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ORIGINAL RESEARCH PAPER

DEGRADATION OF ZEARALENONE BY LACCASE ENZYME

Iuliana Banu*, Anca Lupu, Iuliana Aprodu

"Dunărea de Jos" University of Galaţi, Faculty of Food Science and Engineering, 111 Domneasca Street, 800008 Galaţi, Romania

*Corresponding author: iuliana.banu@ugal.ro

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Abstract: The degradation of zearalenone by pure fungal laccase (*Trametes versicolor*) was investigated in this study. In the experiments different activities of laccase and different initial zearalenone concentrations from liquid medium were used. At 0.4 $mg \cdot mL^{-1}$ laccase concentration, after 240 min time of incubation, was obtained an 81.7 % reduction of zearalenone from liquid medium. The zearalenone degradation depends on the initial concentration of mycotoxin and on the activity of laccase. The results obtained can contribute to the development of preventative strategies to reduce mycotoxin contamination of food by involving enzymes.

Keywords: *zearalenone, laccase, degradation, mycotoxins*

INTRODUCTION

Zearalenone (ZON) is a secondary fungal metabolite produced mainly by *Fusarium graminearum* and *Fusarium culmorum*. It is a strong estrogenic compound, genotoxic and hepatotoxic, and may contribute to the increasing occurrence of cancer [1]. It is most frequently found in corn, but has been also reported in other cereal such as wheat, barley, oat, sorghum and rice. Chemically, ZON is a resorcylic acid lactone described as [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-beta-resorcyclic-acid-lactone [2]. There are numerous ZON derivatives that can be produced by *Fusarium* spp, but only trans-alpha-Zearalenol, has been found to occur naturally in cereal grains.

Laccases are multicopper enzymes which oxidize diphenols and related substances and use molecular oxygen as an electron acceptor [3, 4]. This enzyme is widely found in plants and fungi, in some bacteria and insects, and oxidizes polyphenols, methoxy substituted phenols, aromatic diamines and a range of other compounds [5]. Laccases of fungi have the great potential for industrial and biotechnological applications. In food industry the potential application of laccases are related to bioremediation, beverage processing, ascorbic acid determination, sugar beet pectin gelation, baking and as a biosensor. Other applications of laccases include the detoxification of industrial effluents, use as a tool for soil bioremediation and for medical diagnostics, as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti-cancer drugs and even as ingredients in cosmetics [3].

In the last years the fungal laccases were investigated to be used in developing of preventative strategies to reduce mycotoxin contamination of food and beverages. Alberts et al. [5], and Viksoe-Nielsen and Soerensen [6] suggested that the treatments with fungal laccases and application of genetically engineered microbial strains can improve the quality, safety and acceptability of traditional fermented food and beverages. The enzymatic degradation of aflatoxin B1 by white rot fungi through laccase was investigated in different liquid media by Alberts et al. [5]. Aflatoxin B1 was significantly degraded when treated with pure laccase enzyme from *Trametes versicolor* and recombinant laccase produced by *Aspergillus niger*. Viksoe-Nielsen and Soerensen [6] discovered that the zearalenone in a feed product can be degraded into non-toxic substances by treating the feed product with laccases from *Myceliophthora thermophila*, *Polyporus pinsitus* and *Streptomyces coelicolor*, and as mediators methylsyringate and phenothiazine-10-propionic acid. These results suggested that the degradation of mycotoxins by fungal laccases could be an important bio-control measure to reduce the level of these toxins in food commodities.

In this study degradation of ZON by laccase enzymes was investigated. ZON extracted by corn contaminated with this mycotoxin was treated with pure fungal (*Trametes versicolor*) laccase enzyme.

MATERIALS AND METHODS

Determination of enzyme activity

Laccase activity was determined according to the method described by Jönsson et al. [7] and Alberts et al. [5] by measuring the formation of oxidation products at 434 nm.

Assays were performed at 25 ºC by using 1.6 mM 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) as substrate. The formation of oxidation products was monitored for 10 min. One unit enzyme activity was defined as the amount of enzyme required to oxidise 1 µmol of ABTS per minute.

Mycotoxin quantification

Different concentrations (5041.88; 2519.7; 1276.63; 638.31 şi 319.15 ppt) of ZON were prepared using the reference material (corn) with 59.4 ± 10.5 ppb ZON (R-Biopharm Rhone Ltd.). Each sample was extracted by confinable solvent systems; 5 g of ground sample was suspended in 25 mL extracting solvent methanol:water (7:3). The suspensions were first mixed vigorously for 3 min on a magnetic stirrer (Velp Scientifica) and afterwards the extract was filtered. Ten milliliter of filtrate was passed through Easi-Extract® Zearalenone immunoaffinity columns at about 1-2 drops·s^{-1}. 10 mL water was used to wash the loaded immunoaffinity column at a steady flow rate. ZON was eluted with 1 mL of methanol. The methanol eluate was filtered through a 0.45 µm micro filter and collected in a clean vial for the analysis. Determinations of mycotoxins were performed by the competitive enzyme immunoassay (ELISA-kit). Ridascreen® ZEA tests (R-Biopharm Rhone Ltd.) that are specially designed for quantitative analysis of ZON in cereals, malt, feed, beer and wort, were used. The concentration of ZON was quantified according to the manufacturer's description. The optical density of the final extracts was measured at 450 nm using ELISA 96-well plate reader (Sunrise, Tecan Group Ltd.) and the special software RIDA® Soft Win (R-Biopharm AG, Germany) was afterwards used for mycotoxin content quantification. All samples solutions were analyzed in duplicate. According to the manufacturer's description, the detection limit for ZON by ELISA for cereals was 1.75 µg·kg^{-1}. The mycotoxin content was expressed in ng per kg initial product (ppt).

Experimental design

Effect of laccase concentration on mycotoxins removal

The experiments were carried out in 200 mM sodium acetate buffer *p*H 5.2, using pure laccase enzyme produced by *Trametes versicolor* (Sigma-Aldrich) and ABTS 0.16 mM as mediator. ZON was prepared to final concentrations 5041.88; 3773.83; 2553.26; 1284.03 and 622.46 ppt using references material, and afterwards the laccase was added to obtain solution with 0.4; 0.3; 0.2; 0.1; 0.05 mg enzyme per mL. After maintaining the mixtures at 30°C for 240 min. in a laboratory incubator shaker, the enzyme was inactivated (2 min at 100°C) and then mycotoxin concentrations were determined. Untreated ZON samples used included as control samples.

Effect of incubation time on mycotoxins removal

ZON was prepared to final concentrations 5041.88 ppt using references material, and afterwards the laccase was added to obtain solution with 0.2 mg enzyme per mL. The mixtures were maintained at 30°C in a laboratory incubator shaker for different times. After 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 min the samples were removed from the laboratory incubator shaker and the enzyme was inactivated (2 min. at 100 °C). Then mycotoxin concentrations were determined.

RESULTS AND DISCUSSION

Effect of laccase concentration on mycotoxins removal

The ZON concentration in liquid medium significantly decreased when laccase concentration increased from 0.05 to 0.4 mg·mL^{-1} (Figure 1).
At the highest laccase activity in the medium (0.4 mg·mL^{-1}) were registered the highest reductions of ZON concentration, from 69.6 % to 81.7 %, in case of initial ZON concentration of 5041.88 ppt and 622.47 ppt, respectively. Treatment of the liquid medium with the lowest laccase activity (0.05 mg·mL^{-1}) showed a reduction of the ZON concentration from 30.1 % to 39.1 % when the initial concentrations of the mycotoxin were 5041.88 ppt and 622.47 ppt, respectively. From Figure 1 we can see the ability of pure fungal laccase enzyme to degrade ZON.

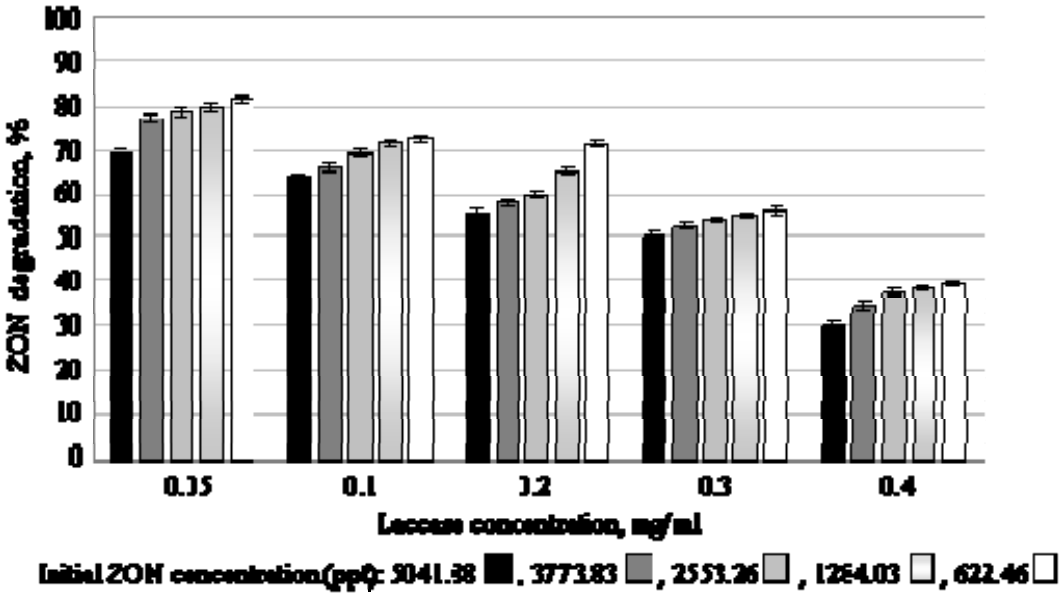

Figure 1. *Degradation of ZON by pure fungal laccase in different concentration in the medium*

At the same enzyme concentration, the most effective ZON degradation was registered at the lowest initial mycotoxin concentration. Alberts et al. [5], in their investigation regarding the enzymatic degradation of aflatoxin B1 by laccase producing white rot fungi, observed that the laccase produced by *Peniophora* sp. SCC0152 and by *P. ostreatus* St2-3 (with a laccase activity in the medium from 0.001 to 0.496 U·mL^{-1}) degraded 40.45 % and 35.9 %, respectively, aflatoxin B1 from the medium. When pure fungal laccase enzyme, with 0.5 U·mL^{-1} activity, from *Trametes versicolor* was used, a significant degradation (87.34 %) of aflatoxin B1, and a decreases of the the fluorescence properties of the aflatoxin B1 molecule were observed by Alberts et al. [5].

Effect of incubation time on mycotoxins removal

After 30 min. the percentage of ZON degraded by laccase (0.2 mg·mL^{-1}) was minimal, 4.7%. When increasing the incubation time from 30 to 240 min, the ZON degraded

increased constantly (Figure 2). After this time the ZON concentration in the liquid medium remained constant (55-58 %). Probably after 240 min the activity of the enzyme is lost and the ZON degradation is not possible.

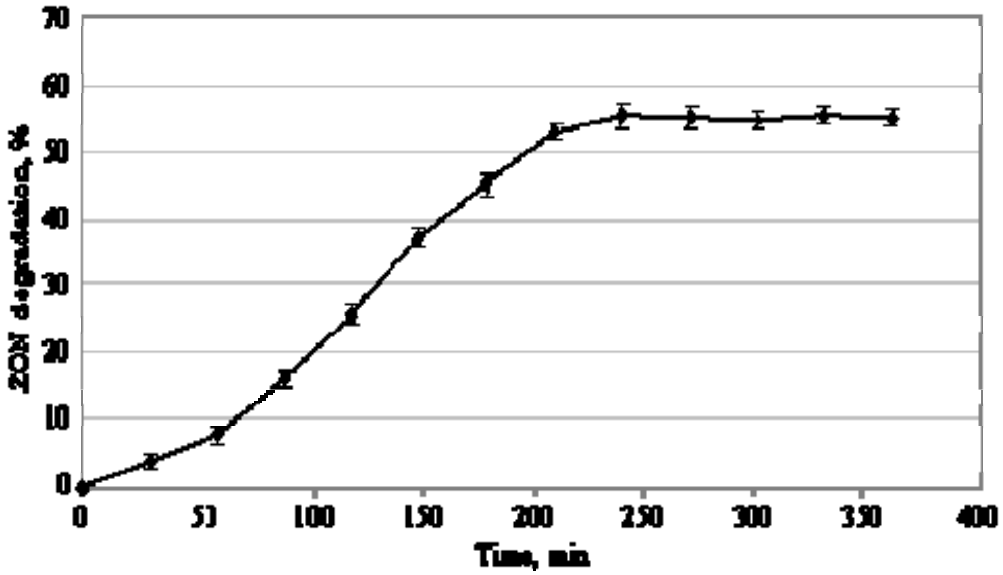

Figure 2. *Degradation of ZON by pure fungal laccase after different time of incubation*

CONCLUSIONS

The results obtained in the experiment regarding the degradation of ZON by different activities of pure fungal laccase enzyme can contribute to the development of preventative strategies to reduce mycotoxin contamination of food by involving enzymes. At 0.4 $mg \cdot mL^{-1}$ pure fungal laccase concentration, after 240 min. time of incubation, was obtained an 81.7 % reduction of ZON from liquid medium. The ZON degradation depends on the initial mycotoxin concentration and bon the activity of pure fungal laccase enzyme.

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