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IN VITRO STUDY OF THE EFFICACY OF MYCOTOXINS DEGRADATION BY FEED ENZYMES

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Abstract

Providing healthy and safe food in terms of mycotoxicological safety is an imperative for not only good and sustainable livestock production, but also for the population that consumes food of both plant and animal origin. Climate change in the temperate regions of southern Europe has led to frequent occurrence of aflatoxins, deoxynivalenol and zearalenone in cereals. In order to reduce harmful effects of these toxins on animal health as well as to avoid large economic losses, various feed additives are increasingly being used. All of them must first of all be safe, and then have certain efficiency in the fight against mycotoxins. Although *in vivo* experiments are mandatory to assess the efficacy, *in vitro* test offers the advantage of rapid screening of the efficacy of a large number of food additives. In this paper, the efficiency of two commercial products belonging to the enzyme group for animal nutrition was investigated for degradation of aflatoxin B1, zearalenone and deoxynivalenol using *in vitro* experiments. For this purpose, two different methodologies were used according to the recommendation of the enzyme manufacturer. The percentage of mycotoxin degradation was recorded by high pressure liquid chromatography and ELISA methods. One of the tested enzymes showed a very high efficiency in zearalenone degradation being as much as 96%. Both tested enzyme samples showed similar percentage of aflatoxin B1 degradation (about 35%). Deoxynivalenol was not significantly degradable under the applied test conditions.

Key words: aflatoxin, deoxynivalenol, zearalenone, degradation, *in vitro*, enzyme

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IN VITRO ISPITIVANJE EFIKASNOSTI RAZGRADNJE MIKOTOKSINA POMOĆU ENZIMA ZA ISHRANU ŽIVOTINJA

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Kratak sadržaj

Obezbeđivanje zdrave i bezbedne hrane u smislu mikotoksikološke bezbednosti imperativ je ne samo dobre i održive stočarske proizvodnje, već i brige o stanovništvu koje konzumira hranu biljnog i životinjskog porekla. Klimatske promene u umerenoj klimi južne Evrope dovele su do česte pojave aflatoksina, deoksinivalenola i zearalenona u žitaricama. U cilju smanjenja štetnog uticaja ovih toksina na zdravlje životinja, kao i izbegavanja velikih ekonomskih gubitaka, sve više se koriste različiti aditivi za hranu. Svi oni moraju pre svega biti bezbedni i imati određenu efikasnost u borbi protiv mikotoksina. Iako su *in vivo* eksperimenti obavezni za procenu efikasnosti, *in vitro* test nudi prednost brzog skrininga efikasnosti velikog broja aditiva za hranu. U ovom radu je ispitivana efikasnost dva komercijalna proizvoda iz grupe enzima za ishranu životinja za razgradnju aflatoksina B1, zearalenona i deoksinivalenola u *in vitro* eksperimentima. U tu svrhu korišćene su dve različite metodologije prema preporuci proizvođača enzima. Procenat razgradnje mikotoksina je izmeren metodama tečne hromatografije visokog pritiska i ELISA metodom. Jedan od testiranih enzima, pokazao je veoma visoku efikasnost u razgradnji zearalenona od čak 96%. Oba ispitana uzorka enzima su pokazala sličan procenat razgradnje aflatoksina B1 (oko 35%). Deoksinivalenol nije bio značajno razgradiv u primenjenim uslovima ispitivanja.

Ključne reči: aflatoksin, deoksinivalenol, zearalenon, razgradnja, *in vitro*, enzim

INTRODUCTION

Enzymes are considered biological catalysts; they are proteins capable of accelerating the speed of chemical reactions, which are essential for the proper cellular functioning of all living beings. Their use has shown benefits in the food industry, while in animal feed could improve the consistency and nutritional value of feed, increase digestibility, animal performance and reduce the effect of antinutrients. (Velázquez-De Lucio et al., 2021). The purpose of adding enzymes in animal feed is to improve food efficiency, production, and consequently to reduce the cost of feeding (Bedford 2018). Enzymes can be obtained from animals, plants or microorganisms. The development of recombinant DNA technology has allowed for the isolation and expression of genes of some microorganisms, and production of enzymes for animal feed (Sarder et al., 2005). Enzymes used in the production of animal feed are considered zootechnical additives in the Republic of Serbia, their use is allowed by the Rulebook on the quality of animal feed (Official Gazette of the Republic of Serbia, 2010).

Mycotoxins are a numerous group of secondary metabolic products of fungi or molds that pose a serious risk to human and animal health. Mycotoxin contamination is widespread in animal feed, especially in cereals (Wu et al., 2015). Currently, more than 300 mycotoxins have been identified, and scientific attention has been focused mainly on mycotoxins that have been shown to be carcinogenic and/or toxic to human and animal health. Aflatoxins (AF), zearalenone (ZEA) and deoxynivalenol (DON) are of great public health concern due to their high prevalence, teratogenic, carcinogenic, mutagenic and immunosuppressive effects (Oueslati et al., 2012). Increased amounts of mycotoxins in animal feed can result in huge economic losses on an annual basis, including declining livestock production as well as increasing human and animal mortality (Zain, 2011). An eight-year study based on the determination of mycotoxins (AF, ZEA, DON, fumonisin and ochratoxin A) in animal feed and feed materials worldwide showed that 72% of the samples were positive for at least one mycotoxin, and 38% is simultaneously contaminated with multiple mycotoxins (Streit et al., 2013).

Prevention of mycotoxin contamination begins in the field. However, when speaking of the protection of cereals from mold and mycotoxins during storage, further measures are necessary. Different treatments (physical, chemical and biological) are used for this purpose (Stoev, 2013; Jevtić et al., 2021). Mycotoxin adsorbents are often used as feed additives (Nešić et al., 2020). Although mycotoxins are stable compounds, research on their degradation is very topical today. Effective degradation of mycotoxins must provide irreversible degradation of mycotoxins to less toxic or non-toxic products. One of the frequently studied techniques is the biological detoxification of mycotoxins, using microorganisms and/or enzymes to degrade mycotoxins into non-toxic or less toxic compounds (Taylor and Draughon, 2001). The advantage of mycotoxin degradation using enzymes is the simplicity of the process, without the potential risk of contamination and operator safety compared to the use of live microorganisms (Loi et al., 2017). The main conversion paths are hydroxylation, hydrogenation, hydrolysis, oxidation, esterification, glucuronidation and glucosidation, de-epoxidation, methylation, sulfation, demethylation and deamination (Nešić et al., 2021). The following enzymes are used for this purpose: oxidase, peroxidase, laccase, reductase (AF), carboxylesterase and aminotransferase (fumonisins), glucosyltransferase (trichotecenes), laccase, lactonohydrolase (ZEA), lypase, protease (ochratoxin) (Loi et al., 2017). Europen Union (EU, 2014; 2017; 2018) and European Food Safety Authority (EFSA, 2020) established the regulations regarding fumonisin esterase (the enzyme that degrade fumonisins) as a feed additive for animal species in accordance with rules for additives for use in animal nutrition (EU, 2003).

Climate change in the temperate climate of southern Europe has led to frequent occurrences of AF, DON and ZEA in cereals and in Serbia. In order to reduce the harmful effects of these toxins on animal health, as well as to avoid large economic losses, various feed additives are increasingly being used. All of them must first of all be safe, and then have a certain efficiency in the fight against mycotoxins. Although *in vivo* experiments are mandatory to assessing the efficacy, the advantage of the *in vitro* test is its capacity to rapidly screen the efficacy of a large number of food additives. In this way, the reduction of mycotoxin toxicity is indirectly confirmed. In this work, the degradation efficiency of AFB1, ZEA and

DON was investigated *in vitro* using two commercial products belonging to the enzyme feed additives.

MATERIAL AND METHODS

Chemicals and enzymes

Two samples of different feed enzymes were provided by INBERG ltd (Belgrade, Republic of Serbia). The tested enzymes are intended for use as feed additives in order to reduce the harmful effects of mycotoxins.

Standard substances were used for degradation tests: AFB1 Cat No A6636, ZEA Cat No Z2125, and DON Cat No D0156. All standards were purchased from "Sigma Aldrich", Saint Louis, USA.

In vitro experiments

For the purpose of in vitro testing of the possibility of mycotoxin degradation by enzymes, two different methodologies were used. The efficiency of sample No. 1 was tested according to the manufacturer's recommendation applying the methodology No. 1. Its efficiency for the degradation of AFB1, ZEA and DON was examined. The efficiency of sample No. 2 for the degradation of the same mycotoxins was tested according to the manufacturer's recommendation and using the methodology No. 2.

The first methodology involved incubation of mycotoxin standards and enzyme in phosphate buffer solution (0.1M PBS, pH 6.5). The test solution for ZEA degradation assay consisted of 1980 µL (1900 µL) of buffer (with appropriate enzyme weighing in suspension), in which 20 µL (100 µL) of ZEA standard solution (100 µg/mL) was added. For testing the degradation of DON 100 µL of standard solution (100 µg/mL in a mixture of ethyl acetate and methanol) was evaporated and reconstituted in 2000 µL buffer (with appropriate weighing of enzymes in suspension). The test solution for AFB1 degradation assay consisted of 1980 µL of buffer (with appropriate enzyme weighing in suspension), in which 20 µL of standard solution of AFB1 (10 µg/mL) was added. All tested solutions were incubated with shaking for 4 h at 37 °C and then centrifuged, filtered, and mycotoxins were determined by liquid chromatography.

The second methodology included incubation of mycotoxin standards and enzyme on LB medium 20 mL (in 1l: peptone 10 g, NaCl 10 g, 5 g yeast extract, adjusted to pH 7.4, autoclaved for 20 min), at 37 ºC for 24 h (0.1 µg/mL AFB1 and 1 µg/mL ZEA) or 1 h (2.5 µg/mL DON). After stopping the reaction, purification was done by solid phase extraction. Purification of samples after treatment on LB medium was performed by using MycoSep 224 AflaZon, and MycoSep 225 TrichMultifunc columns (RomerLabs, USA).

In both cases, the experiment consisted of a toxin and enzyme assay and a control assay with toxins only. The degradation efficiency expressed with standard deviation (STD) is the result of measurement in three replications.

Mycotoxins analysis

After *in vitro* tests, the efficiency of the tested enzymes for mycotoxins degradation was evaluated. The percentage of degradation was recorded by

quantitative measurement of residual mycotoxin in the supernatant by using optimized and validated methods. In the case of ZEA and AFB1, there high pressure liquid chromatography with fluorescence detection (HPLC-FLD) was used, while HPLC-DAD and ELISA were applied for DON.

HPLC Dionex UltiMate 3000 Series system with FLD 3100 and DAD detector (Thermo Scientific, Germany) was used for quantitative measurement of mycotoxins in the solution before and after adsorption. The system was controlled with Chromeleon®7 software (Thermo Scientific, Germany).

For the determination of AFB1 Supelcosil column, 250 x 4.6 mm, 5µm, was used for separation with mobile phase 50% ACN and flow rate 1.2 mL/min. Fluorescence detection was done on λex 365 nm, and λem 435 nm. For ZEA determination Hypersil Gold aQ column, 150 x 3 mm, 3 µm, (Thermo Scientific, Germany), with mobile phase 60% ACN, and flow rate 1 mL/min was used, while for detection the wavelengths λex 275 nm and λem 455 nm were set. The method for the determination of DON used the same column as for ZEA, mobile phase 10% ACN, flow rate 1 mL/min, and detection was done at λ 220 nm.

Determination of the percentage of toxin degradation

After HPLC determination, the peak areas of the determined mycotoxins in the test samples were compared with the corresponding areas of control samples without added enzyme. The percentage of adsorption was calculated by using the equation:

$$\% \text{ degradation} = (1 - PI / P0) \times 100\%$$

Where: PI = peak area of toxins after incubation with enzyme; P0 = surface area of toxin peaks in control solution without enzyme addition.

RESULTS

The tested enzymes are commercially available as feed additives. Their use is based on the effect of reducing the harmful effects of mycotoxins, that is, they degrade mycotoxins in the conditions of the digestive tract of animals. There were no available data on their activity and efficiency, the exact composition and origin. The only available information was about the conditions under which those enzymes work, i.e., the pH values, in which medium, and at what mycotoxins concentrations. These data were used for *in vitro* testing thereof.

Because of different testing conditions as well as different concentrations of mycotoxins and enzymes, the results for each enzyme were presented separately.

Efficiency of mycotoxin degradation by enzyme No. 1

The results of the study of the influence of enzyme number 1 on the degradation of ZEA are shown in Table 1. It can be seen that increasing the mass of the enzyme increases the degradation efficiency to a significant 93%. Given such a high efficiency with a high amount of enzyme, and in order to optimize the ratio of enzyme and ZEA, the possibility of degradation of a larger amount of ZEA was

tested (Table 2). As can be seen, a 96% degradation of ZEA was achieved at a toxin: enzyme ratio of 1 µg: 1.8 mg.

Table 1. Results of testing the degradation efficiency of 2 µg ZEA by using enzyme No. 1.

	Enzyme (mg)	ZEA degraded (%)	STD (%)
TEST 1	5	49.4	4.5
TEST 2	10	66.1	5.5
TEST 3	100	93.6	0.6

Table 2. Results of testing the degradation efficiency of 10 µg ZEA and 10 µg DON using enzyme No. 1.

	Enzyme (mg)	ZEA degraded ± STD (%)	DON degraded (%)
TEST 1	1.8	53 ± 2	2.7
TEST 2	18	96 ± 0.2	6.4

The efficacy of the same enzyme for AFB1 degradation was examined with three different enzyme amounts, and the results showed maximum efficiency of 34.8% (Table 3).

Table 3. Results of testing the degradation efficiency of 0.2 µg AFB1 by using enzyme No. 1.

	Enzyme (mg)	AFB1 degraded (%)
TEST 1	3.27	0
TEST 2	6.53	4.5
TEST 3	32.67	34.8

Efficiency of mycotoxin degradation by enzyme No. 2.

Testing the efficacy of enzyme sample No. 2 was examined by incubating the enzyme and toxin in a nutritionally rich medium Lysogeny Broth (LB). The degradation efficiency of ZEA alone and ZEA and AFB1 in the mixture was examined. The results are shown in Figure 1. There was no difference in the efficiency of ZEA degradation in case where ZEA alone was present in the reaction mixture (13 ± 6%) as compared to the mixture with AFB1 (11%).

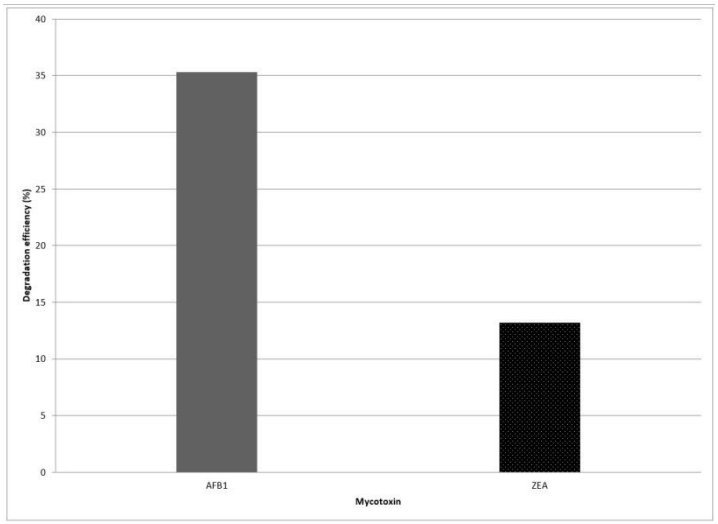

Figure 1. Results of testing the degradation efficiency of 20 µg ZEA and 2 µg AFB1 by using enzyme No. 2. (40 mg).

The enzyme degradation assay of DON was performed in the same medium, but with a shorter incubation time (1 h) and a higher amount of enzyme (200 mg) in comparison to ZEA and AFB1. Due to the low concentration of DON the determination by HPLC-DAD method was not possible, so semiquantitative determination of toxin in the control and solution with enzyme was determined by ELISA. As it can be seen from Figure 2, there is no significant difference in the colour of the cells with the control and test samples. These results reveal no significant difference in DON concentration between control and test samples after enzyme degradation. Enzyme No. 2 has no effect on the degradation of DON. Poor degradation of DON is expected, given its chemical nature (small pollar moiety) and literature data on difficulties in the development of agents for their irreversible detoxification (Nešić et al., 2021).

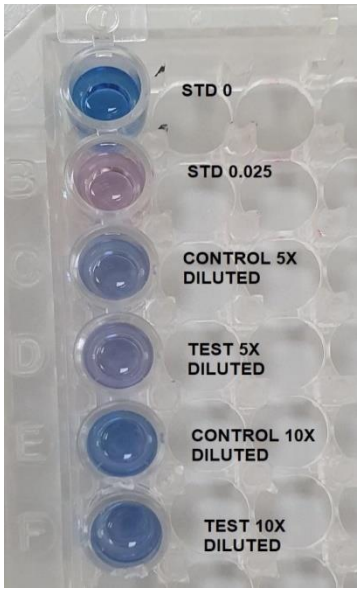

Figure 2. Determination of DON after treatment 50 µg with enzyme No. 2 (200 mg) 1 h at 37 ºC.

DISCUSSION

A modern and economically justified approach to the fight against mycotoxins also involves the use of additives that enzymatically lead to their degradation. Before using these supplements, it is necessary to confirm their activity or efficiency for the decomposition of mycotoxins. Although regulations in the EU on enzymes as additives against mycotoxins are available, national regulations in Serbia and other countries do not include requirements pertaining to the quality and degradation efficiency of enzymes used against mycotoxins in animal feed. Also, there are no unique methodologies for analyzing enzyme efficiency. In *in vitro* tests, it is important to define the conditions under which these experiments are performed. Experimental conditions should mimic the biotransformation of toxins in the animal's body. The levels of toxin and enzyme to be tested in *in vitro* reaction system are also important. A range of various experiments to check the activity of enzymes for the degradation of mycotoxins is described in the literature. Two different methodologies, with different concentrations of toxins and enzymes, were used in this study. Therefore, it is difficult to compare the obtained results with each other. The conclusions of experiments on the efficacy of enzymes should be stated with reference to the conditions under which they were obtained. According to the available literature, effective degradation of AFB1 and ZEA (86% and 100% respectively) is achieved by laccase, using redox mediators, while under the same conditions the degradation of DON was not possible (Loi et al., 2018). A high percentage of AFB1 degradation (90 – 100%) using the enzymes peroxidase and oxidase, while ZEA reduction was achieved by using lactono hydrolase (100%) has also been reported in the literature (Loi et al., 2017).

Here give analyses of the obtained results comparing to the results and opinions of other authors, pointing the importance of this research, without giving a conclusion. The Discussion section is not used to summarize current knowledge. The Discussion should clearly identify the main Conclusions of the study. Authors are to provide a clear explanation of the importance and relevance of these Conclusions. Any new information should be distinguished from the previous findings, and relevant hypotheses can be generated.

CONCLUSION

The result of this research is the confirmation of a quality of the enzyme that has been proven to be highly efficient for the decomposition of ZEA, a toxin often present in cereals and responsible for reproductive disorders in livestock production. Based on the results obtained in *in vitro* studies, the optimal ratio of enzyme and ZEA was obtained, which gives maximum efficiency in application.

Control of enzymes, similar to adsorbents used as feed additives, before mixing into complete mixtures is necessary, and conscientious producers are aware of the cost-effectiveness of such approach and conscious production strategies. The significance of ensuring the health of animals and humans by using proven components of animal feed to prevent the occurrence of mycotoxicosis and mycotoxin-contaminated food is an imperative in the production of safe food.

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Author's Contribution:

SJ -conducted the experiment, design of the study, and paper concept and writing, MZB- revising the manuscript critically, NP- experiment performance, ZM-conducted the experiment, BZ-initial idea and providing material, VP-experiment organization.

Competing interest

The authors declare that they have no competing interests.

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