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Transgenic Soybean Seed as Protein Expression System: Aqueous Extraction of Recombinant β-Glucuronidase

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Abstract Soybean is one of the plant species with potential to be used as seed-based bioreactor. As part of the downstream processing (DSP) of this technology, extraction is a key step, since it defines the composition of the solution from which the recombinant product will be purified. In the present work, the characteristics of soybean seeds used as a bioreactor were evaluated from a process engineering standpoint through analysis of the influence of pH and ionic strength on the extraction of recombinant β-glucuronidase (rGUS). Concentrations of recombinant protein and native soybean compounds were analyzed and compared with similar data from extraction studies using transgenic corn seeds as bioreactor. Efficient rGUS extraction was obtained at pH of around 5.5 with no addition of salt. Soybean seed extracts had lower levels of co-extracted native compounds, than corn seed extracts, and should be considered as a potential plant bioreactor in terms of DSP.

Keywords Downstream processing · Extraction · Recombinant protein · Soyben seed · Transgenic plants

Introduction

Plants have been described as a promising alternative system for the production of recombinant (pharmaceutical) proteins, especially in terms of production cost, when compared with more

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established systems like microbial fermentation and mammalian cell cultures [1]. Among the plant species being evaluated, soybean is considered a potential seed-based bioreactor, since its seed protein content is very high (>40%). Also, among the grain crops, soybean offers the lowest recombinant protein production cost and, from a regulatory standpoint, the risk of contamination through pollen is reduced, since soybeans are largely self-pollinated [2].

From a process engineering standpoint, besides good agronomic characteristics, the native molecular composition of the crop plays an important role in the selection of a species for recombinant protein production. This composition may vary extensively as in the case of the seed composition of soybean and corn—a crop already used as bioreactor on an industrial scale [3]. The latter has four times less protein, two times more carbohydrate, and about the same amount of phenolic compounds [4]. Since the native proteins, carbohydrates, phenolics, and other compounds must be removed during the downstream processing (DSP) operations, the composition of the transgenic plant extract will have a large effect on the final cost of the product. However, the concentrations of these native compounds in the extracts will depend on the extraction conditions employed—especially the pH of the extraction solvent [5] and the need for addition of detergents as in the case of extraction of lipases [6]—which are dependent on the properties of the protein being expressed (such as p*I*, molecular mass, and surface hydrophobicity).

Although there are several advantages in using soybean seeds as bioreactors, there are only a few reports addressing this issue [7–10]. The native proteins of soybean seeds are a complex mixture of proteins of which most have an acidic p*I*. It was assumed that separation of recombinant proteins from those native proteins would be difficult, especially if the recombinant protein had a p*I* below 7.0 [4].

In the present study, we evaluated soybean seeds as a potential bioreactor from the DSP standpoint, analyzing the aqueous extraction of β-glucuronidase (rGUS) and some of its native compounds—proteins, carbohydrates, and phenolic compounds—as a function of pH and ionic strength (in terms of NaCl added to the extraction buffer). Response surface methodology was applied aiming to find an extraction condition that would enable maximization of rGUS extraction and minimization of native soybean components, especially the native proteins, thus favoring DSP operations.

The GUS molecule is a homotetrameric enzyme with a p*I* at approximately pH 5.5 and a monomer molecular mass of approximately 68 kDa [11]. The function of this hydrolase (which is dependent of its tetrameric structure) is to cleave the β-linked terminal glucuronic acids in mono- and oligosaccharides and phenols, and it has been widely used as a visual marker in transgenic plant research [12] and a model protein in DSP studies.

Kusnadi et al. [13] described the expression and recovery of rGUS from transgenic corn seed. They extracted the rGUS by stirring 50 mmol/L sodium phosphate buffer with the seed flour at pH 7.5 and a 1:4 or 1:10 solid-to-liquid ratio for 15 min at ambient temperature. They also reported that the addition of 1% (*w*/*v*) SDS to the extraction buffer resulted in the complete extraction of rGUS. However, the SDS resulted in irreversible enzyme inactivation. A similar rGUS extraction procedure was reported with canola as the expression host [14, 15]. rGUS extracts were obtained by mixing 50 mmol/L sodium phosphate buffer at pH 7.0 with defatted canola meal at a 1:10 solid-to-liquid ratio for 30 min. Bai et al. [16] studied the kinetics of the aqueous extraction of rGUS from transgenic canola in terms of particle size and microstructure. Extraction was carried out with 50 mmol/L sodium phosphate buffer at pH 7.5 using a 1:20 solid-to-liquid ratio. Menkhaus *et al.* [17] used 50 mmol/L sodium phosphate buffer at different pH values varying between 6 and 8 (1:10 solid-to-liquid ratio) for extraction of rGUS from transgenic pea.

Materials and Methods

Materials

Transgenic soybean seeds (cultivar BR-16, lines 8-19) expressing the GUS gene were provided by EMBRAPA, Brazil [18]. Genetic transformation with a constitutive promoter (targeting the rGUS to the cytosol subcellular location) was done using the particle bombardment technique. All chemicals used were of at least analytical grade. A DU 650 spectrophotometer (Beckman, USA) was used for the spectrophotometric measurements.

Methods

Preparation of Soybean Flour

Soybean seeds were ground in a household coffee grinder with intermittent turns in order to avoid heating (the temperature did not exceed 60 °C). The particles were separated using a set of sieves, resulting in flour with particles smaller than 0.5 mm. This flour was defatted in a Soxhlet-type equipment (100 g of flour to 100 mL of hexane) and stored at room temperature until its use in the extraction experiments.

Extraction Protocol

Five-gram samples of soybean flour were mixed with 100 mL of the appropriate extracting solution (1:20 solid-to-liquid ratio) in a 5.5-cm diameter 250 mL beaker. Extraction was carried out at room temperature for 30 min (time to guarantee the stabilization of the concentration of the compounds in the extracts) at a stirring rate of 500 rpm using a mechanical stirrer (Q-251D, IKA Labortechnik, Germany) equipped with an axial-flow impeller (pitched-blade turbine with four blades 4.0 cm in diameter, positioned at a 45° angle 1 cm from the bottom). Extracting solution was prepared by adding sodium chloride (to final concentrations of 0, 30, 100, 170, and 200 mmol/L) to 50 mmol/L citrate–phosphate buffer adjusted to the pH values established in the experimental design described below (pH 3.4, 4.0, 5.5, 7.0, and 7.6). After extraction, each suspension was centrifuged at 15,000×*g* for 20 min at 5 °C and filtered using filter paper.

Determination of rGUS, Protein, Carbohydrate, and Phenolics Concentrations

rGUS activity was quantified with a fluorometric assay based on a method using 4-methylumbelliferyl glucuronide (MUG) substrate [11]. One unit of GUS activity releases 1 nmol of 4-methylumbelliferone (MU) from MUG in 1 mL per minute at pH 7.0 and 37 °C. rGUS specific activity is defined as a rGUS activity divided by concentration of total protein in the solution. Total soluble protein (TSP) concentration in the extracts was determined by Bradford's method [19] using bovine serum albumin (Sigma, USA) as standard. The protein molecular mass profiles for the extracts were evaluated by SDS-PAGE conducted under denaturing conditions as described in [20]. Gels (15%) were stained with Coomassie blue in accordance with [21]. Phenolics were quantified as described in [22] using D-catechin (Sigma, USA) as standard. Soluble carbohydrates were quantified as reducing sugars (RS) and total sugars (TS) using the dinitrosalicylic acid (DNS) method proposed by Miller [23]. Glucose and sucrose (both from Synth, Brazil) were used as standards for RS and TS, respectively.

Experimental Design

A full 2^2 factorial design followed by response surface analysis [24] was used to evaluate the effect of two independent variables—μ (the ionic strength in terms of NaCl added to the extraction buffer) and pH—on the extract composition within a 95% confidence limit. The experimental design selected was a central composite design comprising 11 runs, corresponding to four cube points, four axial points, and three central points (Table 1). In the selected design, a polynomial quadratic model (Eq. 1) is adjusted to the experimental results (responses) obtained at previously determined experimental conditions (independent variables). The parameters not statistically significant are removed from the model, and their effects are added to the overall error of the model.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

In Eq. 1, the Y represents the response surface generated; x_1 and x_2 represent the codified levels of the independent variables; β_1 represents the mean value; β_1 and β_2 are the liner coefficients; β_{11} and β_{22} are the quadratic coefficients; and the β_{12} is the coefficient of interaction between the two independent variables.

The dependent variables (responses) were activity of rGUS, concentrations of total soluble protein, reducing sugars, and phenolic compounds. Statistica software (Statsoft, version 5.5) was used for analysis of the experimental data, generation of the analysis of variance (ANOVA) data and plotting of response surfaces.

Results and Discussion

The experimental conditions and the results for activity and specific activity of rGUS, concentrations of TSP, RS, TS, and phenolics in the aqueous extracts from defatted soybean flour are presented in Table 2. Coefficient values and statistical analysis of the response variables are presented in Table 3.

Recombinant β-Glucuronidase

Recombinant β-glucuronidase (rGUS) activity and specific activity in the extracts were as high as 37,464 U/mL and 7,596 U/mg. The results of the factorial design experiments showed that only the pH of the extraction solution had a statistically significant effect on the extraction of rGUS activity from transgenic soybean seeds. In the case of rGUS activity, the effect of pH was positive, meaning that an increase in pH will increase the rGUS activity in the extracts. A sharp increase of the rGUS activity in the extracts was observed when the pH of the extraction was increased from 4.0 to 5.5 as also verified for TSP (reported ahead). We

Table 1 Values of coded levels and real values for the factors pH and ionic strength used on the complete factorial design.

Factor	−1.41	−1	0	+1	+1.41
pH	3.4	4.0	5.5	7.0	7.6
μ[a]	0	30	100	170	200

[a] μ is the ionic strength in terms of NaCl concentration (mmol/L)

Table 2 Central composite design and responses (rGUS activity, rGUS specific activity, TSP, RS, TRS, and phenolics concentrations) for the aqueous extracts from transgenic soybean seeds.

Run	Independent variable		rGUS activity[b] (U/mL)	rGUS specific activity[b] (U/mg)	Concentration values			
	pH	μ[a]			TSP[b] (mg/mL)	Phenolics[b] (mmol/L)	RS[b] (mg/mL)	TRS[b] (mg/mL)
1	4	30	0	0	1.36	0.10	0.22	7.58
2	4	170	0	0	2.16	0.12	0.22	7.60
3	7	30	37,464	4,738	7.70	0.17	0.58	8.00
4	7	170	35,155	4,498	7.61	0.18	0.58	8.12
5	3.4	100	0	0	1.62	0.08	0.08	7.24
6	7.6	100	36,924	4,423	8.13	0.15	0.65	7.26
7	5.5	0	34,647	7,596	4.44	0.16	0.47	7.75
8	5.5	200	30,572	4,757	6.26	0.24	0.42	7.00
9	5.5	100	30,748	5,147	5.82	0.17	0.48	7.69
10	5.5	100	31,884	5,157	6.02	0.19	0.47	7.75
11	5.5	100	32,866	5,325	6.01	0.18	0.45	7.68

[a] μ is the ionic strength in terms of NaCl concentration (mmol/L)

[b] The standard deviations for the rGUS activity (1,060 U/mL), rGUS specific activity (100 U/mg), TSP (0.11 mg/mL), phenolics (0.01 mmol/L), RS (0.02 mg/mL), and TRS (0.04 mg/mL) are based on repetitions at the central point (runs 9, 10, and 11)

note that the rGUS activity increased as pH got close to the p*I* of rGUS. Generally, the solubility of a protein has its lowest value as the pH gets close to its p*I*. The fact that rGUS activity in the extracts had the same behavior as the TSP regarding pH suggests that the effect of this variable in breaking linkages and structures that hold proteins insoluble (probably attached to the cell solid matrix) is predominant over its effect on rGUS solubility. On the other hand, the ionic strength did not influence the extraction of the rGUS

Table 3 Coefficient values and statistical analysis for rGUS activity, rGUS specific activity, TSP, RS, TRS, and phenolics concentrations of the aqueous extracts.

Coefficients	rGUS activity	rGUS specific activity	TSP	Phenolics	RS	TRS
Mean	31,832.67[a]	5,209.67[a]	5.95[a]	0.18[a]	0.47[a]	7.71[a]
pH	15,604.68[a]	1,936.38[a]	5.25[a]	0.05[a]	0.38[a]	0.24[a]
pH^2	−8,530.58[a]	−1,970.33[a]	−1.28[a]	−0.07[a]	−0.10[a]	−0.20[a]
μ[b]	−1,008.99	−531.87[a]	0.82[a]	0.03[a]	−0.02	−0.23[a]
μ^2	−1,456.83	12.17	−0.80[a]	0.01	−0.02	−0.08
pH μ	−577.25	−60.00	−0.45	−0.01	0.00	0.05
R^2	0.927	0.721	0.94	0.81	0.98	0.06
F value	50.57	13.10	48.66	21.01	86.89	0.77
$F_{calculated}/F_{value}$	11.34	3.01	10.74	4.83	19.48	0.18

R^2 coefficient of determination

[a] Significant at 0.05 level

[b] μ NaCl concentration (mmol/L)

activity, at least within the range studied. The ANOVA of a quadratic model for the rGUS activity resulted in a coefficient of determination of 0.927 and a calculated F of 50.57, which is 11.34 times higher than the F value. The coded model was used to describe the response surface of rGUS activity (Fig. 1a). These results are in accordance with the results on extraction of a rGUS genetically modified with a 6-U histidine tail [GUS(6His)] from transgenic pea [17], except that the drop in GUS(6His) extraction from pea in the acidic region was not as pronounced as that observed for rGUS from soybean. This may be due to the fact that the His residues have a pI around 6.0, which could have contributed to the increase in pI of the protein, thereby lowering its solubility in slightly acidic solutions.

rGUS extraction efficiency was also analyzed in terms of specific activity, and both pH and ionic strength were statistically significant. The response surface of rGUS specific activity (Fig. 1b) shows the negative effect of ionic strength. Therefore, the salt concentration should be minimized as much as possible in the extraction of rGUS in order to maximize the ratio of rGUS to native proteins. Maximum GUS specific activity (7596 U/mg) was obtained at pH 5.5 without the addition of salt.

When comparing the extraction of the rGUS from the soybean seeds to the extractions from corn [13] and canola seeds [16], we noted similar kinetics, since after 30 min, the concentration of rGUS did not increase. Also, the highest rGUS activity achieved for the three seeds were in the pH range from 7.0 to 7.5. In terms of extractibility of rGUS, the three systems can be considered equivalent. Therefore, the advantage of one seed over the other as protein expression system will depend on the presence of contaminants present in the extracts such as TSP, phenolic compounds, reducing and total sugars.

Total Soluble Protein

The pH and ionic strength were statistically significant in the extraction of proteins [total soluble protein (TSP)] from the soybean flour. Both pH and ionic strength had a positive effect on protein extraction, meaning that an increase in any of these variables, within the range studied, favors the extraction of native soybean protein. The term related to the interaction of the variables did not show statistical significance, meaning that there is no synergistic effect between these two variables, at least within the range studied. TSP levels varied from 1.36 mg/mL (for pH 4.0 and 30 mmol/L NaCl) to 8.13 mg/mL (for pH 7.6 and 100 mmol/L NaCl), as pH and ionic strength were changed. These results are very similar to the ones found by Smith and Circle [25], who reported that the concentration of soybean seed protein extract at pH 7.6 was 4.3 times that extracted at pH 4.0 using a solution of 100 mmol/L NaCl.

The response surface plot for TSP of transgenic soybean seed flour (Fig. 1c) was practically the same as the one obtained when using a nontransgenic soybean (data not shown). The conditions for maximum native protein extraction are the higher levels of pH and ionic strength. When comparing the response surface plot of soybean seeds with the one obtained for transgenic corn seeds [5], a similar behavior can be observed, but the levels of protein concentration found when using corn seeds were as much as 14 times lower (considering that the authors used a 1:10 solid-to-liquid ratio).

The molecular mass profiles of the extracted proteins were evaluated through SDS-PAGE analysis (Fig. 2). The protein content of extracts prepared at a pH below the pI of soybean proteins (around 4.0) is basically composed of lipoxygenase (molecular mass of 100 kDa), β-amylase (molecular mass of 55 kDa), agglutinin (molecular mass of 32 kDa), and soybean trypsin inhibitor (molecular mass of 20 kDa). Above this pI, the β-conglycinin (subunit molecular masses of approximately 48, 63, and 76 kDa), glicinin (subunit

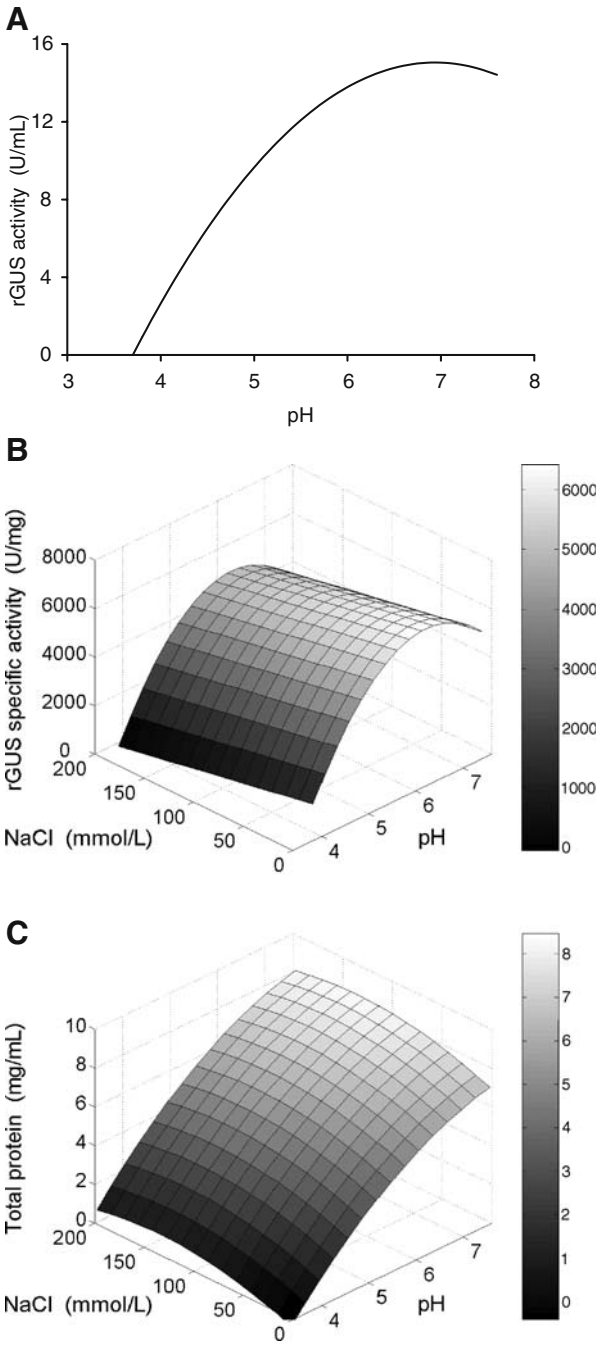

Fig. 1 Response surface plots for concentrations of compounds in the aqueous extracts of transgenic soybean seeds using 50 mmol/L citrate–phosphate buffer with added NaCl at 1:20 solid-to-liquid ratio. **a** rGUS activity; **b** rGUS specific activity; **c** Total protein concentration (TSP). For NaCl, the molar concentration equals the ionic strength of its solutions

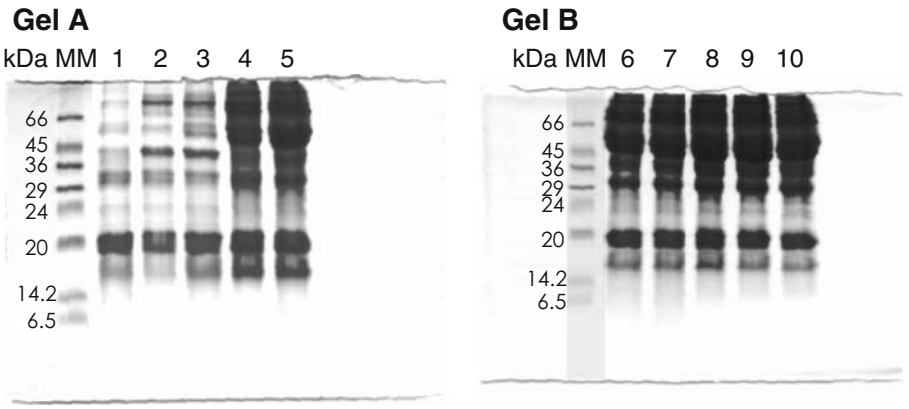

Fig. 2 SDS-PAGE of BR-16 extracts obtained with using 50 mmol/L citrate-phosphate buffer at 1:20 solid-to-liquid ratio at different pH and ionic strength in terms of added NaCl. *MM* molecular mass standards. *Gel A Lane 1* pH 3.4 and 100 mmol/L NaCl, *lane 2* pH 4.0 and 30 mmol/L NaCl, *lane 3* pH 4.0 and 170 mmol/L NaCl, *lane 4* pH 5.5 and 0 mmol/L NaCl, *lane 5* pH 5.5 and 100 mmol/L NaCl. *Gel B Lane 6* pH 5.5 and 100 mmol/L NaCl, *lane 7* pH 5.5 and 200 mmol/L NaCl, *lane 8* pH 7.0 and 30 mmol/L NaCl, *lane 9* pH 7.0 and 170 mmol/L NaCl, *lane 10* pH 7.6 and 100 mmol/L NaCl

molecular masses of approximately 22, 34, and 40 kDa), and soybean trypsin inhibitor are the main proteins extracted. The results obtained are in agreement with the results of Wolf et al. [21]. Since β-conglycinin and glicinin are proteins with high molecular mass (180 and 330 kDa, respectively) and represent 90% of all soybean proteins, the ideal pH of extraction should be near the p*I* of soybean proteins. This extraction condition, besides strongly reducing the native protein content in the extract, also reduces the content of high-molecular-mass native proteins in the extracts and therefore contributes toward simplifying and lowering the cost of recombinant protein DSP.

Reducing Sugars and Total Sugars

The concentration of carbohydrates in the extracts was quantified in terms of reducing sugars (RS) and total sugars (TS). The difference between the RS and TS concentrations corresponds to the acid-hydrolyzable compounds such as polysaccharides and sucrose. The ionic strength of the solution was not statistically significant in the extraction of RS within the range tested (Table 3). In contrast, pH was statistically significant and had a positive effect on the extraction of RS. There was no interaction between these two variables in the extraction of RS from soybean seed flour. Minimum RS extraction could be achieved by using a solution at a low pH value (Fig. 3a). An eight-time reduction in the RS concentration was achieved when extraction pH was lowered from pH 7.6 (0.65 mg/mL) to 3.4 (0.08 mg/mL), since water has a better solvent effect at higher pH values. The concentrations of RS in seed extracts of transgenic soybean are as much as four times lower than those of corn [5].

In terms of TS, the pH and ionic strength of the solution were statistically significant in the extraction of TS. The pH of the solution had a positive effect on the extraction of TS, while the effect of ionic strength was negative. The ANOVA analysis for TS showed that the coefficient of determination and the *F* test were not satisfactory for the prediction of a model (0.06 and 0.77, respectively). The concentration of TS in the soybean flour extracts varied between 7.24 and 8.12 mg/mL, which are around five times the levels of TS in corn

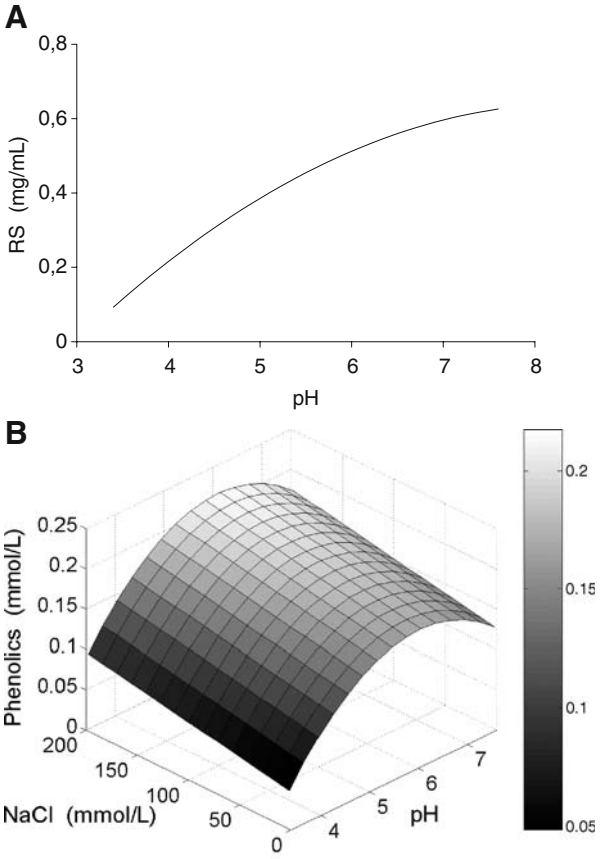

Fig. 3 Response surface plots for concentrations of compounds in the aqueous extracts of transgenic soybean seeds using 50 mmol/L citrate–phosphate buffer with added NaCl at 1:20 solid-to-liquid ratio. **a** RS concentration; **b** Phenolics concentration. For NaCl, the molar concentration equals the ionic strength of its solutions

extracts [5]. An experimental condition to minimize carbohydrate co-extraction would be a lower pH and a higher ionic strength. However, this condition does not favor maximization of rGUS.

Phenolic Compounds

pH and ionic strength were statistically significant in the extraction of phenolic compounds from soybean seed flour (Table 3). Both variables had a positive effect on the extraction of phenolics. Lower concentrations of phenolics were obtained when the pH and the ionic strength of the solutions were at their lowest level, as can be observed in the response surface plot (Fig. 3b).

According to Sosulki *et* al. [26], the majority of phenolic acids is bound to insoluble residues and can be solubilized with aqueous extraction under alkaline conditions. The results presented here corroborate this information. When comparing our results with the ones obtained with corn seeds [5], the levels of phenolic compounds in the soybean seed extracts were found to be about two times higher. But when comparing the ratio of phenolic

compounds to total protein, we concluded that for the case of soybean, it is about two times lower. Also, ionic strength had the opposite effect on the extraction of phenolic compounds, which is positive in the case of soybean and negative in the case of corn. Canola, on the other hand, is known to contain very large amounts of phenolic compounds, as much as 30-fold higher than the soybean seeds [4], while at the same time containing about 30% less protein.

Conclusions

The use of soybean seeds as a bioreactor was evaluated from a process engineering standpoint through analysis of the influence of the variables pH and ionic strength on the extraction efficiency of rGUS together with the concentrations of native proteins, carbohydrates, and found phenolics. The condition to obtain a high efficiency rGUS extraction (high-specific activity) was a pH of around 5.5 with no addition of salt. Also, the extraction of native proteins, phenolic compounds, and soluble carbohydrates is minimized when using a condition of low pH and ionic strength of the extraction buffer. Therefore, the complexity of the native soybean proteins and the fact that most of them have an acidic p*I* did not interfere negatively with the extraction efficiency of the recombinant protein, even for the case of a recombinant protein having a p*I* below 7.0. Besides, soybean seed extracts had lower levels of co-extracted native compounds than corn seed extracts, the crop already used on industrial scale for recombinant protein production. These findings concerning DSP corroborate the proposition of soybean being considered as a potential plant bioreactor.

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