



A novel technique to evaluate interactions between *Saccharomyces cerevisiae* cell wall and mycotoxins: application to zearalenone

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Abstract

Three models based on sigmoidal plotting were tested for their ability to describe zearalenone adsorption on *Saccharomyces cerevisiae* cell walls *in vitro*. All three models closely fitted the experimental data, but Hill's equation gave the most accurate parameters, and provided information on the physical and chemical mechanisms involved in the adsorption of mycotoxin on yeast cell walls.

Introduction

Mycotoxins are acutely toxic, carcinogenic, mutagenic, teratogenic and estrogenic secondary metabolites secreted by moulds, mostly of the genera *Aspergillus*, *Penicillium* and *Fusarium*. Food contamination occurs directly on grains, forages and fruits and on their by-products formed during production, processing, transport and storage. Food safety is also concerned by the carry-over of mycotoxins and their metabolites in animal tissues, milk and eggs after intake of contaminated feed, via the bioactivation and detoxification pathways (Galtier 1998, 1999). Owing to the marked carcinogenic properties of some mycotoxins, authorities in many countries are encouraging research into mycotoxins to set tolerance limits, develop prevention programmes and draw up regulatory guidelines for the control of mycotoxins in food and feeds (Boutrif & Canet 1998).

One of the most effective methods for controlling mycotoxin hazards in animal husbandry is based

on the use of specific materials that adsorb mycotoxins, thus limiting their bioavailability in the body. The binding of inorganic adsorbents such as hydrated sodium calcium aluminosilicates to mycotoxins in aqueous media has been thoroughly described (Grant & Phillips 1998). These models are mainly based on the Langmuir and derived equations, which allow the determination of a capacity (maximal amount bound) and an affinity parameter (K_D). This approach has also been used to determine the heterogeneity of interactions between binder and toxins and some thermodynamic data for the adsorption (Grant & Phillips 1998).

The aim of the present work was to adapt a reliable method to explore, using an *in vitro* test, the nature and efficacy of mycotoxin adsorption on a natural organic adsorbent made of isolated *Saccharomyces cerevisiae* cell wall fraction. A ligand-toxin interaction model was developed and proposed to plot experimental data on the amount of mycotoxin adsorbed

against increasing mycotoxin concentrations, and to compare our method with models generally found in the literature. This approach was used quantitatively and qualitatively, respectively to reduce the experimental data needed and to increase the accuracy of adsorption capacity and affinity determination through better computation. We expected the model to provide mechanistic information on the physical and chemical interaction between zearalenone (ZEN), the toxin used in the modelling trials, and yeast cell walls.

Materials and methods

Strains

Saccharomyces cerevisiae strain 1026 (Alltech Inc., Catnip Hill Pike, Nicholasville, KY) was grown in shake flasks at 30 °C and 200 rpm in YPD medium [1% (w/v) yeast extract, 2% (w/v) bacteriological peptone and 2% (w/v) glucose]. Yeast cultures were collected at 2×10^7 cells ml⁻¹ for cell wall extraction.

Isolation of cell walls

Cell walls were disrupted with glass beads ('micro method') and then isolated as described by Dallies *et al.* (1998).

Mycotoxin quantification

The stock solution of 8.72 mM zearalenone (ZEN, Sigma) in 100% acetonitrile was stored at -20 °C. ZEN was analysed by HPLC (Hewlett-Packard HP-1090 series II) using a UV diode array detector coupled with an HP-1046A fluorescence detector. A C18 Nucleosil column (150 mm × 4 mm internal diam) set at 50 °C and a mobile phase of acetonitrile at 0.8 ml min⁻¹ were used. Twenty μ l of sample were injected by means of a 6-port sample valve. The UV detector was set at 280 nm and the fluorescence detector at 280 nm (excitation) and 460 nm (emission) (Lauren & Agnew 1991).

In vitro binding capacity test

Yeast cell wall, 100 μ g per ml water, was placed in each test tube in the presence of 2, 4, 6, 8, 10, 20 μ g ZEN per ml. The tubes were agitated on a rotary shaker at 37 °C at 200 rpm for 1.5 h, and then centrifuged at 5000 g. Free toxins were assayed in the supernatants. The amount of bound toxin was

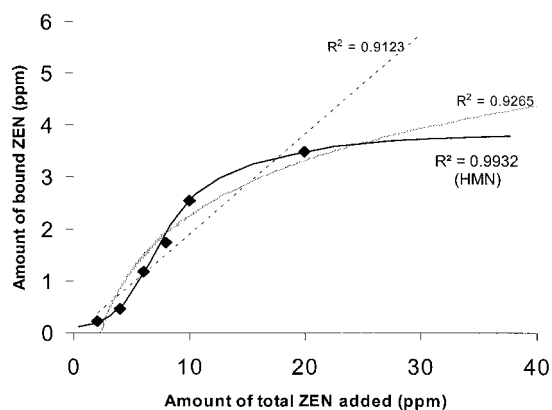


Fig. 1. Adsorption properties of ZEN on 100 μ g ml⁻¹ of *S. cerevisiae*; the experiment was carried out at 37 °C. Linear (---), logarithmic (···) and sigmoidal (—) regression plots for experimental data are shown with their correlation coefficient (R^2).

calculated by subtracting the amount of toxin in experimental tubes from the amount found in control tubes.

Data calculation and curve fitting

DataFit 7.1 (Oakdale Engineering) was used to plot experimental data to perform regression (curve fitting) and statistical analysis of the binding capacity test results.

Results and discussion

The *in vitro* tests and curve modelling were carried out with ZEN, which was used as a mycotoxin model because of its high affinity for yeast cell wall, low toxicity compared with aflatoxins for laboratory manipulation and its spectroscopic properties, allowing easier HPLC quantification.

Experimental data for the amount of toxin adsorbed (T_{bound}) against the amount of total toxin added to the medium (T_{total}) did not fit an isothermal curve, which is proposed in the literature for interaction of toxin with inorganic material (Grant & Phillips 1998). Plotting the function [$T_{\text{bound}} = f(T_{\text{total}})$] with linear and logarithmic models resulted in lower correlation coefficients than with a sigmoidal model (Figure 1). Unlike the other models, this model was not suited to linear plotting owing to saturation of the curve at very high mycotoxin concentrations. Trials were carried out using classical sigmoidal equations with and without intercepting 0 (the origin; SM0 and SM, respectively), and a biological model already applied to

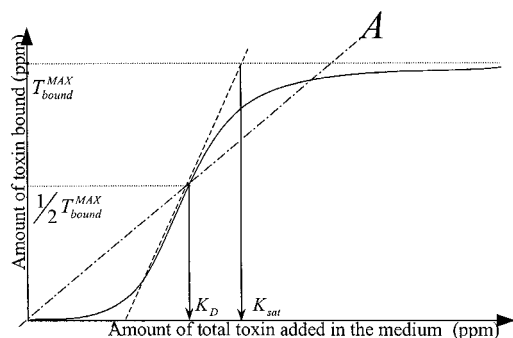


Fig. 2. Theoretical representation of the adsorption of a mycotoxin to a binder with HMN (fixed concentration of binder and fixed temperature): T = free or total amount of toxin; K_{sat} = saturation point (ppm); A = free or total affinity (%).

interactions between molecules. In this study, Hill's model (HMN), which is generally applied to identify enzymatic reactions between active sites on different sub-units of regulatory enzymes with a substrate, was applied to interactions between mycotoxins and cell wall components (Table 1a). Hill's equation yielded sigmoidal plots of fractional saturation level (Y) (Table 1a). This equation can be derived assuming that the binder has n binding sites for the toxin T and that the binding process of T is co-operative. Simulated models were built from this theoretical model. For its application to enzymatic reactions, interactions were expressed according to the relation [$T_{bound} = f(T_{free})$] and as is usual for isothermal representation of adsorption as [$T_{bound} = f(T_{total})$]. Equations and graphical application are shown in Table 1b and Figure 2. SM and SM0 were defined by parameters a , b , and c , which have no biological meaning.

HMN not only improved the fit of our model to our experimental data, but also provided some hints on the biological interpretation of the adsorption process. An association coefficient for the entire molecule (K_D) and per site (K_D), maximal amount of toxin bound (T_{bound}^{max}), saturation point (K_{sat}), and number of sites (n) were calculated according to the definitions given in Table 1b and Figure 2. The affinities (A) relative to free or total toxin (Table 2) were evaluated and compared with isothermal models which gave a rate of adsorption (Grant & Phillips 1998).

Figure 3 shows plots of experimental data obtained for the three models. Expression of [$T_{bound} = f(T_{free})$] and [$T_{bound} = f(T_{total})$] accounted for the adsorption capacities of ZEN on yeast cell walls. The different models showed correlation coefficients ranging from 0.99 to 0.997 (Table 3) with no signif-

icant differences between them. [$T_{bound} = f(T_{total})$] may be preferable to the first [$T_{bound} = f(T_{free})$] because of accuracy in the determination of the adsorption parameters. T_{total} is an accurate parameter with very small errors resulting from the quantification of control tubes, whereas T_{free} and T_{bound} characterise adsorption between toxin and binder added together to the media, respectively measured by HPLC and calculated by subtracting T_{free} from T_{total} . Thus, T_{free} and T_{bound} have higher standard errors in their determination than T_{total} . In the expression [$T_{bound} = f(T_{total})$], errors came only from (T_{bound}) and correlation coefficients were more accurate (0.993 and 0.997) than from [$T_{bound} = f(T_{free})$], which gave a range of 0.99 to 0.993. Accordingly, the error in a , b and c was lower with [$T_{bound} = f(T_{total})$] than with [$T_{bound} = f(T_{free})$] (25 to 28% against 38 to 44%). [$T_{bound} = f(T_{total})$] also favoured T_{bound}^{max} determination with smaller errors for HMN, SM and SM0 models and thus higher accuracy for K_{sat} , K_D and A determination.

No differences were noted between models for T_{bound}^{max} determination, but HMN exhibited a smaller standard deviation (5.8%) than sigmoidal models (errors between 50 to 60%). Therefore, accuracy will be lower with SM and SM0 than with HMN equations. Differences between SM and SM0 were also found with, surprisingly, lower accuracy with SM0 (Table 3). The HMN model enabled us to make some inferences about the number n of binding sites per glucose unit, which was 3.44 ± 0.56 and 2.75 ± 0.39 for [$T_{bound} = f(T_{free})$] and [$T_{bound} = f(T_{total})$] respectively. In several investigations on active ion channel modulation or protein interactions (Hackos & Korenbrot 1997, MacRae *et al.* 2000), n was considered more as an indication of the occurrence of a co-operative interaction than as a number of active sites.

Our mode of data representation led us to the conclusion that HMN model is probably the best model for the interaction of ZEN with yeast cell walls. Also, the model provides clues for the biological interpretation of this interaction that are difficult to obtain with the SM and SM0 models. Few results are available on the assessment of mycotoxin adsorption by *S. cerevisiae* cell wall components. Only the commercial Mycosorb product has been tested (Dawson *et al.* 2001) where K_D or T_{bound}^{max} values were expressed using an isothermal model.

In the graphical representation of adsorption (Figure 4), estimates for K_{sat} and K_{bound}^{max} were taken as the points on the x - and y -axes, respectively, of the break displayed in the curve obtained by increasing

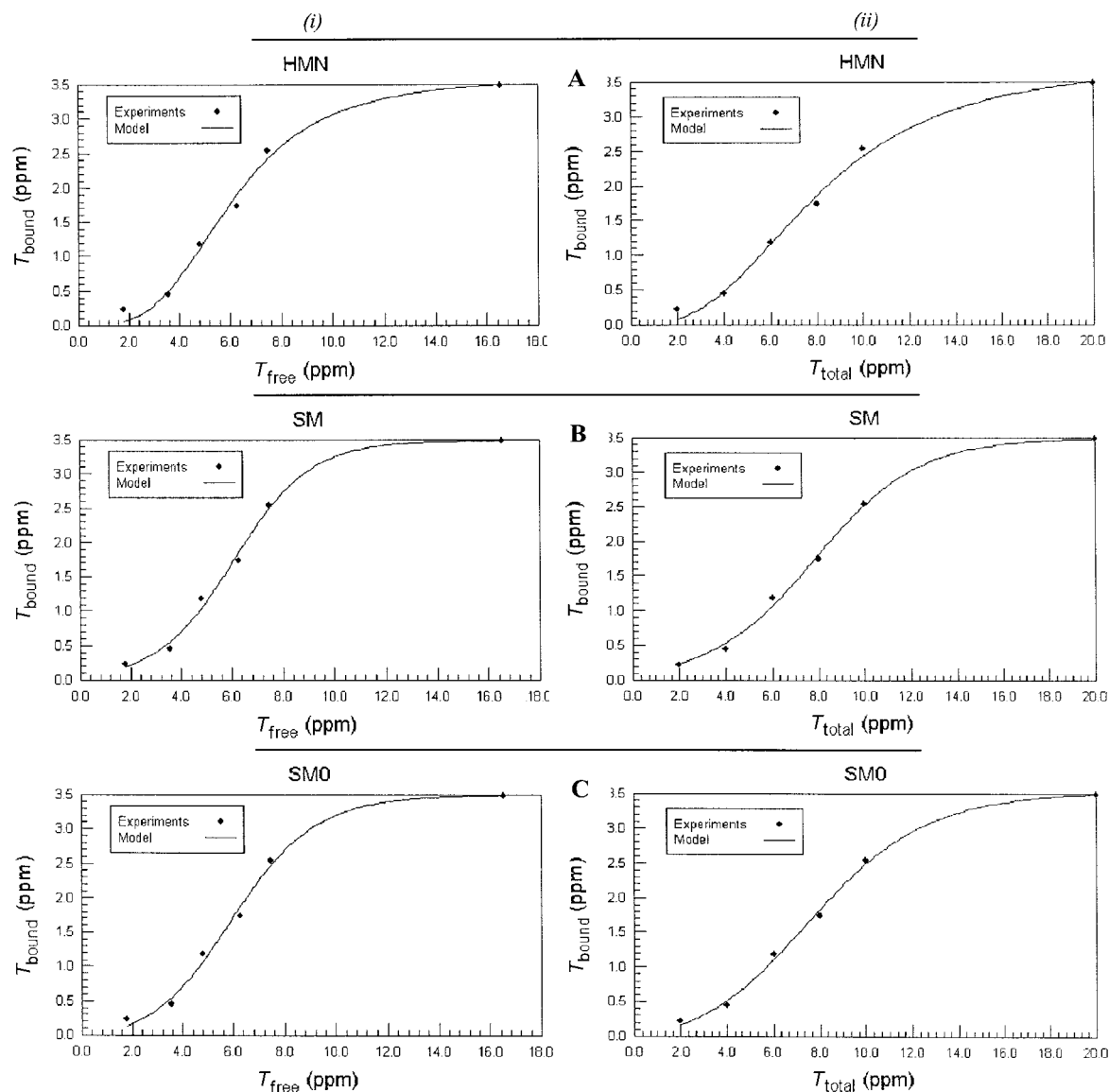


Fig. 3. Calculation of binding capacity at 37 °C of ZEN to 100 $\mu\text{g ml}^{-1}$ of yeast cell walls. Calculation was performed according to $[T_{\text{bound}} = f(T_{\text{free}})]$ (i) or to $[T_{\text{bound}} = f(T_{\text{total}})]$ (ii). Values are expressed in $\mu\text{g ml}^{-1}$ and the data were fitted by the mathematical model described in Tables 2 and 3. A. = HMN; B. = SM; C. = SMO.

Table 1a. Hill's theoretical model with n sites accounting for biological interactions^a.

Theoretical biological basis:

$$\text{Hill's model with } n \text{ sites} \quad Y = \frac{[B]^n}{KD + [B]^n} \quad KD = \sqrt[n]{KD}$$

^a Y = fractional saturation (concentration of sites on the binder that are actually occupied, divided by the total concentration of such sites); B = free binder or substrate; KD = association constant; n = number of binding sites; K_D = association constant per site.

Table 1b. Models used to fit plots to evaluate ZEN binding to yeast cell walls of *S. cerevisiae* according to Hill's equation^b.

Model expressions:	$T_{\text{bound}} = f(T_{\text{free}})$	$T_{\text{bound}} = f(T_{\text{total}})$
Sigmoid model intercepting origin (SM0)	$T_{\text{bound}} = \frac{a(T_{\text{free}})}{b(T_{\text{free}}) + \exp^{-cT_{\text{free}}}}$	$T_{\text{bound}} = \frac{a(T_{\text{total}})}{b(T_{\text{total}}) + \exp^{-cT_{\text{total}}}}$
Sigmoid model with free origin (SM)	$T_{\text{bound}} = \frac{a}{b + \exp^{-cT_{\text{free}}}}$	$T_{\text{bound}} = \frac{a}{b + \exp^{-cT_{\text{total}}}}$
Hill's model with n sites (HMN)	$T_{\text{bound}} = \frac{T_{\text{bound}}^{\text{max}}(T_{\text{free}})^n}{K D_{\text{free}} + (T_{\text{free}})^n}$	$T_{\text{bound}} = \frac{T_{\text{bound}}^{\text{max}}(T_{\text{total}})^n}{K D_{\text{total}} + (T_{\text{total}})^n}$

^b T_{bound} = amount of bound toxin; T_{total} = amount of total toxin added in the medium; T_{free} = amount of free toxin in the medium; $T_{\text{bound}}^{\text{max}}$ = maximal amount of bound toxin; $K D_{\text{free}}$ = association constant compared with free toxin; $K D_{\text{total}}$ = association constant compared with total toxin added; a, b, c = model parameters for sigmoid models.

Table 2. Affinity formula for [$T_{\text{bound}} = f(T_{\text{free}})$] and [$T_{\text{bound}} = f(T_{\text{total}})$] expressions used according to $T_{\text{bound}}^{\text{max}}$ and $K D$ evaluated with HMN^a.

Model expressions:	$T_{\text{bound}} = f(T_{\text{free}})$	$T_{\text{bound}} = f(T_{\text{total}})$
Affinity rate formula	$A_{\text{free}} = \frac{T_{\text{bound}}^{\text{max}}}{2K D_{\text{free}}}$	$A_{\text{total}} = \frac{T_{\text{bound}}^{\text{max}}}{2K D_{\text{total}}}$
Correspondence between total and free association constant	$K D_{\text{free}} = K D_{\text{total}} - 1/2T_{\text{bound}}^{\text{max}}$	

^a A_{total} = total affinity; A_{free} = free affinity; $K D_{\text{free}}$ = association constant per site relative to free toxin; $K D_{\text{total}}$ = association constant per site relative to total toxin added.

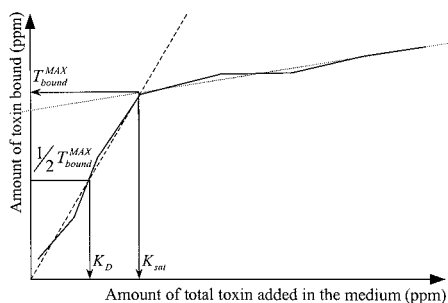


Fig. 4. Plot of isothermal adsorption of substrate to binder in water for estimation of $T_{\text{bound}}^{\text{max}}$, K_D and K_{sat} (ppm) from experimental data.

the amount of toxin added. The affinity constant (K_D) was the value on the x -axis of $1/2 T_{\text{bound}}^{\text{max}}$ on the y -axis. Using this mode of calculation, Mycosorb, which is made of processed cell walls of *S. cerevisiae* 1026, was shown to have an adsorption coefficient on ZEN of 66.7% (Dawson *et al.* 2001). In contrast, using pure cell walls obtained from *S. cerevisiae* 1026, the

affinity was about 23.4% by HMN. This discrepancy could be accounted for by the nature of the binder, and by the use of the HMN approach. Affinity, calculated from the isothermal model, expressed the maximal adsorption capacities of the adsorbent but had poor significance for the biological phenomenon. Also, this model tended to over-estimate the adsorption efficacy since the value of this parameter was calculated from the highest slope of the curve.

Another approach was applied without isothermal or sigmoidal models (Newman 2000), but by computing the affinity rate for each concentration of ZEN. It showed that the affinity of Mycosorb for ZEN ranged from 22 to 40% when ZEN was added from 0.25 $\mu\text{g ml}^{-1}$ to 2 $\mu\text{g ml}^{-1}$ respectively (Newman 2000). This method gave values closer to those from the HMN model than those obtained with isothermal curves. Thus HMN seemed to be a more reliable model of the adsorption of ZEN on yeast cell walls.

Values of K_{sat} , $T_{\text{bound}}^{\text{max}}$ and K_D obtained using HMN representation could be used together with the

Table 3. Regression coefficients calculated for HMN, SM and SM0 models and model parameters evaluation for $T_{\text{bound}} = f(T_{\text{free}})$ and $T_{\text{bound}} = f(T_{\text{total}})$ for $100 \mu\text{g ml}^{-1}$ of *S. cerevisiae* cell walls per sample placed at $37^\circ\text{C}^{\text{a}}$.

Model	HMN		SM			SM0	
$T_{\text{bound}} = f(T_{\text{free}})$							
Parameters	Values	Errors (%)	Parameters	Values	Errors (%)	Values	Errors (%)
n	3.44	16.2	a ($\mu\text{g ml}^{-1}$)	0.063	38.1	0.034	43.6
–		–	b	0.018	38.9	0.01	42.8
–		–	c ($\text{ml } \mu\text{g}^{-1}$)	0.662	10.1	0.469	16
$T_{\text{bound}}^{\text{max}}$ (ppm)	3.61	5.8	$T_{\text{bound}}^{\text{max}}$ (ppm)	3.5	54.3	3.5	60
K_{sat} (ppm)	9.6		K_{sat} (ppm)	12.1		7.3	
K_{Dfree} (ppm)	6.2		K_{Dfree} (ppm)	6.1		5.0	
A_{free} (%)	30.6		A_{free} (%)	28.8		47.8	
R^2	0.99		R^2	0.994		0.993	
$T_{\text{bound}} = f(T_{\text{total}})$							
Parameters	Values	Errors (%)	Parameters	Values	Errors (%)	Values	Errors (%)
n	2.75	14.3	a ($\mu\text{g ml}^{-1}$)	0.105	25.6	0.014	27.6
–		–	b	0.03	25.1	0.447	26.7
–		–	c ($\text{ml } \mu\text{g}^{-1}$)	0.048	7.8	0.288	13
$T_{\text{bound}}^{\text{max}}$ (ppm)	3.79	6.4	$T_{\text{bound}}^{\text{max}}$ (ppm)	3.5	37.1	3.5	40
K_{sat} (ppm)	14		K_{sat} (ppm)	16.8		11.3	
K_{Dfree} (ppm)	8.1		K_{Dfree} (ppm)	7.8		7.7	
A_{free} (%)	23.4		A_{free} (%)	20.8		31.2	
R^2	0.993		R^2	0.997		0.997	

^aErrors are expressed in percent of the values.

affinity (A) to define and describe the entire phenomenon. HMN improved the reliability of these values (Figure 1) and showed that interaction between the mycotoxin and the binder is co-operative. This supports the hypothesis that the three-dimensional conformation mobility of yeast cell wall is probably important in the adsorption event.

Several isothermal adsorption studies have been carried out on HSCAS clay or montmorillonite silicate and AFB1, but only few have been conducted on organic adsorbents (Grant & Phillips 1998, Ramos & Hernandez 1996). Unlike yeast cell walls, the inorganic binders display not co-operative but isothermal behaviour ($R^2 = 0.997$, Grant & Phillips 1998), which can be ascribed to their rigid structure. This work, previously carried out on clays, was hardly comparable with HMN because of the different nature of the parameters calculated. Isothermal curves mainly based on the Langmuir and derived equations, allowed

determination of the capacity, the number of sites, the strength and thermodynamic properties of the adsorption phenomena, whereas HMN provides $T_{\text{bound}}^{\text{max}}$, K_{sat} , K_{D} and n values expressing the co-operativity and the number of binding sites. By fitting the biological interaction between organic material and ZEN more closely, this latter representation favours the biological interpretation that cannot be easily obtained using isothermal modelling.

Owing to large differences in molecular weight (100 to 1000 g mol^{-1}), stereochemistry, and hydrophilic or hydrophobic properties, mycotoxins may have different relationships with yeast cell wall and hence may exhibit various curve shapes that have to be investigated. Further work must therefore be done using HMN modelling to evaluate adsorption of mycotoxins other than ZEN, to find an explanation from a mechanistic viewpoint for mycotoxin adsorption on the yeast cell wall.

Conclusions

HMN modelling appears to be more reliable than the isothermal approach for studies of the adsorption of ZEN on *S. cerevisiae* cell walls. This modelling method, used for the first time on the binder-toxin interaction, provides better quantitative characterisation of the adsorption. This mode of data representation is more informative of the physical and chemical mechanisms involved in the linkage between binder and toxins. Thus HMN should prove a useful tool for comparing the interaction of molecules or structures implicated in this process, and should help to standardise the calculation of adsorption parameters.

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References

- Boutrif E, Canet C (1998) Mycotoxin prevention and control: FAO programmes. *Rev. Med. Vet.* **149**: 681–694.
- Dallies N, François, J Paquet V (1998) A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of *Saccharomyces cerevisiae*. *Yeast* **14**: 1297–1306.
- Dawson KA, Evans J, Kudupoje M (2001) Understanding the adsorption characteristics of yeast cell wall preparations associated with mycotoxin binding. In: Lyons TP, Jacques KA, eds. *Science and Technology in the Feed Industry*. Nottingham, UK: Nottingham University Press, pp. 169–181.
- Galtier P (1998) Biological fate of mycotoxins in animals. *Rev. Med. Vet.* **149**: 549–554.
- Galtier P (1999) Biotransformation and fate of mycotoxins. *J. Toxicol. Toxin. Rev.* **18**: 295–312.
- Grant PG, Phillips TD (1998) Isothermal adsorption of aflatoxin B1 on HSCAS clay. *J. Agric. Food Chem.* **46**: 599–605.
- Hackos DH, Korenbrot JI (1997) Calcium modulation of ligand affinity in the cyclic GMP-gated ion channels of cone photoreceptors. *J. Gen. Physiol.* **110**: 515–528.
- Lauren DR, Agnew MP (1991) Multitoxin screening method for *Fusarium* mycotoxins in grains. *J. Agric. Food Chem.* **39**: 502–507.
- MacRae IJ, Hanna E, Ho JD, Fisher AJ, Segel IH (2000) Induction of positive cooperativity by amino acid replacements within the C-terminal domain of *Penicillium chrysogenum* ATP sulfurylase. *J. Biol. Chem.* **275**: 36303–36310.
- Newman K (2000) The biochemistry behind esterified glucomannans – titrating mycotoxins out of the diet. In: Lyons TP, Jacques KA, eds. *Biotechnology in the Feed Industry*. Nottingham, UK: Nottingham University Press, pp. 369–382.
- Ramos AJ, Hernandez E (1996) *In vitro* aflatoxin adsorption by means of a montmorillonite silicate. A study of adsorption isotherms. *Anim. Feed Sci. Technol.* **62**: 263–269.