DEACTIVATION OF MYCOTOXINS. I. AN IN VITRO STUDY OF ZEARALENONE ADSORPTION ON NEW POLYMERIC ADSORBENTS

Key Words: In vitro adsorption, zearalenone, cryogels of cross-linked PVP

A.K. Alegakis¹, A.M. Tsatsakis¹, M.I. Shtilman², D.L. Lysovenko² and I.G. Vlachonikolis³

¹ Center of Toxicology, ³ Biostatistics Unit, Medical School, University of Crete, Voutes, Iraklion 71409, Crete, Greece
² Mendelejev University of Chemical Engineering, Miusskaya pl. 9, 125047, Russia
Moscow, Russia

ABSTRACT

This study describes the elimination of zearalenone concentrations in vitro using two new polymeric forms of cross-linked polyvinylpyrrolidone (cryogels of cross-linked PVP). Adsorption of zearalenone was studied under isothermal conditions and simulating pH of intestinal environment. A Freundlich isotherm was used to describe the adsorption data obtained. The results showed significant decrease of zearalenone concentrations, ranging from 33.5 - 66.2 % per 25 mg of polymer. Adsorption capacity (k) was estimated to be higher than that of previously tested adsorbents, including crospovidone. The data indicate the need to
investigate structure peculiarities in order to improve mycotoxin deactivation procedures using PVP derivatives.

INTRODUCTION

Zearalenone (ZRL) 3,4,5,6,9,10 hexahydro- 14,16 - dihydroxy-3- methyl-1H- 2- benzoxyclotetradecin- 1,7 (8H) dione (Figure 1) is a mycotoxin produced by several species of the genus *Fusarium*, including *Fusarium graminearum* and *Fusarium roseum*. It is formed in white crystals of molecular weight 318.4 and melting point 164-165 °C. It dissolves easily in organic solvents (acetone, ethanol, methanol, dimethylethyl ether), but is only sparingly soluble in water.

ZRL affects plants such as corn, barley and wheat in the field or during storage (Ramos et al., 1996a; 1996b). According to the International Agency for Research on Cancer (IARC) there is evidence for carcinogenicity in animals, but ZRL is not classified as a human carcinogen. It is also considered as a teratogen when administered during pregnancy.

The methods proposed for preventing the mycotoxins problem can be categorized. One group of methods is focused on the use of mycostatic or antifungal agents like propionic acid, sorbic acid, nystatin etc. which prevent the growth of fungi and the production of mycotoxins (Gareis et al., 1984; Skrinjar, 1995; Tzatzarakis, et al., 1998).

The other group of methods concern mycotoxin deactivation and are based on the physico-chemical properties of materials like aluminosilicates, activated charcoal, crospovidone, polymeric resins etc. (Doyle et al., 1982; Scheideler, 1993;
Ramos et al., 1996b). The deactivation of mycotoxins is thus mainly due to the adsorption phenomenon. Most of the aforementioned adsorbent materials have been studied in vitro or in vivo for aflatoxins inactivation. On the contrary ZRL adsorption on these materials has not been thoroughly studied.

The decrease of mycotoxins concentration is usually estimated indirectly, measuring the bulk (free) concentration of the toxin. Many analytical techniques have been used for detecting or measuring mycotoxins concentration, like high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), etc. (Musuku et al., 1994; Tzatzarakis and Tsatsakis, 1996).

The purpose of this in vitro study is to test the efficiency of two new polymeric derivatives of vinylpyrrolidone (VP) with methylene-bis-acrylamide (MBAA) (cryogels of cross-linked PVP) in eliminating ZRL. The experimental conditions used, simulate the intestinal environment in animal organism (aqueous solution, pH= 7.35, T= 37 °C).
MATERIALS AND METHODS

Materials

Zearalenone was supplied by Sigma Chemical Co., (St. Louis, USA). Water, methanol, acetonitrile all of HPLC grade were supplied by Merck Co., (Darmestaad, Germany). Acetone, VP, MBAA, tetramethylene-ethylene-diamine (EDTA), and other reagents used were of analytical grade.

Synthesis of Polymeric Derivatives of PVP

The two polymeric forms synthesized were copolymers of MBAA with VP. K2S2O8 and EDTA (2% solutions in water) were used as initiators. The polymerization process was carried out in sealed reaction tubes at -12 °C for 6 hours. The derived gels were extracted with water, washed with acetone and dried under vacuum. The two cryogels of cross-linked polyvinylpyrrolidone (CPVP1 - CPVP2) synthesized differed in the %age composition of MBAA.

Instrumentation - HPLC Conditions

A Spectra Physics SP 8800 HPLC model with a SP 8450 UV-VIS detector at 235 nm wavelength, was connected to a Spectra Physics SP4270 integrator and was used to determine the free concentration of zearalenone. A Silasorb reverse phase C18 column was used (25 cm x 4.6 mm, 10 μm particle size, Rigas Labs, Thesaloniki, Greece). The mobile phase consisted of methanol: water: acetonitrile (5:4:2) and flow rate was 1.0 ml/min. This method is a slightly modified version of the method described by Prelusky et al. (1989).

A P-Selecta (Barcelona, Spain) shaking water bath temperature controlled of ±0.1 °C was used. Centrifugation was performed using an Econospin centrifugger (Sorvall Instruments). pH was adjusted using an Consort P107 pH-meter.
Preparation of Stock Solution and Standards

A stock aqueous solution of ZRL was prepared by dissolving 5 mg of ZRL in 30 ml acetone, the pH adjusted to 7.35 ± 0.01 by dropwise addition of NaOH and HCl and the final volume adjusted to 400 ml. The stock solution was capped firmly and stored at 0°C.

Ten standards of ZRL were prepared, by diluting the initial stock ZRL solution with water to a final volume of 3 ml. The concentration of standards ranged from 0.1 to 12.5 μg/ml. The HPLC calibration curve gave an excellent linear fit ($r^2=0.9989$). The calibration equation was $y= 0.077+ 0.00000588x$ (where $y$ is ZRL concentration in μg/ml and $x =$ Area under the curve of ZRL peak). Area was determined as a mean value of five injections for each ZRL concentration.

Adsorption Experiments

Eight ZRL standard solutions with concentrations ranging from 2.1 to 10.5 μg/ml (10 ml final volume) were prepared, using the initial stock solution. In each tube a quantity of 0.025 gr of polymer was added. Two blank samples were prepared: a water solution with polymer and a ZRL standard solution without polymer.

All tubes were placed in a thermostatically controlled water bath at 37.0 ± 0.1 °C and shaken at 100 rev/min for 2 hours. The polymer was separated from solution by filtration through a 2-fold filter. Polymer traces were eliminated by centrifugation at 3000 rpm/min for 15 min and 50 μl of each solution was directly injected to HPLC.

The determination of free concentration of ZRL is done using the previously described HPLC conditions.
Mathematical Modeling of Data

A double log expression of Freundlich isotherm is used:

\[ \log\left(\frac{q_{ads}}{m}\right) = \log(k) + n \log(C_{free}) \]

where \( n, k \) empirical constant, \( k \) constant are measured in \( \mu g \) of adsorbate per gr of adsorbent, \( n \) - constant is dimensionless. The \( k \) constant is related to the capacity of adsorbent to various adsorbates while the \( n \) constant describes the connection among free concentration of adsorbate and the adsorbent quantity. The \( C_{free} \) is the concentration of ZRL remaining after adsorption process in bulk solution and \( q_{ads}/m \) is the quantity of ZRL adsorbed per gram of adsorbent.

Freundlich isotherm is an empirical formula which is widely applied to evaluate many adsorption data. Excel 7.0 for Windows 95 was used for statistics and graphic representation of the adsorption data.

RESULTS AND DISCUSSION

Table 1 depicts quantitative data of the reagents used for the polymerization process. Cryogels synthesized differed in MBAA content according to UV measurements.

In Figure 2 two typical chromatograms of ZRL standard (left) and ZRL treated solution (right) are shown. ZRL peak is eluted at \( Rt= 20.30 \) min. Chart speed is modified between \( Rt= 8.00 \) to \( 18.00 \) min in the figure. A new peak marked with (*) was observed at \( Rt= 3.85 \), on the right chromatogram which was attributed to polymer low molecular weight impurities. The same peak was
DEACTIVATION OF MYCOTOXINS. I

TABLE 1
Quantities of Reagents (in gr) Dissolved in 10 g of Bidistilled Water for the Preparation of Cryogels of Crosslinked PVP

<table>
<thead>
<tr>
<th>Polymer</th>
<th>VP (gr)</th>
<th>MBAA (gr)</th>
<th>EDTA (gr)</th>
<th>K₂S₂O₈ (gr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPVP1</td>
<td>0.95</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>CPVP2</td>
<td>1.42</td>
<td>0.075</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

FIGURE 2
A typical chromatogram of ZRL standard solution (left) and ZRL solution after adsorption experiment (right).
detected in blank water samples with polymer. The other blank sample (ZRL solutions without polymer) do not show any decrease in ZRL concentration during adsorption experiments which indicates that the total decrease in ZRL concentration may be attributed solely to adsorption on polymers.

The ratio $q_{ad}/q_o$ -adsorbed ZRL quantity per initial ZRL quantity- as estimated from the experiment showed that 25 mg of polymers used in different initial concentrations of ZRL solutions decrease the concentration of ZRL in a range of 33.5 - 63.9 % for CPVP1 and 49.2 % - 66.2 % for CPVP2.

In Figures 3 and 4 double-log forms of $q_{ad}/m$ vs $C_{free}$ are shown for CPVP1 and CPVP2. Intercept of plot according to Freundlich isotherm indicates values of log($k$) (2.7624 for CPVP1 and 2.8709 for CPVP2). $k$ value can be calculated as a tenth power of estimated log($k$)'s. The $n$ values are easily estimated as the slope of regression plots (0.6345 and 0.6441 respectively).

As can be seen from Figures 3 and 4 very good data fitting were obtained when using a Freundlich isotherm. This fitting indicates a heterogeneous sorbent surface which means that more than one adsorption mechanisms exist. This was an expected result because it also appeared at batch adsorption studies using commercial forms of crospovidone (McMurrough, et al., 1995).

Adsorption parameters for Freundlich isotherms are shown in Table 2. Over 95% of the total variation of data are simulated using a Freundlich isotherm ($r^2 = 0.9598$ and 0.9713). $k$- values for each polymer estimated from Freundlich isotherm were at 578.6 and 742.9 µg/ml respectively.
DEACTIVATION OF MYCOTOXINS. I

FIGURE 3
Adsorption data fitted using a Freundlich isotherm for CPVP1.

FIGURE 4
Adsorption data fitted using a Freundlich isotherm for CPVP2.
TABLE 2
Adsorption Parameters Using Freundlich Isotherm for Polymers CPVP1 and CPVP2

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>n</th>
<th>SE*(n)</th>
<th>log(k)</th>
<th>SE*(log(k))</th>
<th>k (µg/g)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPVP1</td>
<td>0.6345</td>
<td>0.0530</td>
<td>2.7624</td>
<td>0.0265</td>
<td>578.6</td>
<td>0.9598</td>
</tr>
<tr>
<td>CPVP2</td>
<td>0.6441</td>
<td>0.0452</td>
<td>2.8709</td>
<td>0.0201</td>
<td>742.9</td>
<td>0.9713</td>
</tr>
</tbody>
</table>

* SE: Standard error of the mean

There is a great variety of crospovidone formulations in trade differing mainly in physicochemical characteristics (particle size, bulk density etc). Many uses have been suggested especially in mycotoxins deactivation (Ramos et al., 1996b), in brewery (McMurrough et al., 1995). There is also a great interest on adsorption characteristics of crospovidone to various other chemicals or pharmaceuticals (Doner et al., 1993; Fromming et al., 1981). ZRL is not as extensively studied as other mycotoxins in adsorption experiments.

A commercial form of crospovidone used for ZRL deactivation was studied by Ramos et al. (1996b). The examined crospovidone form proved to have a greater adsorption capacity per gram than other adsorbents like bentonite, magnesium trisilicate, montmorillonite and sepiolite and lower than cholysteramine. Crospovidone adsorption capacity ($k$) was estimated at 313.7 µg/ml. In our study, $k$ values estimated for each polymer (Table 2) were greater than those of Ramos et al., 1996b. The greater $k$ value of the second polymer than of the first polymer indicates that the second polymer could have a greater surface area.
Taking into account the estimated SE of $n$-values, 0.0530 and 0.0452 of each polymer, the differences between the $n$-values of Table 2 are not statistically significant. Such a result probably shows that surface characteristics are quite similar in both polymers (McMurrough et al., 1995).

CONCLUSIONS

As a result of our study we conclude that the two novel polymeric PVP derivatives synthesized, which are copolymers of PVP and MBAA may be characterized as having high adsorption capacities. Our in vitro experiments showed that the above cryogels of crosslinked PVP can be efficiently used for ZRL deactivation. We suggest that these cryogels be further studied in ZRL contaminated animal feed.

Further studies will involve in vitro and in vivo tests to examine ZRL adsorption in gastric fluid. The adsorption of other mycotoxins on our prepared cryogels must also be examined. The synthesis of new cryogels using our proposed method will further elucidate structure peculiarities and adsorption relationship. In conclusion such total studies will result in new efficient products to be used in food industry, veterinary and human medicine.

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REFERENCES


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