Co.) using a UV diode array detector coupled to an HP-1046A fluorescence detector. A C18 Nucleosil Spherisorb ODS-2 column (4 × 150 mm) equipped with a guard column was used at a flow rate of 0.8 mL/min of mobile phase.

2.3.6 AN ORIGINAL *IN VITRO* TECHNIQUE TO ESTIMATE TRE THE BINDING CAPACITY (Yiannikouris *et al.*, 2003)

For each *in vitro* test, 100 µg/mL of adsorbent were placed in tubes together with either 2, 4, 6, 8, 10 or 20 µg/mL of ZEN dissolved in water and agitated at 200 rpm for 1.5 h at + 37°C. Control tubes with no adsorbent were used in parallel. Then, the suspension was centrifuged at 5,000 *g*. The amount of bound toxin was calculated for each mycotoxin supplementation by subtracting the amount of free toxin found in the supernatant of the experimental tubes from the amount found in the control tubes with no adsorbent. DataFit 7.1 software (©Oakdale) was used to plot the experimental data, set up the regression curve (curve fitting) and calculate the statistical data (*Yiannikouris et al., 2003*) (Figure 3).

2.3.7 IDENTIFICATION OF THE CHEMICAL BONDS EXISTING IN COMPLEXES FORMED BETWEEN β-D-GLUCANS AND MYCOTOXINS (*Yiannikouris et al., 2004a*).

NMR spectra of mycotoxins and β-D-glucans were realized according to the following conditions. Fifty micrograms/mL of ZEN and soluble purified ◎-D-glucans were mixed in 10 mL of a Milli-Q^{uf+} water, shaken at 640 rpm for 1.5 h at + 39°C and then cooled on a bath of 2-propanol kept at - 30°C before freeze-

drying. The powder collected after freeze-drying contained the [◎-D-glucans + ZEN] complexes, which were solubilized in 500 ◎L of DMSO-d_6 to reach a concentration of 1 mg/mL for each molecule. The solution was analyzed using an NMR Bruker Avance 400 spectrometer (300 K). The ^{1}H-NMR spectra of the [◎-D-glucans + ZEN] sample were recorded and compared with the spectra of each of the two components analyzed separately.

Wide-angle X-ray diffraction patterns were assessed on pure laminarin and curdlan used as two different β-D-glucan models (laminarin is is a soluble linear β-(1,3)-D-glucan with some branched β-(1,6)-D-glucans; curdlan is made only of β-(1,3)-D-glucans), in both semicrystalline and hydrated forms at ambient temperature using Inel X-ray equipment (XRG 3,000) operating at 40 kV and 30 mA. Diffracted intensities were monitored over a 2h exposure period at 120° curve position sensitive detector.

2.3.8 MOLECULAR MODELING OF MYCOTOXINS AND β-D-GLUCANS, SEPARATELY AND IN COMBINATION

Molecular modeling was carried out on Silicon Graphics computers running Accelrys software packages (Accelrys. Inc, CA, USA) and InsightII, Biopolymer, Analysis, Docking and Discover modules. A CFF91 force field adapted to "polysaccharide/binder" interaction studies was used under vacuum (◎ = 1).

Construction of the most highly probable conformations of ◎-(1,3)-D-glucan chains was performed as described in a previous study (*Yiannikouris et al., 2004a*). [◎◎◎◎◎◎dihedral angle values of a ◎-(1,3)- bond between two glucoses were explored through their rotation (from –180° to +180°) and their minimum energy conformations evaluated before further elongation to a 5,868 Da polymer. ◎-D-glucan structure was assessed using X-ray diffraction under several hydration levels (*Yiannikouris et al., 2004a*) in order to identify the influence on the packing of yeast cell wall components (*Nuessli et al., 2003*).

The ZEN molecule was constructed from our NMR and X-ray data and from those obtained by *Cordier et al., (1990)*. A [ZEN + ◎-D-glucans] complex was constructed by manually positioning ZEN molecules in cavities on the helix of the◎-(1,3)-D-glucan polymer. Translations plus rotations as well as up and down positioning of ZEN were achieved inside the ◎-(1,3)-D-glucan to explore all the possible spatial orientations of the interaction within a 10,000 iterations minimization procedure.

Results

3.1 CHEMICAL COMPOSITION OF YEAST CELL WALL

The cell wall fraction accounted for 24.8, 22.3, 21.4, and 13.2% of the dry weight of the total cell for *wt292*, *fks1*, *mnn9* and *sc 1026,* respectively. It was composed of various glucans, mannans and chitin contents. *Wt292* and *mnn9* strains had high

◎-D-glucan contents, respectively 45% and 75% of the total cell wall, compared to *fks1* and *sc1026* strains (~30%). As a consequence, mannan/Glucan ratios varied to a large extent between strains: 1.25, 2.09, 2.18, and 0.21 in the cell walls of *wt292*, *fks1*, *sc1026*, and *mnn9*, respectively. Both *mnn9* and *fks1* strains had high chitin contents, 9.7% and 5.8%, respectively, while *wt292* and *sc1026* had only 2%. These differences in cell wall carbohydrate and chitin content among strains were related to their binding capacity to assess the cell wall component which is chiefly involved in the binding process.

3.2 MATHEMATICAL APPROACH FOR IDENTIFICATION OF THE COMPONENT INVOLVED IN BINDING PROPERTY OF YEAST CELL WALLS

Curves representing the amount of bound toxin *versus* the amount of added toxin were plotted according Hill's model with *n* sites (HMN) (Figure 3), which gave a better representation of our experimental data ($R^2 = 0.993$) compared to isothermal models or sigmoid models, the latter being generally used for inorganic adsorbents (*Grant and Phillips, 1998*). HMN was used to set up three sub-models: (*i*) The HMN equation evaluated the ZEN adsorption efficacy of total cell wall, alkali-soluble and alkali-insoluble fractions from each strain of *S.cerevisiae* and was used to discriminate the strains; (*ii*) The HMN-2 equation took into account the amounts of total ◎-D-glucans in the cell wall and related them to the adsorption properties for ZEN (*Yiannikouris et al., 2004b*); (*iii*) The HMN-3 equation took into account the respective amounts of alkali-insoluble and alkali-soluble fractions of ◎-D-glucans and their respective roles in the adsorption process (*Yiannikouris et al., 2004c*). Calculations of $T_{\text{bound}}^{\max}$ (maximal amount of toxin bound) and K_D (association constant) were used to evaluate the affinity rates (*A*) given in percentages as: $A = T_{bound}^{MAX} / 2.K_D$.

The sigmoid shapes of the adsorption curve revealed a cooperative interaction between toxin and binder (*Yiannikouris et al., 2003*). This means that few molecules of mycotoxins must be bound before the binding capacity of yeast cell wall is optimized.

3.3 IDENTIFICATION OF YEAST CELL WALL COMPONENT(S) INVOLVED IN COMPLEX FORMATION

A strong correlation between the amount of ◎-D-glucans in cell walls and binding capacity ($R^2 = 0.889$; RSD = 0.534 µg/mL; $A \approx 30\%$) (Figure 4) was established. Cell walls of *wt292* and *mnn9* strains, which have the highest levels of ◎-D-glucans, were able to complex larger amounts of ZEN with higher affinity rates than the *fks1* and *sc1026* strains. On the contrary, the strains *mnn9* and *fks1,* with high chitin content, had a lower binding capacity than expected from their ◎-D-glucan content

(*Yiannikouris et al., 2004b*). Chitin increased the insolubility of ◎-D-glucans and decreased the flexibility of the overall structure, which consequently limited the accessibility of ZEN to the chemical sites of the ◎-D-glucans.

We showed that the alkali-insoluble fraction had a greater affinity (up to 50%) than the alkali-soluble fraction (about 16%). From the results obtained with each isolated fraction or with HMN-3 equation, taking into account both fractions (R^2 = 0.969; RSD = 0.296 µg/mL), we confirm that *mnn9* and *wt292* strains (*A* = 35.9 and 50.4% for the alkali-insoluble fractions, respectively) had the highest adsorption efficacy for ZEN. Partial elimination of chitin during alkali extraction led to a 1.5-fold improvement in the capacity of the alkali-insoluble fraction of ◎-D-glucans in the *mnn9* strain to adsorb ZEN compared to *wt292 (Yiannikouris et al., 2004c)*.

Pure ◎-D-glucans such as laminarin, curdlan, pachyman and pustulan, which are made with various "◎-(1,3)-D-glucan/◎-(1,6)-D-glucan" ratios were used to determine the individual role played by each of these ◎-D-glucans. ◎-(1,3)-D-glucans were responsible for the main complexing activity since they had greater affinity than ◎-(1,6)-D-glucans, but were less stable in extreme environmental conditions such as pH>8. As a consequence, complexes obtained from binders made of ◎-(1,3)-D-glucans highly branched with ◎-(1,6)-D-glucans are less sensitive to alkaline pH than ◎-D-glucans made of pure chains of ◎-(1,3)-D-glucans.

3.4 CHEMICAL BONDS INVOLVED IN THE INTERACTION BETWEEN ◎-D-GLUCANS AND ZEN

◎-D-glucans are defined as a complex 3D structure with alternating regions of random coil, single helices and triple helices *(Kogan, 2000; Lipke and Ovalle, 1998)*. Increasing the alkali-insoluble ◎-D-glucan fraction makes the yeast cell walls more rigid and more resistant to the external environment. In contrast, yeast cell walls with high content of alkali-soluble ◎-D-glucans are more flexible *(Fleet, 1991; Kopecka et al., 1974)*. As discussed previously, encrustation of chitin into the glucan network increases its mechanical resistance and its rigidity.

A wide-angle X-ray diffraction study carried out on purified fraction of ◎-D-glucans indicated that they are spatially organized as a cluster of four triple-helix chains of ◎-(1,3)-D-glucans (*Chuah et al., 1983*). These helical chains were 1.56 nm apart, with a fiber interval of 0.60 nm, consequently defining six ◎-D-glucopyranose units per turn of helix (Figure 5). Such organization was compared with pure ◎-D-glucans such as paramylon, curdlan and laminarin, with reticular distances of 1.36 (*Chuah et al., 1983*), 1.50 and 1.62 nm, respectively, as calculated during our X-ray diffraction measurements. The increase in reticular distance between ◎-D-glucan molecules involved an increase in the relaxation level of their triple helical conformation *(Marchessault and Deslandes, 1979; Marchessault et al.,*

1977). For example, the laminarin molecule that was used for further investigations on the interaction with ZEN has a highly relaxed triple-helix and (or) single-helix organization as indicated by its high reticular distance. These conformations with different degrees of relaxation can be found together in the same highly complex structure of *S. cerevisiae* cell wall. Molecular mechanics confirmed that the most stable conformation of the ◎-(1,3)-D-glucan single helical chain was found for glycosidic linkage of [◡◎◡] = (- 100°, + 140°) with six ◎-D-glucopyranose units per turn. Two distinct low-energy conformations of ◎-(1,6)-D-glucans were found for the dihedral angles [◡◎◡◡◡] = (- 87.7°; + 179.9°; - 72.6°) and [◡◎◡◡◡] = (- 85.5°; + 179.7°; - 177.9°), underlining the high flexibility of ◎-(1,6)-D-glucan side chains, as previously reported in the literature *(Kim et al., 2000; Manners et al., 1973; Stevens and Sathyanarayana, 1987)*. Among all the conformations including random coil or densely-packed triple helix, the single helix conformation evidenced for pure ◎-D-glucans is probably the structure giving the highest potential sites for binding interactions with mycotoxins. That is why we investigated the single helix macromolecular conformation to know the chemical mechanisms involved in the complexing properties of ◎-D-glucans toward ZEN.

Macromolecular studies carried out *in vitro* with bound and free toxins in equilibrium here and there of a dialysis membrane, indicated that chemical bonds are weak. Also, environmental conditions such as pH and nature of the solvent have been shown to alter the stability of the ◎-D-glucan structure, thus decreasing their ability to complex ZEN *(Yiannikouris et al., 2004b, 2004c; Yiannikouris et al., 2004d; Yiannikouris et al., 2003)*. These results indicate that non-covalent bonds are involved in the interactions between ◎-D-glucans and ZEN, making them more of "adsorption type" than of "binding type".

^{1}H-NMR spectra showed that the peaks of the two hydroxyl groups of ZEN phenol moiety were strongly reduced after reaction with ◎-D-glucans, which thus indicates that they are involved in chemical bonds with ◎-D-glucans. Molecular mechanics showed that the ◎-(1,3)-D-glucan chain promoted a very stable intra-helical association with ZEN (Figures 6 and 7). Two types of bonds were identified in the chemical association of ZEN and glucans: (*i*) hydrogen bonds involving hydroxyl, ketone and lactone groups of ZEN on one hand, and hydroxyl groups of glucose units in ◎-D-glucans on the other hand; (*ii*) van der Waals bonds between ◎-D-glucopyranose rings and ZEN phenol moiety. The geometrical symmetry of ZEN molecule and the open site in the helix of ◎-D-glucans favors a close association between the two molecules. As a result, the ZEN molecule is totally entrapped inside the ◎-D-glucan structure (*Yiannikouris et al., 2004a*).

3.5 APPLICATION TO OTHER MYCOTOXINS (AFLATOXIN B1, PATULIN, DEOXYNIVALENOL)

The binding efficacy with ◎-D-glucans strongly depends on both molecular structures of glucans and mycotoxins as indicated in the previous paragraph. Using the molecular modeling technique, we defined the first molecular indices of the binding ability of a single helix of ◎-D-glucans for AFB1, PAT and DON. As shown for ZEN, the geometry of AFB1 molecule allows it to easily enter the open structures of the helical structure of ◎-D-glucans. The aromatic ring, the lactone and ketone groups of AFB1 form polar or electron bonds with the glucose units in the single helix of ◎-D-glucans, which maintain the toxin linked to the glucans. The small size of PAT enables it to penetrate deep within the helix of ◎-(1,3)-D-glucans. Then, hydrogen bonds are formed between hydroxyl groups from glucose units and lactone and hydroxyl groups from PAT. Less geometrical similarities were found between the DON molecule and the single helix of ◎-D-glucans when compared to ZEN and AFB1 molecules, leading to a lower contribution of van der Waals bonds. However, DON was able to interact with ◎◎◎◎◎◎-D-glucan molecule through at least two hydroxyl bonds.

We have identified for the first time the chemical mechanism of the interaction between mycotoxins and ◎-D-glucans at the molecular level. Although this study demonstrates the difficulty in extending our conclusions obtained on ZEN to all the mycotoxins, it indicates that some chemical structures play a major role during the binding process with ◎-D-glucans. All aflatoxins, citrinin, ergotamine, T-2, paspalitrems, slaframine and verrucarin can probably bind to ◎-D-glucans due to their "aflatoxin-like", "deoxynivalenol-like" or "zearalenone-like" structures. For example, it has been reported that T-2 has an affinity of about 33%, as determined with a commercial product based on yeast cell wall according to *Devegowda et al. (1998a)* and *Devegowda et al. (1998b)*.

Conclusions

This study underlines the major role played by ◎-D-glucans from yeast cell wall and their 3D-structure in the adsorption of ZEN. The similarity of molecular geometry of the binder and the toxin, associated with the existence of electrostatic and hydrophobic interactions between glucose units in a single helix of ◎-D-glucans and mycotoxins were identified as key factors in adsorption efficiency. As far as we know, this is the first attempt to clear up the chemical mechanisms involved in the sequestration of mycotoxins by yeast cell wall components.

Also, we describe an original *in vitro* method that can objectively discriminate yeast strains for their ability to adsorb mycotoxins..

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Table 1: Molds and associated mycotoxins.

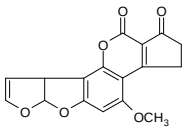
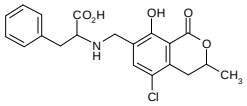
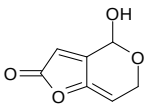
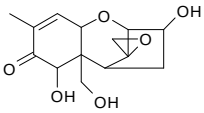
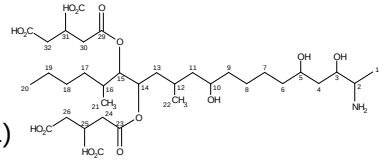
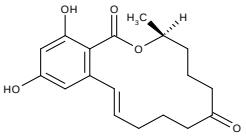
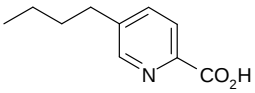
Molds	Mycotoxins
Aspergillus flavus, A. parasiticus, A. nomius,	Aflatoxins B1, B2, G1, G2 **(AFB1)** 
Penicillium verrucosum, Aspergillus clavatus, A. ochraceus	Ochratoxin A 
Penicillium expansum, P. urticae, Aspergillus clavatus, Byssochlamys nivea	Patulin 
Fusarium sporotrichioides, F. graminearum, F. culmorum, F. poae, F. roseum, F. tricinctum, F. acuminatum	Trichothecenes (DON) 
Fusarium moniliforme, F. proliferatum	Fumonisins B1, B2, B3 (FB1) 
Fusarium graminearum, F. culmorum, F. crookwellense	Zearalenone 
F. moniliforme, F. crookwellense, F. subglutinans, F. sambucinum, F. napiforme, F. heterosporum, F. oxysporum, F. solani, F. proliferatum	Fusaric acid 

Table 2 : Natural occurrence of mycotoxins from *Fusarium* spp. in cereals grown in France on two successive years (1996-1997) (from Richard-Molard 1999)

Cereal	n samples	Mycotoxin	Positive samples	Toxin content (µg/kg)	
				Mean	Range
Wheat 1996	46	DON	40	39	trace-580
		NIV	28	24	trace-60
		ZEN	12	9	trace-16
Corn 1996	17	DON	84	400	trace-2800
		NIV	78	276	trace-1300
		ZEN	95	335	trace-1750
		FB1	72	370	trace-3300
Wheat 1997	69	DON	90	87	trace-650
		NIV	92	32	trace-232
		ZEN	12	7	trace-9
Corn 1997	24	DON	76	100	trace-558
		NIV	47	69	trace-250
		ZEN	90	15	trace-40
		FB1	66	320	trace-1100

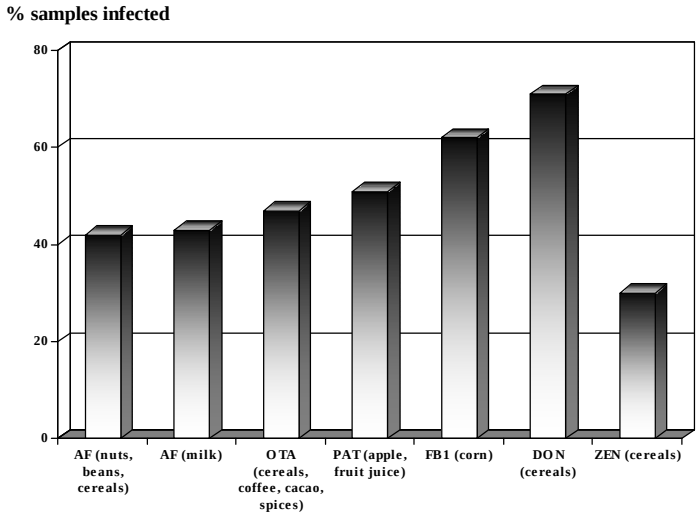


Figure 1: Recent data on the occurrence of mycotoxins in feeds (from Pittet 1998)

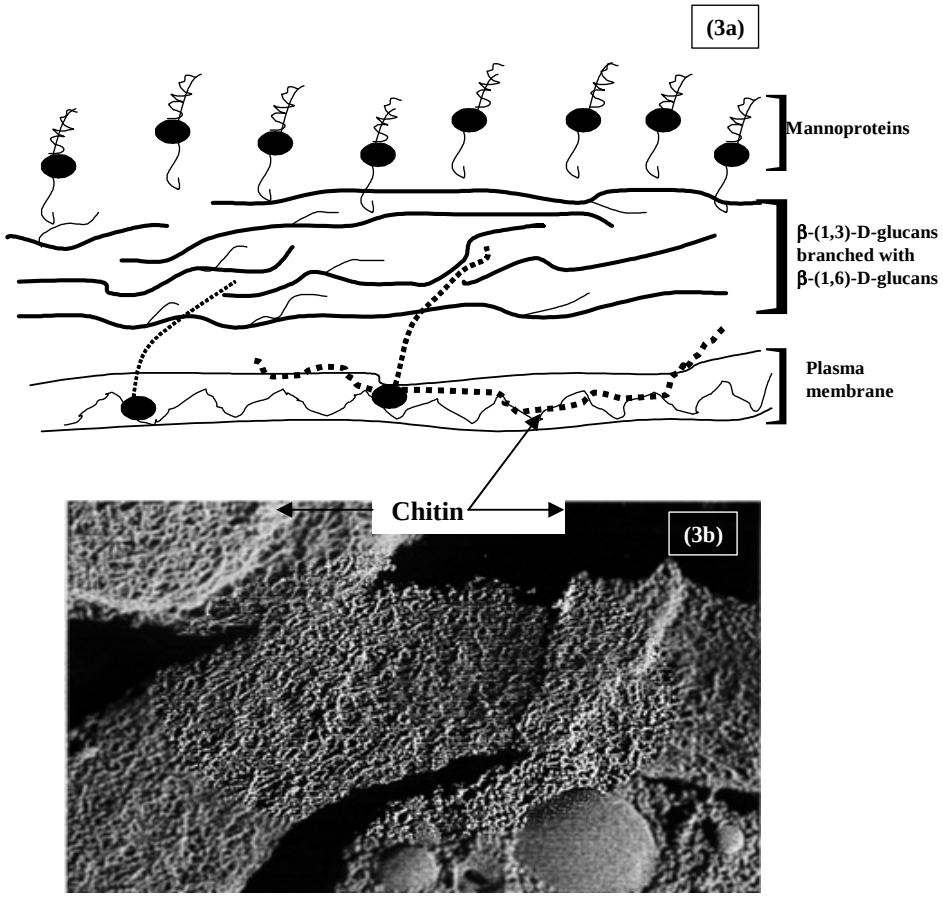
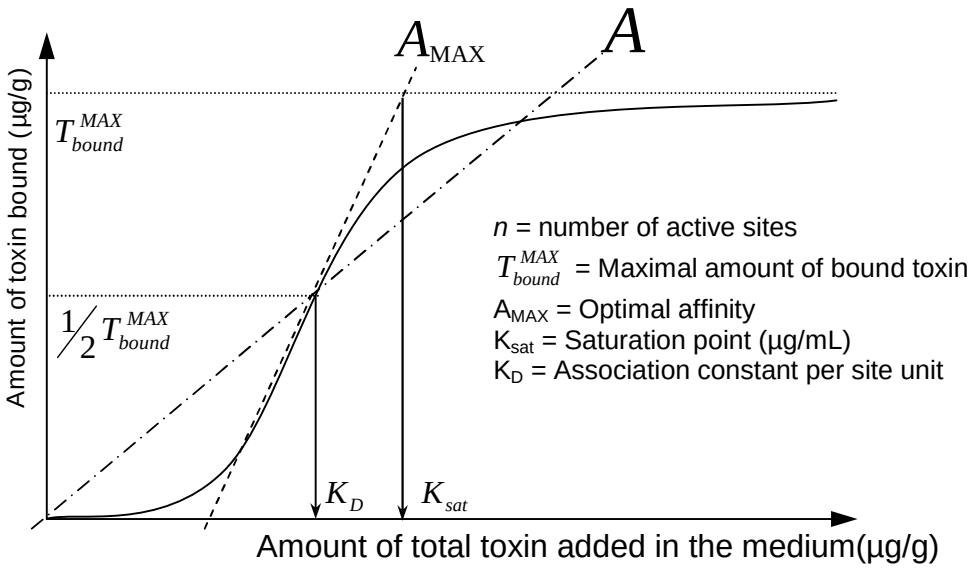


Figure 2: (a) Schema of the chemical organization of the cell wall of *Saccharomyces cerevisiae*
(b) Scanning microscopy of yeast cell wall



$$T_{bound} = (Sol\ Glucans + Insol\ Glucans) \times \frac{T_{bound}^{MAX} . (T_{initially\ added})^n}{(Sol\ Glucans + a \times Insol\ Glucans).KD + (T_{initially\ added})^n}$$

Figure 3: Modeling of an adsorption curve and physical parameters calculated from Hill's model

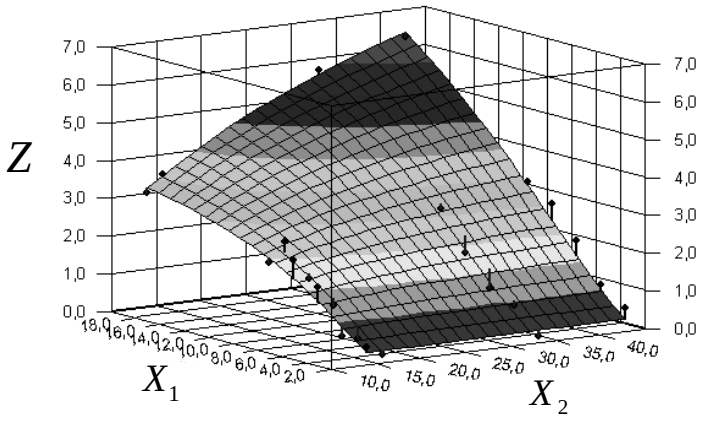


Figure 4: Influence of β-D-glucans on the adsorption efficacy of yeast cell walls
[X1: amount of added mycotoxin; X2: amount of β-D-glucans ; Z: amount of adsorbed mycotoxin]

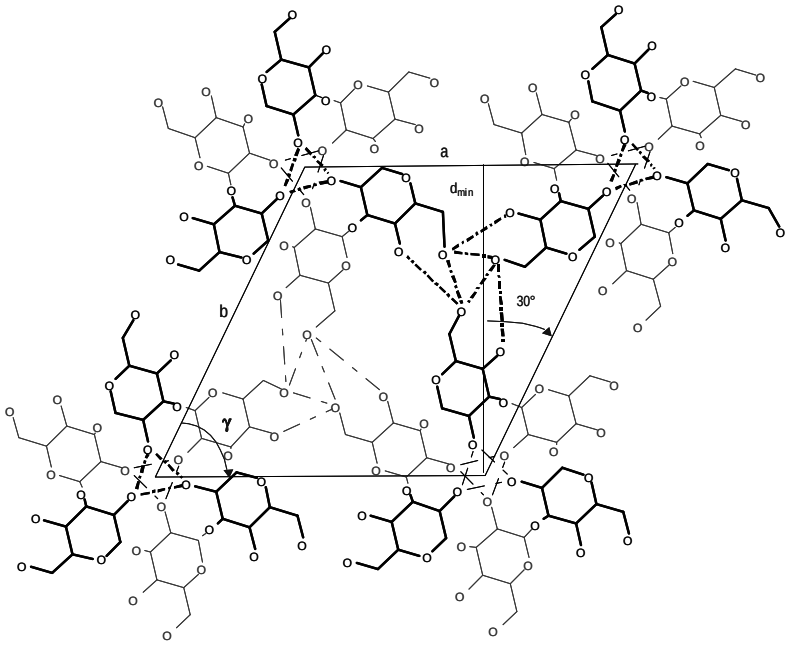


Figure 5: Diagram of the projection following "a" and "b" axes of the hexagonal crystal made of 4 x [3 dimers of β-D-glucopyranose organized in triples helices] (Chuah et al., 1983; Pelosi, 2002). Glucopyranose residues in bold are forward to the plane "a-b" and the residues in grey are backward to the plane "a-b". Stabilization of the helix involved intra and inter-helical hydrogen bonds. Size of the diagram: d_{min} = 1.36 nm ; a = b = 1.56 nm; fiber period c = 0.60 nm; γ = 120°.

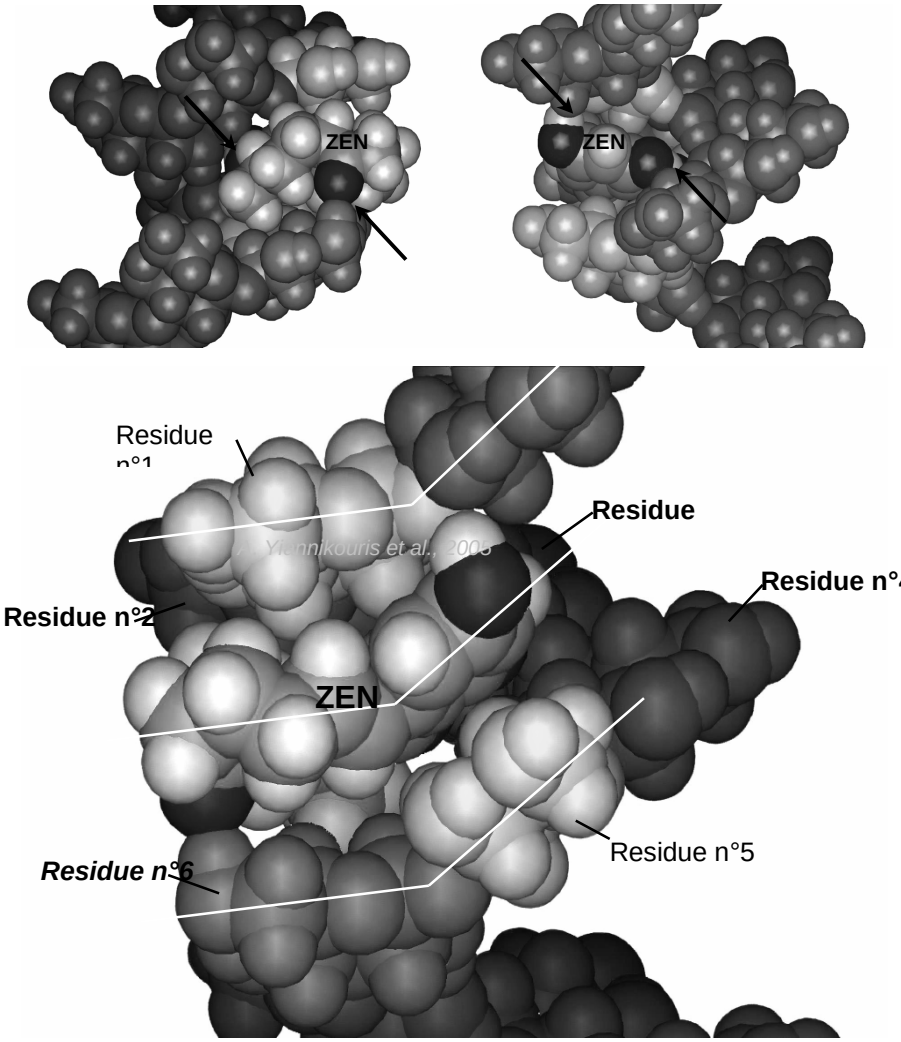


Figure 6: Computer-generated views with the Insight II program of the energy-minimized structure of the docking of the most favorable conformation of ZEN (B) into the single-helix of ◎-(1,3)-D-glucan chain. Arrows indicate hydrogen bonds involved in the interaction. Lines highlights the steric complementarity between ZEN and ◎-(1,3)-D-glucan geometry.

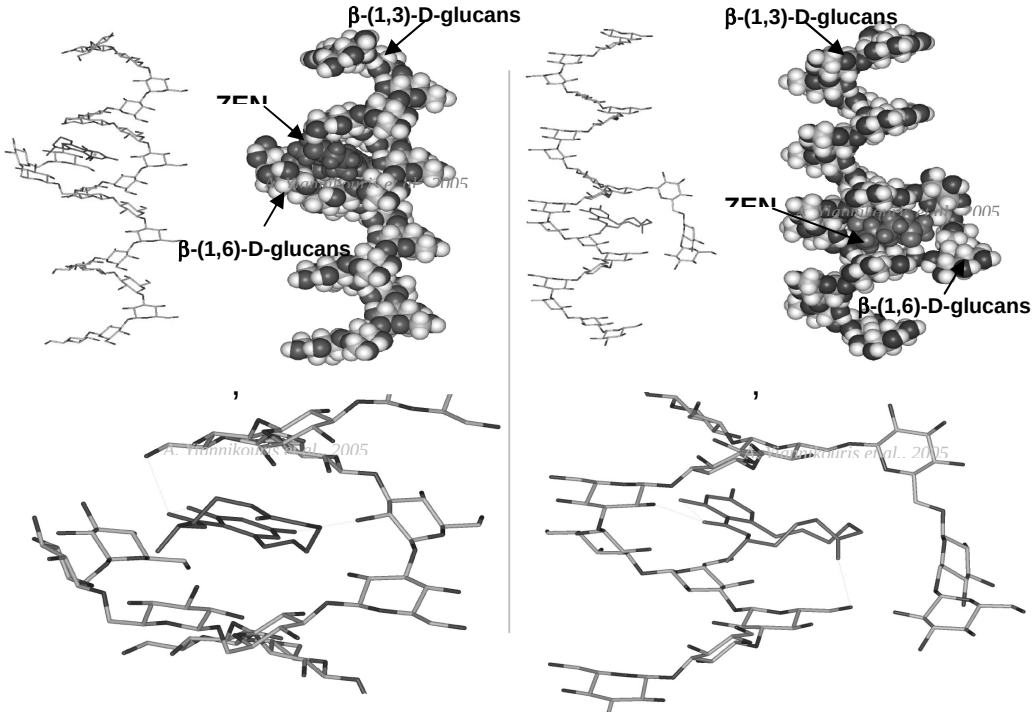


Figure 7: Computer-generated views (Insight II program) of the energy-minimized structure of the docking of the most favorable conformation of ZEN into the single-helix of ◎-(1,3)-D-glucan chain branched with ◎-(1,6)-D-glucan side chain, in two conformations (' and ').