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Full Length Research Paper

Extraction of fungal amylase inhibitors from cereal using response surface methodology

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This study involved the application of response surface methodology to recover amylase-inhibiting protein fractions from oats, rice and wheat, in order to promote strategic procedures to reduce the risk of fungal contamination. The parameters studied were ethanol concentration, agitation time and type of grain using fractional factorial design 3^{3-1}. Fungal amylase (Fungamyl®) inhibition was used as the response. The results showed that in oats and wheat, optimum recovery of protein extracts capable of inhibiting Fungamyl® activity was achieved using 70% ethanol for 12 hours' agitation, whereas for rice, the best condition was obtained with 95% ethanol and 7 hours' agitation. It was shown that statistical treatment to combine process variables and enzyme activity measurement can be adopted for the selection and subsequent purification of enzyme inhibitors with antifungal activity.

Keywords: Ethanol, extraction time, percentage inhibition, planning experiment.

INTRODUCTION

Protein inhibitors found in vegetable tissues can be grouped into six classes according to their similarity of primary sequence and their tertiary structure: lectin, knottin, cereal, kunitz, g-purothionin and thaumatin (Svensson et al., 2003). In certain species these can account for 5 to 15% of the total seed proteins (Jongsma et al., 1995; Pompermayer et al., 2001) and they have shown to be capable of inhibiting α-amylase of some insect species and other organisms (Payan et al., 2004). As well as their presence in wheat, α-amylase inhibitors have also been reported in beans (Mosca et al., 2008), corn (Marsaro-Júnior et al., 2005), rye (Gibbs and Alli, 1998), rice, barley, oats (Nakase et al., 1996) and other species.

Understanding the natural defence mechanisms of plants can help control crop productivity and ensure food safety, thus reducing losses and public health risks. Another approach is to obtain these inhibitors from grains not fit for sale and use them for food product conservation, or as medication for people with innate metabolism disorders (Boniglia et al., 2008). The recovery of these natural inhibitors from cereals and the industrial use of their inadequate portions also serves the desirable purpose of reducing secondary contamination levels in the ecosystem caused by phytosanitary products used on crops (kadosawa et al., 2002; Motomura et al., 1996).

Simple tools that enable the identification of varieties and residues with greater antifungal resistance can help prevent damage to crops, the environment and public health, by allowing intervention at several points in the production chain (Jouany, 2007).

The use of factorial design and response surface analysis is important for establishing the quantification and extraction conditions of fungal enzyme Inhibitors

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(Aragão et al., 2011), as such methodology requires few experiments to determine the primary factors with significant effect on analytic efficiency (Mitchell et al., 2001; Morais et al., 2001). The factorial design of a limited set of variables is advantageous in relation to a series of conventional methods that manipulate one parameter by selection and often result in a flawed determination of the optimum conditions due to their inability to consider the effects of any cross-factorial interaction (Kalil et al, 2001). This information is of fundamental importance in the extraction of protein compounds from grains, in view of the structural and physical-chemical differences of the tissues and of the inhibitors.

A fast extraction procedure associated to the determination of the inhibitory quality makes the use of commercial fungal amylase a suitable indicator of inhibition for acquiring consistent and satisfactory results, providing useful information for quick testing and immediate pathogenic control.

The objective of this study was to apply response surface methodology to recover the protein fraction from oats, rice and wheat capable of inhibiting amylase activity, with a view to developing simple and strategic procedures to reduce the risk of fungal contamination and optimizing the natural defence mechanisms of the tissues.

MATERIALS AND METHODS

Stock solution of fungal alpha amylase extracted from *Aspergillus oryzae* (Fungamyl® 800L, CEE 2006/ 121, Novozymes Latin America, Paraná, Brazil) was prepared (1.26 g mL^{-1}), stored at 4 ºC and removed upon use to indicate the presence of enzymatic inhibitors in the protein extracts.

The sources of inhibitors studied were: a) oat grains (*Avena sativa* L.) varieties A (UPFA20 Teixeirinha), B (UPFA22 Temprana) and C (UPFA Pampa) supplied by Passo Fundo University, b) wheat (*T. aestivum* L.) varieties A (Ônix), B (Safira) and C (Pampeano), supplied by *OR Melhoramentos* and c) rice (*O. sativa* L.) varieties A (BR410), B(BR 417) and C (BR 424) supplied by the Rio Grandense Institute of Rice (IRGA). The samples were ground and the granulometric fraction used was a mixture of the fractions that passed through and that were retained in a 32 mesh sieve, at a ratio of 1:4 (p/p), respectively.

The oat flour was defatted with petroleum ether (Synth, São Paulo, Brazil), homogenized for 15 minutes and centrifuged at 2,240 g for 20 minutes. This procedure was repeated three times. The defatted flour was dried at room temperature and stored at - 20 ºC.

Experimental design

Previous experiments with ethanol, sodium acetate buffer and sodium phosphate buffer (data not presented) showed ethanol as the most suitable solvent for extracting amylase inhibitors from these cereal grains. Therefore, to improve extraction yield, the independent variable tested in the fractional factorial design $3^{(3-1)}$ were ethanol concentration (70-95%), cultivar type (A-C) and extraction time (2-12 hours), with percentage of fungal amylase inhibition (% I) as the response. The experiments were conducted in duplicate, with a total of 18 tests (Table 1).

Analysis of variance was applied to test the significance of the model. Non-significant variables were eliminated in the adjusted models, leaving the final equation containing only those significant to the level of probability (< 0.05), where Y is the dependent variable (response variable) to be modeled; X_1-X_3 are the independent variables. The lack-of-fit test was used to determine whether the constructed model was adequate to describe the observed data (Montgomery 2000). The R^2 indicates the optimization percentage of the variability parameters explained by the model (Box and Hunter, 1978). Three-dimensional response surface plots were drawn to illustrate the main and interactive effects of the independent variables on the response variable.

Extraction of enzymatic inhibitors

The inhibitors were extracted by homogenizing the sample with solvent at a ratio of 1:5 (p/v) for the design period. The 12-hour extraction time was divided into 8 h, 2 h and 2 h; 7-hour extraction into 4 h, 2 h, 1 h, with the addition of 25 mL, 10 mL and 10 mL of solvent, respectively (Figueira et al., 2003; Mosca et al., 2008). The aliquots of each extraction were gathered, centrifuged, filtered and quantified for protein content, and their inhibitory activity subsequently tested.

α-Amylase activity

Iodometric titration was used to determine initial amylase activity (Baraj et al., 2010), where 1 mL of fungal alpha-amylase (0.137 mg of protein mL^{-1}) was added to 1 mL of starch solution (5 µg mL^{-1}) and diluted in 1 mL of pH 7 sodium acetate buffer. The reaction was triggered at 30 ºC and interrupted by the addition of 1 mL of hydrochloric acid solution 0.1 M.

The required amount of enzyme for starch hydrolization without the inhibitor was considered at the maximum reaction speed (v), expressed in µg of starch mL^{-1} min^{-1},

Table 1. Values of coded levels real values (in parenthesis) used in fractional factorial design 3 $^{(3-1)}$ and percentage inhibition (%I) of fungal amylase

	Variable levels			Percentage inhibition (%)		
Trial	**X_1 (%)**	**X_2**	**X_3 (hours)**	**Oat**	**Wheat**	**Rice**
1	-1 (70)	-1 (A)	-1 (2)	74	66	43
2	-1 (70)	0 (B)	+1 (12)	96	71	67
3	-1 (70)	+1 (C)	0 (7)	82	89	41
4	0 (82,5)	-1 (A)	+1 (12)	69	65	53
5	0 (82,5)	0 (B)	0 (7)	68	25	43
6	0 (82,5)	+1 (C)	-1 (2)	60	52	39
7	+1 (95)	-1 (A)	0 (7)	26	74	96
8	+1 (95)	0 (B)	-1 (2)	35	21	44
9	+1 (95)	+1 (C)	+1 (12)	28	43	60
10	-1 (70)	-1 (A)	-1 (2)	61	49	42
11	-1 (70)	0 (B)	+1 (12)	98	81	64
12	-1 (70)	+1 (C)	0 (7)	79	76	37
13	0 (82,5)	-1 (A)	+1 (12)	65	62	50
14	0 (82,5)	0 (B)	0 (7)	67	44	51
15	0 (82,5)	+1 (C)	-1 (2)	55	45	40
16	+1 (95)	-1 (A)	0 (7)	16	61	96
17	+1(95)	0 (B)	-1 (2)	24	7	43
18	+1 (95)	+1 (C)	+1 (12)	34	42	51

X_1 = ethanol concentration, X_2= cultivar, X_3= extraction time

as per equation 1. One unit of amylase was defined as the enzyme quantity required to hydrolize 0.06 mg of starch per minute.

$$v = \left(\frac{absTimeZero - abs\,\text{Samplefromenzymeactivity}}{a} \right) \div 30$$

(Equation 1)

where: a = specific absorptivity (µg mL-1)

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α-Amylase inhibitor activity

The extracts containing amylase inhibitors were incubated with commercial fungal α-amylase and pH 7.0 sodium acetate buffer for 30 minutes at 25 ºC. Subsequently, 1 mL of starch solution (5 µg mL-1) was added and the solution incubated for 30 minutes. The reaction was interrupted by the addition of 1 mL of HCl 0.1M. Iodometry was applied to measure residual iodine, at 620 nm, in quadruplicate. The amylase inhibitory activity was obtained via equation 2, expressed in µg of hydrolyzed starch mL^{-1} min^{-1}.

RESULTS AND DISCUSSION

Reducing the particle size facilitates extraction by increasing the contact area between the sample and solvent (De Souza, 1998). The sample aliquots obtained from fractions smaller than 32 mesh (3 parts) and larger than 32 mesh (1 part) were mixed to ensure representation of the different part of the grain that might contain inhibitors, especially considering that the outer portions are the first to act as protection systems; physical barriers against fungal attack. Moreover, the particle size of the samples could influence efficiency of the extraction, as regards interference by sugars, lipids and other molecules in the grain (Luz et al., 2005).

The variables studied in the extraction process were solvent concentration, time and cultivar of each cereal

Table 2. Effects on percentage of inhibition (%I) caused by oat extract (%I)

Factors	CR	EE	EP	t (9)	p
Average	57.601	57.601	1.177	48.955	0.000
Ethanol (x_1)	-27.444	-54.887	2.883	-19.044	0.000
Cultivar (x_2)	2.246	4.492	2.883	1.559	0.1535
Extraction time (x_3)	6.860	13.720	2.883	4.760	0.0010
X_1x_2	5.662	11.325	4.324	2.6220	0.0278
X_1x_3	-7.055	-14.109	4.324	-3.264	0.0098
X_2x_3	-5.814	-11.629	4.324	-2.689	0.0248

CR=coefficient of regression, EE=estimated effect, EP=pure error, t=coefficient, p=level of significance

Table 3. ANOVA for the response percentage of inhibitions (%I) caused by oat extract

Model	Degrees of freedom	Sum of squares	Mean square
Regression	6	10333.31	1722.22
Residual	11	451.02	41.00
Lack of fit	2	226.75	
Pure error	9	224.27	
Total	17	10784.33	

R= 0.958, R^2= 0.935, tabulated F value= 42, calculated F value= 3.39.

species, as the constituents of each grain affect the recovery to a greater or lesser extent (Table 1). In this case, the efficiency of the water-alcohol ratio and contact time is closely related to the chemical composition of the sample.

Other authors have investigated the effects of ethanol concentration of 0 to 95% during 12 hours of inhibitor extraction from corn samples (Figueira et al., 2003); while inhibitors have been extracted from wheat by using a sodium phosphate buffer for 20 hour (Mundy, Hejgaard and Svendsen, 1984) and from rye using 70% ethanol for 3 hours (Lulek et al., 2000). These and other studies have all proven the importance of the solvent and the extraction time for efficient inhibitor recovery.

The selected cultivars are recommended for planting in the south of Brazil and were genetically modified to improve their productivity and resistance against severe climate conditions and fungal attack.

The estimated effect (equation 3) of the ethanol concentration (x1), cultivar (x2) and extraction time (x3) indicated the influence of each factor on the inhibition percentage generated through the oat extract, demonstrating that the increased ethanol concentration and extraction time resulted in 14% higher amylase inhibition (Table 2).

The ANOVA data (Table 3) enabled the construction of the adjusted first order model, as per Equation 3. Regression was significant (p (0.05), demonstrating that the model explains the results found. Calculated F (42) was greater than tabulated F (3.39), indicating good agreement between the experimental values and those predicted by the model and enabling the construction of the response surfaces and definition of the interest regions for inhibitor extraction from each variety.

Y (% I) = 57.6 – 27.5x1 +6.9x3 +5.7x1x2 -7.1x1x3 -5.8x2x3 (3)

The lowest ethanol concentration (70%) and the 12-hour agitation time presented the most promising results, with the oat variety UPFA 22 Temprana standing out. The combined effect of extraction time and ethanol concentration presented a negative effect on inhibitor recovery, regardless of the cultivar, as shown by the percentage values of fungal amylase inhibition. A general assertion can be made that all the oat varieties were good source of enzymatic inhibitors of fungal amylase (Figure 1).

Figure 1 Response surface of inhibition percentage as a function of extraction time and ethanol concentration for the oat cultivars: (a) UPFA 20 Teixeirinha, (b) UPFA 22 Temprana and (c) UPFA Pampa.

The wheat extract displayed similar inhibition in treatments 3 (Pampeano wheat) and 11 (Sapphire wheat), with an average value of 85% when 70% ethanol was used with higher contact times between the solvent and sample (7 and 12 hours). Table 4 shows the analysis of variance results for the inhibition percentage caused by wheat extracts obtained under the different conditions.

The inhibition caused by rice extracts showed linear

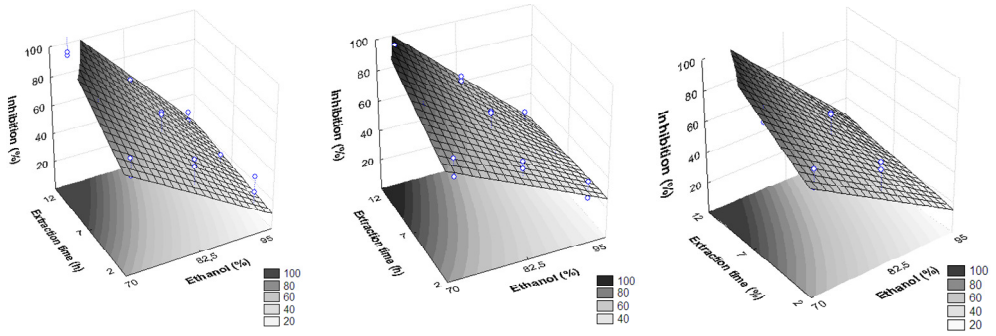

Figure 1 . Response surface in relation to the combined effect of the variable extraction times and ethanol concentration for oat cultivars UPFA 20 Teixeirinha (A), UPFA 22 Temprana (B) and UPFA Pampa (C). The α-amylase inhibitor activity from oat cultivars was determined by iodometry and the results expressed in percentage inhibition.

Table 4. Effects on percentage of inhibition (%I) caused by wheat extract

Factors	CR	EE	EP	t (9)	p
Average	51.918	51.918	2.059	25.209	0.000
Ethanol (X_1)	-15.469	-30.938	5.045	-6.133	0.000
Cultivar (X_2)	-2.558	-5.115	5.045	-1.014	0.3370
Extraction Time (X_3)	10.443	20.886	5.045	4.140	0.0025
X_1x_2	-18.526	-37.052	7.568	-4.897	0.0008
X_1x_3	11.517	23.034	7.568	3.044	0.0139
X_2x_3	0.436	0.872	7.568	0.116	0.9108

CR=coefficient of regression, EE=estimated effect, EP=pure error, t=coefficient, p=level of significance

Table 5. Effects on percentage of inhibition (%I) caused by rice extract

Factors	CR	EE	EP	t (9)	p
Average	53.369	53.369	0.749	71.295	0.0000
Ethanol (x_1)	8.101	16.200	1.834	8.835	0.0000
Cultivar (x_2)	-9.479	-18.957	1.834	-10.339	0.0000
Extraction time (x_3)	7.692	15.383	1.834	8.389	0.0000
x_1 X x_2	-13.716	-27.431	2.593	-10.578	0.0000
x_1 X x_3	-0.312	-0.624	2.750	0.227	0.8256
x_2 X x_3	8.485	16.969	2.593	6.544	0.0001

CR=coefficient of regression, EE=estimated effect, EP=pure error, t=coefficient, p=level of significance

variation in accordance with the increase in ethanol concentration and extraction time. The combination of ethanol and cultivar presented a negative effect; whereas cultivar and extraction time had a positive effect on inhibition, showing that despite belonging to the same species, the behaviour of the inhibitor sources varied in the extraction process.

No mathematical model could be constructed from the ANOVA data due to lack-of-fit of the data for wheat (R2 = 0.61) and rice (R2 = 0.68).

The experiments revealed that increasing the contact time between solvent and sample led to greater efficiency in the inhibitor extraction from the wheat and rice samples, especially when the solvent is renewed at each interval to prevent saturation. Rice extracts behaved differently to the other cereals examined, as the best inhibitor extraction results for rice were obtained with 95% ethanol, similar to that reported for corn (Figueira et al., 2003), indicating that these protein inhibitors greater hydrophobicity when compared to oat and wheat inhibitors, which could be extracted with more hydrated alcohol.

Considering Osborne's classification (1924), the inhibitors extracted from oat, wheat and rice can be classed as prolamins, as they present average specific inhibition of cultivars of 62, 33 and 18 for rice, oats and wheat, respectively, expressed in % I min-1 mg protein-1.

The fractional factorial design was important for establishing the conditions for extracting compounds with inhibitory activity, without denaturation of the protein structure, demonstrated by its capacity to inhibit commercial fungal alpha-amylase.

All the cultivars proved to be good sources of enzymatic inhibitors, with the prominence of BR 410 rice, the protein extract of which demonstrated inhibitory activity 2.5 time greater than the other cereal extracts examined.

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