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Effect of Deoxynivalenol and T-2 Toxin in Malt Amylase Activity

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ABSTRACT

The objective of this work was to evaluate the effect of DON and T-2 toxin levels on the malt amylase activity. The malt was contaminated in accordance with central composite design experiment with DON and T-2 toxin levels until 1000 ng/g. The activities of the enzymes were evaluated by Bernfeld method. The increase in T-2 toxin concentration inhibited the α-amylase activity. However, the increase of both toxins concentration caused inverse effect. The interaction between toxins indicated synergetic effect on the β-amylase activity. An increased activity occurred when the toxins contamination levels in malt were higher (1000ng/g malt). The trichothecenes interfered with the performance of aminolitic enzymes in the stage of malting, resulting in a significant model for enzymatic activity of β-amylase.

Key words: Aminolitic enzymes, fermentation, trichothecenes, barley

INTRODUCTION

Trichothecenes are a group of sesquiterpenoid produced by genera *Fusarium, Myrothecium, Trichothecium, Cephalosporium, Verticimosporium* and *Stachybotrys* (Mello, Placinta and Macdonald, 1999; Schapira and Whitehead, 1989; Ueno, 1986; Ueno, 1983). This class of mycotoxins contaminates the cereals produced in field and remain post-harvest (Scott, 1990; Yoshisawa, 2001). Deoxynivalenol (DON) has been the most frequent trichothecene detected in worldwide since 1970s. T-2 toxin trichothecene has been constantly studied due to its toxicity (Tanaka, 2001; FAO, 2003; Ueno, 1983, 1986; Garda, Macedo and Badiale-Furlong, 2004). Trichothecenes are relatively stable during the main processes used in food production (Scott, 1984; Lauren and Ringrose, 1989; Hazel and Patel, 2004; Pronik, et al., 2005; Wolf-Hall et al., 1999). These mycotoxins have been detected in beer, which can be originated from natural contamination of barley, or other associated cereals, such as rice and wheat, as well as fungal contamination during malting (Scott, 1990, 1992, 1995, 1996; Flannigan et al., 1985; Bennett and Richard, 1996; Trucksess, 1996; Garda et al.., 2005).

Malt is the source of α- and β-amylases, phosphorylases and α-glycosidases that degrade the barley starch to fermentable sugar (Hough, 1990). The activity of amylases is the main factor responsible for the diastatic power of malt, wich

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define the quality of fermentation (Osman, 2002; Taylor, Robbins, 1993; Agu, Palmer, 1997).

The competitive marketing of beverage production of the beer, as well as quality production and low cost has been of high concern. The use of contaminated barley can decrease the income in beer production, as well as it is hazardous to consumers (Schapira, et al., 1989; Sydenham, Thiel, 1996; Trucksess, 1996, Scott, Lawrence, 1997; Torres et al., 1998).

This work aimed to evaluate the effect of DON levels and T-2 toxin in beer malt on the activity of amylases, using the central composite design, CCD (surface response method).

MATERIALS AND METHODS

Materials

The reagents used were analytical degree of Merck Chemical Co., UK, and Sigma Chemical Company, USA. The stock solutions of DON and T-2 toxin were prepared in benzene: acetonitrile (95: 5) to concentration of 100μg/ml. The working solution was prepared from the stock solution to 50μg/ml. The mycotoxin concentration was evaluated as described in AOAC (2000) procedure.

Malt

The commercial malt from industry of Rio Grande do Sul state was previously screened for aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A and zearelenone by multimethod of thin layer chromatography. The detection limit of zearalenone was improved using the reaction with aluminum chloride (Soares and Amaya, 1989). DON and T-2 toxin were determined by thin layer chromatography (Furlong and Baraj, 2003), confirmed and quantified by gas chromatography after derivation with trifluoroacetic anhydride (TFAA) (Garda-Buffon and Badiale-Furlong, 2008).

Methodology

The effect of the artificial contamination of DON and T-2 toxin in α and β-amylase activity was evaluated by central composite design, CCD (surface response method) described in Table 1.

Planning Experiment Procedure

Malt was homogenized, ground (28 mesh) and 5g were used for enzymatic activity assay. For the central composite design experiment, the sample was contaminated with levels of DON and T-2 toxin at 0 to 1000ng/g range, as described in Table 1, and stored during 24 h prior to the determination of the α and β-amylase activity.

Table 1 - Central composite desing planning for the determination of the effect of DON and T-2 toxin in the malt amylase activity.

Treatment	X 1	X 2	DON (ng/g)	T-2 (ng/g)
1	-1	-1	146	146
2	1	-1	854	146
3	-1	1	146	854
4	1	1	854	854
5	0	0	500	500
6	0	0	500	500
7	-1.414	0	0	500
8	1.414	0	1000	500
9	0	-1.414	500	0
10	0	1.414	500	1000
11	0	0	500	500
12	0	0	500	500

Being: $X_1 = (\xi_1 - 500) / 354$ and $X_2 = (\xi_2 - 500) / 354$, where ξ_1 is the contamination value of DON (ng/g) and ξ_2 is the contamination value of T-2 toxin (ng/g). 354 (500/1,414) is the value of α for CCD (2 factors).

α-amylase Assay (EC3.2.1.1)

The enzymatic assay was carried out as described by Bernfeld (1955). The α-amylase was extracted with 50 ml of 0.5% NaCl at 30°C by shaking. The suspension was centrifuged at 1,820 g to 10 min and filtered (5°C). The dextrinization employed a mixture of 0.5ml of starch solution, 0.1 ml of solution of 0.1 M citric acid/sodium phosphate

(pH 6.0) and 0.3 ml of distilled water (5°C for 5 min). Then, 0.1 ml of enzymatic extract was added and after 5 min, the reaction was stopped adding of 0.5 ml of 1M HCl. The hydrolyze was determined by complex of 0.1 ml of iodine solution (0.3% I_2 in 3% KI solution) and 13.4 ml of distilled water. The absorbance was read at 620 nm. One unit of dextrinizant activity (UD) was defined as the capacity of the enzyme to hydrolyze 1 mg of starch in 1 min at 5°C and pH 6.0 in the presence of 1.0 mg of protein.

β-amylase Assay (EC 3.2.1.3)

The β-amylase assay was carried out as described by Bernfeld (1955), which measured the increase in the reducing power of the starch solution by enzymatic hydrolysis. An aliquot of 0.5 ml of 1% starch solution at 5°C with 0.1 ml of the enzymatic extract was centrifuged at 1,820 g to10 min. The mixture remained at 5°C for 3 min. The reaction was stopped by adding 1 ml of 3.5- dinitrosalicylic acid, warmed in boiling water (5 min), then cooled (5°C) and 8 ml of distilled water was added. The absorbance was read at 546 nm. The reducing sugar was determined using a calibration curve with maltose standard. One unit of Saccharification activity (UE) was defined as the capacity of the enzyme to break 1 mg of maltose in 1 min at 5° C and pH 6.0.

RESULTS AND DISCUSSION

The previous analysis of the malt sample used in this assay were negatives for aflatoxins, ochratoxin A, zearalenone and trichothecenes. The performance of the thin layer multimethod for this mycotoxins determination are presented at Table 2. The extraction of the amylases was carried out during 4 hours in 0.5% NaCl that corresponded the maximum activity when different times were compared.The analysis of variance (ANOVA) and the effect of DON and T-2 toxin in dependent variable UD and UE, as well as its coefficients are presented in Tables 3, 4 and 5 and Fig. 1.

Schapira et al., (1989) reported that the synthesis of α-amylase, during the process of barley germination could be inhibited by trichothecenes. The T-2 toxin at 10μg/g levels reduced 23 % of the enzymatic activity, while DON caused a minor influence. The UD values decreased with the increase of concentration of the T-2 toxin as a first-order term (146 to 854ng/g), exerting a negative effect on its activity. The values are shown in Table 4.

Table 2 - Performance of the mutimethod determination.

Mycotoxins	Detection limit (ng/g)	Recovery* (%)	Variation Coefficient (%)
Aflatoxins[1]	0.8	88	-
Zearalenone[1]	60	79.5	-
Ochratoxin A[1]	6	80	-
DON[2]	21	83	4
T-2 toxin[2]	60	103	2

Method: (1) Soares and Amaya (1989) and (2) Furlong and Baraj (2003).
* Three replicates in four levels.

Table 3 - α and β-amylases activities in malt artificially contamined with DON and T-2 toxin.

Treatment	UD	UE
1	628.48	305.94
2	633.98	348.14
3	616.19	364.96
4	621.75	328.91
5	619.27	312.00
6	623.48	311.60
7	644.86	357.88
8	639.16	319.92
9	656.74	324.74
10	636.78	336.95
11	625.08	311.06
12	613.72	314.91

UD: α-amylases activity expressed as the unit of dextrinizant activity.
UE: β-amylases activity expressed as the unit of dextrinizant activity.

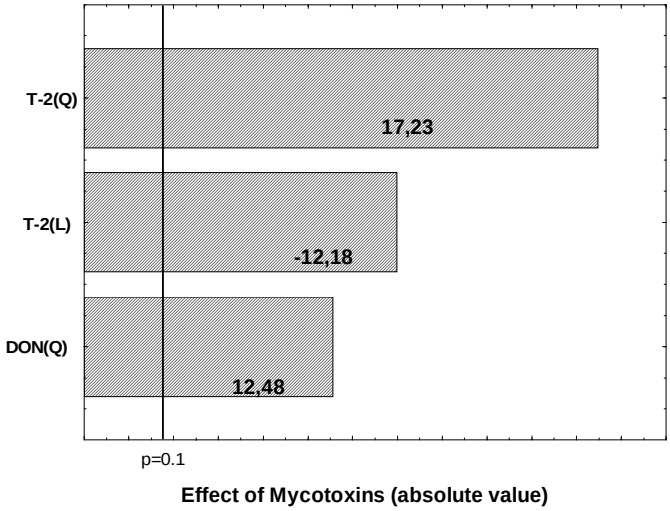

Figure 1 - Effect of the mycotoxins (DON and T-2 toxin) on the enzymatic activity response of α-amylase (UD). DON- deoxinivalenol; T-2 – T-2 toxin, L – linear; Q-quadratic

Table 4 - Values of the effects and coefficients for the preview equation for enzyme activities.

Enzyme		Effect	Standard deviation	p-value	Coefficients
UD	Average	620.3875	2.537838	0	620.3875
	DON(Q)	12.48	4.012675	0.052876	6.24
	T-2 (L)	-12.1869	3.589045	0.042599	-6.0935
	T-2(Q)	17.23	4.012675	0.023228	8.615
EU	Average	312.3925	0.860982	0	312.3925
	DON(L)	-11.8834	1.217612	0.002286	-5.9417
	DON(Q)	27.565	1.361332	0.000263	13.7825
	T-2 (L)	14.2644	1.217612	0.001336	7.1322
	T-2(Q)	19.51	1.361332	0.000736	9.755
	DON(L) . T-2(L)	-39.125	1.721964	0.000187	-19.5625

Q: planning quadratic or second order (0 to 1000ng/g)
L: planning linear or first order (146 to 854ng/g); UD - α-amylase activity; UE - β-amylase activity

Table 5 - ANOVA for the enzyme activities response.

Enzyme	Variation Source	Quadratics Totals	Degrees of Allowance	Quadratic Averages	F Test (*p*)
UD	Regression	908.062	3	302.69	2.92
	Residue	830.462	8	103.81	
	Lack of Adjustment	753.175	5	150.64	(0.003686)
	Pure error	77.287	3	25.76	
	Total	1738.524	11	158.05	
EU	Regression	3762.394	5	752.48	8.53
	Residue	528.756	6	88.13	
	Lack of Adjustment	519.861	3	173.28	(0.088458)
	Pure error	8.895	3	2.965	
	Total	4291.,150	11	390.10	

$F_{Tabled}(95\%, 3, 8) = 4.07$; $R^2 = 0.52232$; $R = 0.34319$; $F_{Tabled}(95\%, 5, 6) = 4.39$; $R^2 = 0.87678$; $R = 0.7741$; UD - α-amylase activity; UE - β-amylase activity.

However, the increase of the concentration of the DON and T-2 toxin, presented as terms of second order (0 to 1000ng/g), increased the activity of the enzymes. The concomitant adition of T-2 and DON showed no significant, additive or synergistic effect on the activity of α-amylase in malt ($p>0.05$). Whitehead and Fannigan (1989) forward an increase in the activity of the enzymes associated with the collapse of toxics agents in the cells exposed to T-2 toxin.

The ANOVA`s data indicated significant lack of adjustament (p = 0.003686) (Table 5). The data variability around the central point differed from the variability of experimental regions. Therefore, the construted model could have low reability, and could not predict the influence of α-amylase activity.

$$UD = 620{,}39 + 6{,}24 \times DON^2_{COD} - 6{,}09 \times T\text{-}2_{COD} + 8{,}62 \times T\text{-}2^2_{COD} \quad (1)$$

Tables 4 and 5 also show the data of effect and ANOVA's analysis for the dependent variable β-amylase (UE). The two factors DON and T-2 toxin, being first or second order, and the interaction between them was significant in the enzymatic activity of β-amylases. These data indicated that the highest effect was caused by this interaction of both the toxins, a synergetic effect (Table 4). The increase of the DON concentration, in the first-order term (146 to 854ng/g), inhibited the enzymatic activity (-11.9 UE). However, when the concentration of this toxin was highest, term of second order (0 to 1000ng/g), the enzymatic activity increased (27.6 UE). T-2 toxin caused the highest effect with an increase in concentration, either in first or second order, resulting an increase in the activity (14.3 and 19.5 UE, respectively). Thus, independent of the toxin, the effect of second order increased with the toxin concentration, increasing the enzymatic activity.

In this case, probably an increase in DON and T-2 toxin allowed a physical association that caused the change of the tertiary configuration of other soluble proteins of malt. Thus, in the presence of higher amounts of toxins, there were a high number of small proteins that associated themselves to toxins more easily and let amylases free; in addition to other factors that influenced the α-amylase activity (UD).

$$UE = 312{,}39 - 5{,}94 \times DON_{COD} + 13{,}78 \times DON^2_{COD} + 7{,}13 \times T\text{-}2_{COD} + 9{,}76 \times T\text{-}2^2_{COD} - 19{,}56 \times T\text{-}2_{COD} \times DON_{COD} \quad (2)$$

The data obtained from ANOVA allowed concluding that the β-amylase activity (UE) presented a behavior in presence of the toxins that would be described by a significant and predictive equation considering the reliable interval of 95% (Fig. 2). An increase of activity occurred when the toxins contamination levels in malt were high (1000ng/g malt), indicated by the UD and UE value.

These results showed that the trichothecenes interfered with the performance of amylases in the stage of malting, but not following a linear behavior in the inhibition the enzymes. However, a significant and predictive model to β amylase activity was obtained when the toxins concentration increased.

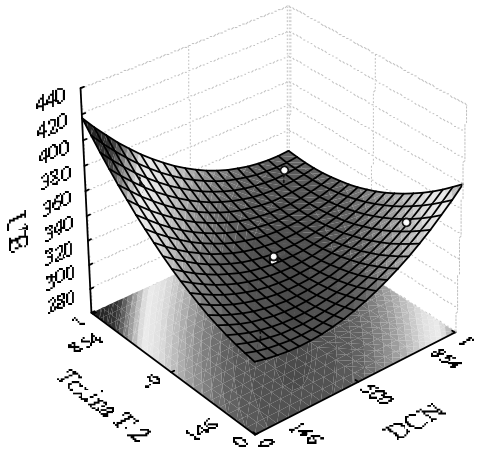

Figure 2 - Surface design of enzymatic activity of β-amylase (UE).

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RESUMO

O objetivo deste trabalho foi avaliar o efeito de deoxynivalenol e toxina T-2 na atividade de α e β-amilase em malte. A contaminação artificial foi de acordo com planejamento experimental completo nos níveis de 0 a 1000ng/g para cada micotoxina. O aumento da concentração da toxina T-2 inibiu a atividade da α-amilase. Entretanto, a coocorrência a altas concentrações das duas toxinas indicou efeito sinergistíco. Um aumento da atividade enzimática ocorreu na presença de altos níveis das micotoxinas avaliadas (1000ng/g de malte). Os tricotecenos interferiram na performance das enzimas aminolíticas na etapa de malteação, permitindo determinar um modelo preditivo para a atividade da β-amylase.

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