Aqueous Extraction of Recombinant Human Proinsulin from Transgenic Maize Endosperm

Cristiane S. Farinas,[†] Adilson Leite,[‡,§] and Everson A. Miranda*[,†]

LEBp: Laboratório de Engenharia de Bioprocessos, Departamento de Processos Biotecnológicos, Faculdade de Engenharia Química, Universidade Estadual de Campinas, CP 6066, CEP 13083-970, Campinas SP, Brazil, and Laboratório de Plantas, Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, Campinas SP, Brazil

Different plant species have been used as systems to produce recombinant proteins. Maize is a crop considered to have a large potential to produce high levels of recombinant proteins and is the host for the recombinant proteins from plants currently available on the market. In the development of a plant system to produce a recombinant proteins it is important to consider the costs related to downstream processing. Also, the steps necessary to achieve the protein purity required will be highly influenced by the quality of the extract obtained. In this study, we analyzed aqueous extracts from the endosperm of transgenic maize expressing recombinant human proinsulin (rhProinsulin). A study of the effects of the variables pH and ionic strength on the extraction efficiency was carried out using experimental design and response surface methodology. Besides the concentration of the recombinant protein, the characteristics of the extracts were evaluated in terms of concentration of native components (proteins, carbohydrates, and phenolic compounds) and extract filterability. The highest rhProinsulin concentration (97.33 ng/mL) was found with a 200 mM NaCl pH 10.0 extraction solution. Under this experimental condition the concentrations of total soluble proteins, carbohydrates, and phenolics were 2.01 mg/mL, 2.21 mg/mL, and 0.11 mmol/L, respectively.

Introduction

The use of transgenic plants to produce recombinant proteins, also known as molecular farming, has been reviewed by several authors (*1*−*6*). The advantages of plants as expression hosts for recombinant protein over other production systems are mainly related to practicality, cost, and safety (*4*). There are a large variety of plants being used as host for recombinant proteins, such as maize, canola, tobacco, soybean, rice, wheat, alfalfa, and potato (*2*). Among the major cereal crops, maize has been the bioreactor preferred by companies (*7*). In addition, maize is one of the crop species most extensively studied, both genetically and physiologically. The success of large-scale use of plants as bioreactors depends on many aspects such as genetic and agronomical characteristics, and especially in the case of recombinant protein products that require a high level of purity, it also depends on the downstream processing (DSP). Extraction is a key step for efficient DSP, since it defines concentration and complexity of the solution from which the recombinant protein must be purified. Plant species contain different levels of compounds that can be co-solubilized with the recombinant product during extraction (e.g., native proteins, soluble carbohydrates, phenolic compounds, and lipids). Since these compounds are impurities that can be deleterious to the separation media and equipment, they can have a significant effect on DSP in terms of type and sequence of the separation steps. Therefore, they can affect capital and manufacturing costs, and, consequently, the viability of the process.

In this study, we analyzed the aqueous extraction of recombinant human proinsulin (rhProinsulin) from the endosperm of transgenic maize seeds as a function of pH and ionic strength (in terms of NaCl concentration). Human proinsulin is an α-helical protein with a molecular mass of 9,500 Da and an isoelectric point of 5.5. Proinsulin is the precursor in insulin production (it is enzymatically converted to the biologically active insulin used in the treatment of diabetes mellitus).

In addition to concentration of rhProinsulin, other parameters associated with DSP were analyzed: concentrations of the native components of maize endosperm (proteins, carbohydrates, and phenolic compounds) and the filtration flux of the extract. Analysis of these parameters was based on the following considerations: (a) the physicochemical properties and concentration of the native proteins extracted may influence the choice of separation methods employed for purification of recombinant proteins from transgenic plant extracts (*8*); (b) phenolics are one of the most chemically active secondary metabolites and can form complexes with proteins in an aqueous extract that can result in the formation of aggregates (*9*); (c) soluble carbohydrates may cause fouling of filtration membranes and chromatographic resins and can also contribute to microbial growth; and (d) the filtration flux of the extracts may indicate possible problems in equipment such as filters

* To whom correspondence should be addressed. Ph: 55 (19) 3788-3918. E-mail: everson@feq.unicamp.br.
[†] Laboratório de Engenharia de Bioprocessos.
[‡] Laboratório de Plantas.
[§] In memoriam.

10.1021/bp050103r CCC: $30.25
Published on Web 08/17/2005

and chromatographic columns caused by impurities in the extract.

The compositions of the extraction solutions were planned according to a complete factorial design used to investigate the effects of two independent process variables: ionic strength and pH. The response surface methodology was applied, aiming to find an extraction condition that would combine maximization of recombinant protein extraction and minimization of maize native components, thus reducing the burden on subsequent DSP operations.

Materials and Methods

Materials. Transgenic maize seeds expressing the human proinsulin gene were provided by the Plant Laboratory group at CBMEG (Centro de Biologia Molecular e Engenharia Genética), Brazil. Production of the heterologous protein was directed toward the endosperm of the maize seed by using a γ-kafirin gene promoter from sorghum and a signal peptide for α-coixin (*10*). All chemicals used were of at least analytical grade. A DU 650 spectrophotometer (Beckman, USA) was used for the spectrophotometric measurements and a microplate reader (Multiskan II MS, Finland) was used for the ELISA assay measurements at 492 nm.

Methods. ***Preparation of Maize Endosperm-Rich Flour.*** Maize kernels were degermed using a knife mill (Renard, Brazil), and the endosperm-rich fraction was then separated from the germ-rich fraction with a 0.5 mm sieve. The endosperm-rich fraction (fraction with particles larger than 0.5 mm) was then broken down further in a roller mill (Quadrumat, Germany). The hulls were separated using a set of sieves in the outflow of the roller mill, leaving endosperm-rich flour with particles smaller than 0.5 mm. This flour was stored at 4 °C until used in the extraction experiments.

Extraction Protocol. Ten-gram samples of endosperm-rich flour were mixed with 100 mL of the appropriate extraction solution (1:10 solid-to-liquid ratio) in a 5.5 cm diameter 250 mL beaker. Extraction was carried out at room temperature by stirring for 30 min at 270 rpm with a mechanical stirrer (Q-251D, IKA Labortechnik, Germany) equipped with an axial-flow impeller (pitched-blade turbine with four blades with 4.0 cm diameter and 45° angle, positioned at 1 cm from the bottom). Extraction solution was prepared by adding sodium chloride to water (60, 100, 200, 300, and 340 mmol/L) and adjusting the pH to the values established in the experimental design (pH 3.0, 4.0, 6.5, 9.0, and 10.0) using 1.0 mol/L NaOH or 1.0 mol/L HCl (the volumes of these chemicals were measured to ensure that they did not cause a significant increase in the ionic strength of the extraction solution). During extraction, the pH was constantly monitored and adjusted to the desired value. After extraction, each suspension was allowed to stand for 5 min prior to filtering.

Determination of Filtration Flux. The upper portion of the aqueous extracts (approximately 50 mL) was poured onto a 3 μm pore filter paper membrane in a Buchner funnel with a 12 cm diameter. The funnel was positioned over a graduated cylinder and the filtration time was recorded after each 5.0 mL volume was filtrated. Volume of filtrate versus time was plotted, and the filtration flux was calculated as the ratio of the slope of the linear portion of this curve to the area of the filter.

Determination of Protein, Carbohydrate, and Phenolics Concentrations. Total soluble protein (TSP) concentration in the extracts was determined by Bradford's method (*11*) using bovine serum albumin (Sigma, USA) as standard. The protein molecular mass profiles of the extracts were evaluated by SDS−PAGE electrophoresis conducted under denaturing conditions as described by Laemmli (*12*). Gels (15%) were stained with silver nitrate in accordance with Morryssey's method (*13*). rhProinsulin was quantified by ELISA using a commercial kit (DakoCytomation, UK). Phenolics were quantified as described by Price and Butler (*14*) using D-catechin (Sigma, USA) as standard. Soluble carbohydrates were quantified as reducing sugars (RS) and total reducing sugars (TRS) using the dinitrosalicylic acid (DNS) method proposed by Miller (*15*). Glucose and sucrose (both from Synth, Brazil) were used as standards for RS and TRS, respectively.

Table 1. Values of Coded Levels and Real Values for the Factors pH and Ionic Strength Used in the Complete Factorial Design

factor	−1.41	−1	0	+1	+1.41
pH	3.0	4.0	6.5	9.0	10.0
u[a]	60	100	200	300	340

[a] u = NaCl concentration (mmol/L).

Experimental Design. A full factorial design followed by response surface analysis was used to evaluate the effect of two independent variables, ionic strength (in terms of NaCl concentration) and pH, and their possible interaction in the aqueous extract composition and filtration flux. The experimental design selected was a central composite design comprising 11 runs, corresponding to four cube points, four axial points, and three central points, with the experiments carried out in a random order. The factors and levels investigated are shown in Table 1. The dependent variables (responses) were concentrations of rhProinsulin, total soluble protein, reducing sugars, and phenolic compounds as well as filtration flux. 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

Table 2 contains the experimental conditions and the results for concentrations of rhProinsulin, total soluble protein (TSP), reducing sugars (RS), total reducing sugars (TRS), and phenolics as well as filtration flux of the aqueous extracts from maize endosperm. Coefficient values and statistical analysis of the response variables are presented in Table 3.

Recombinant Human Proinsulin (rhProinsulin). Both pH and ionic strength as well as the term related to their interaction were statistically significant within a confidence level of 95% in the extraction of rhProinsulin from transgenic maize endosperm. The pH had a positive effect, whereas the effect of ionic strength was negative (Figure 1). The positive effect of pH means that an increase in pH will favor the extraction of rhProinsulin, whereas the negative effect of ionic strength means that an increase in the value of this parameter will hinder rhProinsulin extraction. The statistical significance of the interaction term is that the effects of pH and ionic strength cannot be interpreted separately, i.e., the effect of these factors together cannot be explained by the sum of their individual effects. The ANOVA of a quadratic model for the rhProinsulin concentration resulted in a correlation coefficient of 0.979 and a calculated F of 48.86, which is 9.7 times higher than the F-value at a confidence level of 95%, 5.05. The coefficients of the coded model

Table 2. Central Composite Design and Responses (rhProinsulin, TSP, RS, TRS, and Phenolics Concentrations and Filtration Flux) for the Aqueous Extracts

	independent variable		concentration values					
run	pH	u	rhProinsulin (ng/mL)	TSP (mg/mL)	RS (mg/mL)	TRS (mg/mL)	phenolics (mmol/L)	filtration flux ($mL \cdot min^{-1} \cdot cm^{-2}$)
1	9.0	300	40.71	4.02	0.80	2.81	0.03	0.006
2	9.0	100	72.74	1.68	0.74	2.27	0.14	0.008
3	4.0	300	0.45	0.85	1.17	2.77	0.08	0.302
4	4.0	100	0.49	0.23	1.02	2.57	0.06	0.420
5	6.5	200	2.29	1.33	1.36	3.03	0.07	0.139
6	6.5	200	2.30	1.29	1.17	2.67	0.06	0.128
7	6.5	200	2.07	0.96	1.26	2.69	0.08	0.134
8	10.0	200	97.33	2.01	0.49	2.21	0.11	0.020
9	3.0	200	0.25	0.28	0.47	2.28	0.03	0.240
10	6.5	340	2.47	1.13	0.94	2.52	0.06	0.130
11	6.5	60	1.35	0.98	0.95	2.53	0.09	0.100

Table 3. Coefficient Values and Statistical Analysis for rhProinsulin, TSP, RS, TRS, and Phenolics Concentrations and the Filtration Flux of the Aqueous Extracts

coefficients	rhPro-insulin	TSP	RS	TRS	phenolics	filtration flux
mean	2.22^b	1.19^a	1.26^b	2.79^a	0.069^a	0.134^b
pH	31.23^b	0.88^a			0.016^a	-0.128^b
pH^2	24.10^b		-0.34^b			0.014^b
u	0.66^b	0.39^a			-0.014^a	0.009^b
u^2	-3.81^b					
pH·u	-7.99^b	0.43^a			-0.033^a	-0.029^b
R^c	0.979	0.773	0.667	0.602	0.871	0.818
F-value	48.86	7.96	18.05	1.52	15.73	6.75
F_{calcd}/F_{value}	9.7	2.59	3.52	0.44	3.62	2.12

[a] Significant at 0.10 level. [b] Significant at 0.05 level. [c] R = coefficient of determination.

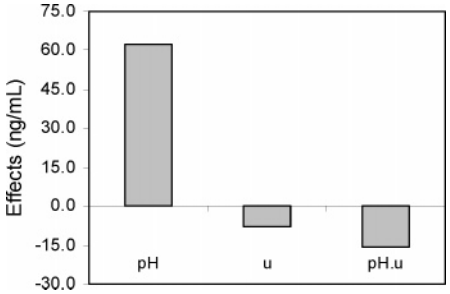

Figure 1. Effects of pH and ionic strength of the solution on the concentration of rhProinsulin in the extracts of transgenic maize endosperm.

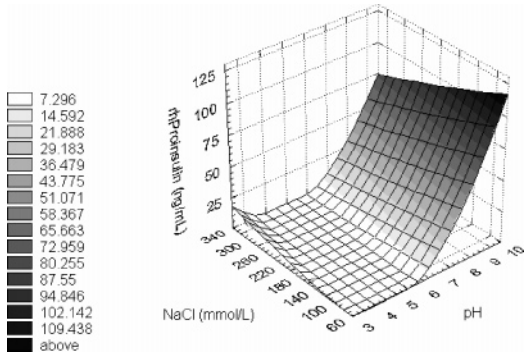

Figure 2. Response surface plot for concentration of rhProinsulin in the aqueous extracts of transgenic maize endosperm.

used to describe the response surface of rhProinsulin concentration (Figure 2) are listed in Table 3.

The effect of pH was more significant than the effect of ionic strength (Figure 1), as can also be verified by the higher coefficient values for pH in Table 3. However, even in the optimum range on the response surface, with pH at the higher level and ionic strength at the lower level, the concentration of rhProinsulin in the extracts was relatively low (97.33 ng/mL) compared to semiquantitative Western blot results obtained by De Lucca (*10*). He estimated rhProinsulin concentrations around 20 μg/mL (200 times higher) in the maize endosperm extracts obtained with a complex solvent (50 mM Tris-HCl pH 8.5 buffer with 0.2% Triton X-100, 2.0 mM EDTA, 5.0 mM benzamidine, and 5.0 mM DDT) and a high shear rate mixing.

The extracts with low rhProinsulin concentrations reported here were rather unexpected, since proinsulin is soluble under most of the extraction conditions used. De Lucca (*10*) suggested that rhProinsulin is associated with the maize endosperm protein bodies formed by zeins. One possible reason for the low concentrations obtained in this work is this association between rhProinsulin and protein bodies. These protein bodies seem to be solubilized under the drastic conditions used by De Lucca (*10*), but not under the mild conditions used in this work. Bailey et al. (*16*) had similar difficulties with the solubilization of recombinant laccase produced in transgenic corn. The expression of this enzyme was targeted to the cell wall. Aqueous extraction at optimum conditions left up to 90% of the laccase activity retained in the cell debris pellet, indicating that the laccase was immobilized in the cell wall matrix. Rees and Singer (*17*) found indications that insulin and zeins have structural similarities. Given the hydrophobic nature of zeins, the rhProinsulin could be attached to them through hydrophobic interaction. The negative effect of ionic strength on rhProinsulin extraction corroborated this fact.

Thus, process development must address the possible association of the heterologous protein with native host components. Here we showed that an extraction condition under which the heterologous protein is soluble may not be sufficient for its extraction from the plant material. This is a reason for special attention in the case of DSP studies using the spiking method—the addition of the target protein to an extract from a nontransgenic plant in order to emulate the protein extract from transgenic plants, an interesting strategy since producing transgenic seed is time-consuming and expensive (*18*–*21*).

Total Soluble Protein (TSP). The degree of the effects of pH and ionic strength of the solution as well as the effect of interaction between these two variables on TSP extraction is shown in Figure 3. The linear terms for pH and ionic strength as well as the term related to their interaction were statistically significant within a confidence level of 90%, with the effects of both pH and ionic strength being positive. By increasing ionic strength, besides maize albumins, globulins should also be solu-

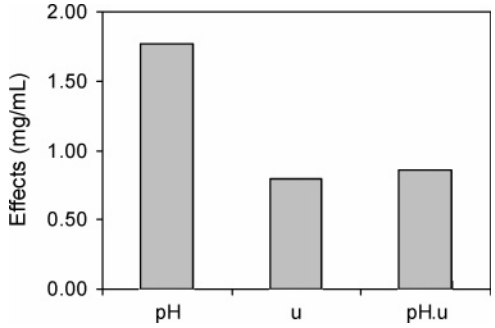

Figure 3. Effects of pH and ionic strength of the solution on the TSP concentration in the extracts of transgenic maize endosperm.

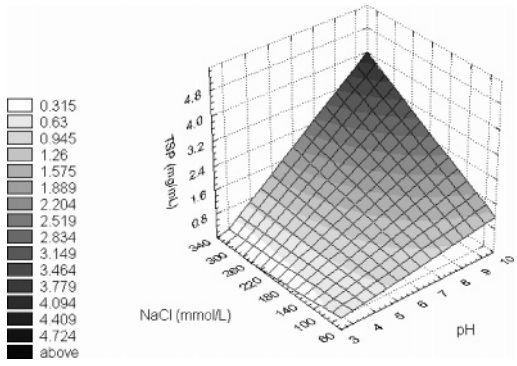

Figure 4. Response surface plots for concentration of TSP in the aqueous extracts of transgenic maize endosperm.

bilized, since it is known that they are associated with ionic interactions that are weakened by salt. When using an alkaline pH some glutelins could also be found in the extracts.

TSP levels varied from 0.28 to 4.02 mg/mL as pH and ionic strength were changed. Balasubramaniam et al. (*21*) found that the pH of the buffer used for protein extraction from tobacco had a significant effect on protein concentration and that by increasing the pH from 3.0 to 9.0 the total protein extracted increased from 1.0% to 1.6% of the biomass. Azzoni et al. (*22*) showed that the protein concentration extracted from transgenic maize seeds increased from 0.25% to 2.0% (w/w) when the pH increased from 3.0 to 10.0.

The response surface plot for TSP is shown in Figure 4. The conditions for maximum native protein extraction are at the upper levels of pH and ionic strength. Nevertheless, it was the aim of this study to find a condition that could maximize extraction of the heterologous protein while minimizing extraction of the native proteins. In terms of pH, extraction of both native and the heterologous protein would be favored at an alkaline pH. However, in terms of ionic strength, it is possible to minimize the native proteins and maximize rhProinsulin extraction by using a low salt concentration (60 mmol/L NaCl).

The molecular mass profiles for the extracted proteins were evaluated through SDS−PAGE analysis (Figure 5). One of the extracts that showed a highly complex protein profile (lane 1, for pH 9.0 and 300 mmol/L NaCl, level +1 for both variables) corresponds to run 1 (Table 2), which also had a maximum value of TSP concentration (4.02 mg/mL). Extracts obtained at a pH higher than 6.5 also showed highly complex protein profile (lanes 2, 5, 6, 8, and 9). Therefore, the low pH solutions resulted in extracts with less complex protein profiles, besides low protein concentrations. Thus, a lower pH can be advantageous when the heterologous protein is basic (pI >7) and

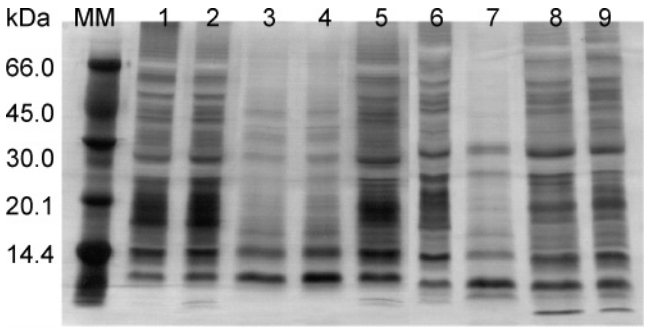

Figure 5. Protein molecular mass profile for the extracts obtained under conditions planned according to the experimental design. Lane MM: molecular mass markers; lane 1: 300 mmol/L NaCl at pH 9.0; lane 2: 100 mmol/L NaCl at pH 9.0; lane 3: 300 mmol/L NaCl at pH 4.0; lane 4: 100 mmol/L NaCl at pH 4.0; lane 5: 200 mmol/L NaCl at pH 6.5; lane 6: 200 mmol/L at pH 10.0; lane 7: 200 mmol/L NaCl at pH 3.0; lane 8: 340 mmol/L NaCl at pH 6.5; and lane 9: 60 mmol/L NaCl at pH 6.5. Sample volume, 10 μL.

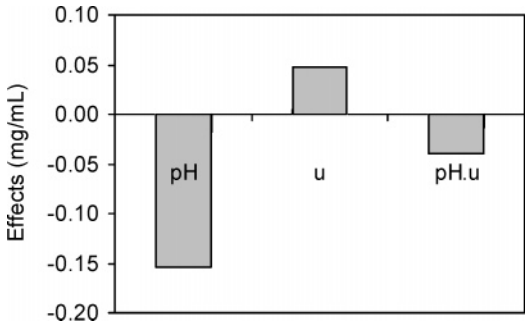

Figure 6. Effects of pH and ionic strength of the solution on the concentration of RS in the extracts of transgenic maize endosperm.

therefore has a higher solubility at pH <7.0, since a higher ratio of recombinant protein to native proteins could be achieved, simplifying the subsequent purification steps, as verified by Azzoni et al. (*22*). However, that was not the case in this work, since rhProinsulin required an alkaline pH for better extraction. Some bands of molecular mass smaller than 14,400 Da could be assigned to proteins with the molecular mass of proinsulin (9,500 Da). However, they are not related to rhProinsulin, since the intensity of these bands is too strong for the concentration determined with the ELISA method.

Reducing Sugars (RS) and Total Reducing Sugars (TRS). The concentration of carbohydrates in the extracts was quantified in terms of RS and TRS. D-Glucose and D-fructose are the major RS in corn seeds, and phytoglycogen (a branched polysaccharide formed of up to 30 glucose units with a structure similar to mammalian glycogen) is the most common water-soluble polysaccharide (*23*). The difference between the RS and TRS 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 within a confidence level of 95% and had a negative effect on the extraction of RS (Figure 6). There was no interaction between these two variables in the extraction of RS. Minimum RS extraction (around 0.2 mg/mL) could be achieved by using a solution at extreme pH values (Figure 7).

In terms of TRS, none of the independent variables was statistically significant in the extraction of TRS within a confidence level of 90%. The pH of the solution had a

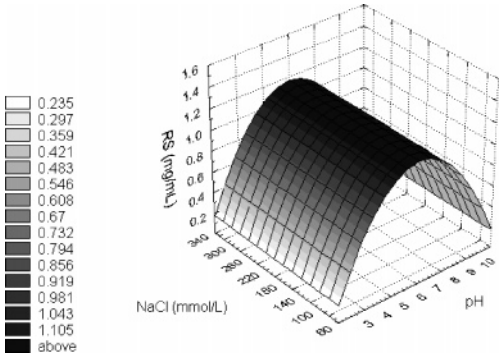

Figure 7. Response surface plots for concentration of RS in the aqueous extracts of transgenic maize endosperm.

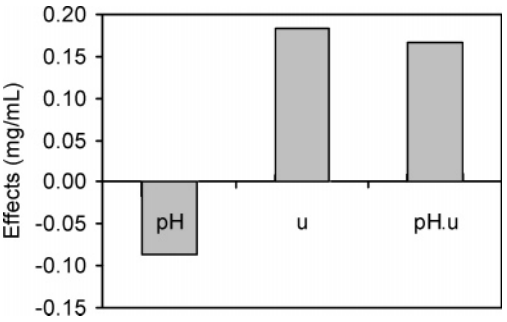

Figure 8. Effects of pH and ionic strength of the solution on the concentration of TRS in the extracts of transgenic maize endosperm.

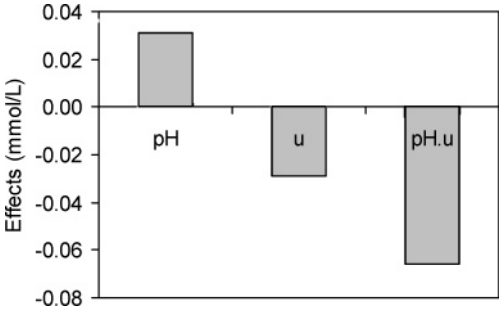

Figure 9. Effects of pH and ionic strength of the solution on the concentration of phenolics in the extracts of transgenic maize endosperm.

negative effect on the extraction of TRS, whereas the effect of ionic strength was positive (Figure 8). The ANOVA for TRS showed that the coefficient of correlation and the *F*-test were not satisfactory for the prediction of a model (0.602 and 1.52, respectively).

As in the case of TSP, an experimental condition to maximize rhProinsulin extraction and minimize carbohydrate co-extraction would be an alkaline pH and a low ionic strength.

Phenolic Compounds. The linear terms for pH and ionic strength as well as their interaction were statistically significant in the extraction of phenolic compounds within a confidence level of 90% (Table 3). The pH of the solution had a positive effect, whereas the ionic strength had a negative one (Figure 9). The effect of the interaction between the two variables can be observed in the response surface plot (Figure 10). Lower concentrations of phenolics were obtained when the pH and the ionic strength of the solutions were at their lower levels, whereas an alkaline pH resulted in a higher phenolics extraction, which was minimized only with an increase in the ionic strength of the solution.

According to Sosulki et al. (*24*), the majority of the phenolic acids (which are the predominant type of

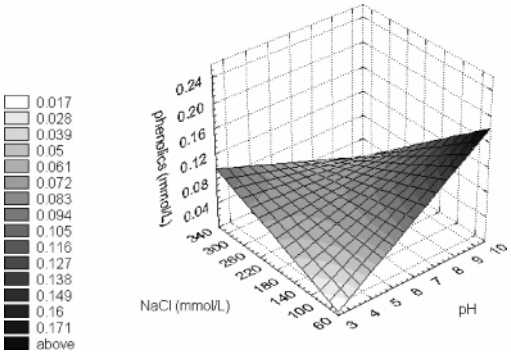

Figure 10. Response surface plots for concentration of phenolics in the aqueous extracts of transgenic maize endosperm.

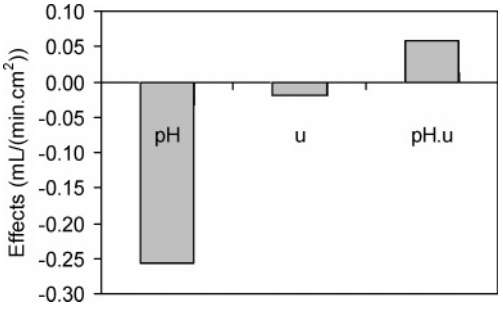

Figure 11. Effects of pH and ionic strength of the solution on the filtration flux of the aqueous extracts of transgenic maize endosperm.

phenolics in maize) are bound to insoluble residues and could be solubilized with aqueous extraction under alkaline conditions. The results presented here corroborate this information. The negative effect of ionic strength on the extraction of phenolics at an alkaline pH was also observed by Price and Butler (*14*). They studied the effect of NaCl in the extraction of tannin from sorghum and found that a relatively small proportion of high-molecular-mass phenolics was extracted by salt solutions.

The condition that maximizes phenolics extraction coincides with that which maximizes rhProinsulin extraction (high pH and low ionic strength). However, even the highest phenolic concentration obtained, 0.14 mmol/L or 0.038 mg/mL (at pH 9.0 and 100 mmoL/L NaCl), would probably not cause protein aggregation, since according to Maa and Hsu (*25*), the concentration needed for that would be in the range of 1.5−15 mg/mL. The maximum value found (0.038 mg/mL or 38 ppm) is well below the phenolic acid content in maize flour reported by Sosulki et al. (*24*), 309 ppm.

Extract Filterability. The linear and quadratic terms for pH, the linear term for ionic strength, and the term for interaction between pH and ionic strength were statistically significant for the filtration flux of the extracts, within a confidence level of 95% (Table 3). The pH of the extraction solution had a negative effect on extract filterability, i.e., at an alkaline pH the filtration flux was lower than at an acidic pH. Ionic strength also had a negative effect on extract filterability (Figure 11). The conditions that resulted in lower filterability were the same as those that resulted in higher protein concentration in the extracts. However, the relatively small increase in protein concentration from 0.23 to 4.02 mg/mL cannot be the sole reason for this low filterability. The main reason for the low filtration flux observed is not clear but is certainly associated with solubilization of other native compounds and generation of small particles during extraction.

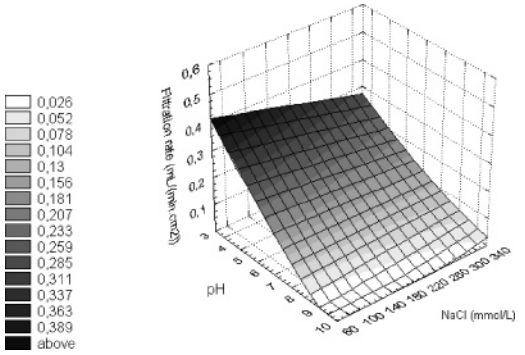

Figure 12. Response surface plots for filtration flux of the aqueous extracts of transgenic maize endosperm.

The response surface plot for the filtration flux is shown in Figure 12. Ideally, a favorable condition for recombinant protein extraction would be one of higher filterability, i.e., keeping the pH and ionic strength at their lower levels. Therefore, since rhProinsulin extraction was better achieved by using solutions of alkaline pH and low ionic strength, it was not possible to find a condition for both high rhProinsulin extraction and high filterability.

Conclusions

Aqueous extraction of rhProinsulin from endosperm of transgenic maize seeds was evaluated in association with concentration of native proteins, carbohydrates, and phenolics and extract filterability. The condition to maximize rhProinsulin extraction was found to be a low ionic strength and a high pH. However, even at the optimum condition, rhProinsulin was not efficiently extracted, suggesting that the protein could be attached to some native maize component that is not readily soluble in the extraction solutions used. Nevertheless, this study showed that certain impurities, such as native proteins and reducing sugar, are worth investigating, since it could be possible to find a condition that minimizes these components in the extracts while maximizing the heterologous protein concentration, thereby reducing the burden during the downstream purification process.

Acknowledgment

The authors would like to thank Dr. Zivko L. Nikolov (Texas A&M University, USA), Dr. José A. Yunes (C.I. Boldrini, Brazil), and Dr. Elíbio Rech (EMBRAPA, Brasília, Brazil) for their comments on the manuscript, Dr. Paulo C. De Lucca (currently at Alellyx Applied Genomics, Brazil) for the donation of transgenic maize seeds, and CNPq and FAPESP (both in Brazil) for their financial support.

References and Notes

(1) Fischer, R.; Emans, N. Molecular Farming of Pharmaceutical Proteins. *Transgenic Res.* **2000**, *9*, 279−299.

(2) Daniell, H.; Streatfield, S. J.; Wycoff, K. Medical Molecular Farming: Production of Antibodies, Biopharmaceuticals and Edible Vaccines in Plants. *Trends Plant Sci.* **2001**, *6*, 219−226.

(3) Ma, J. K.; Drake, P. M. W.; Christou, P. The Production of Recombinant Pharmaceutical Proteins in Plants. *Nat. Rev. Genet*. **2003**, *4*, 794−805.

(4) Twyman, R. M.; Stoger, E.; Schillberg, S.; Christou, P.; Fisher, R. Molecular Farming in Plants: Host Systems and Expression Technology. *Trends Biotechnol.* **2003**, *21*, 570−578.

(5) Gomond, V.; Sourrouille, C.; Fitchette, A. C.; Bardor, M.; Pagny, S.; Lerouge, P.; Faye, S. Production and Glycosilation of Plant-made Pharmaceuticals: The Antibodies as a Challenge. *Plant Biotechnol. J.* **2004**, *2*, 83−100.

(6) Horn, M. E.; Woodard, S. L.; Howard, J. A. Plant Molecular Farming: Systems and Products. *Plant Cell Rep*. **2004**, *22*, 711−720.

(7) Giddings, G.; Allison, G.; Brooks, D.; Carter, A. Transgenic Plants as Factories for Biopharmaceuticals. *Nat. Biotechnol.* **2000**, *18*, 1151−1155.

(8) Menkhaus, T. J.; Bai, Y.; Zhang, C.; Nikolov, Z. L.; Glatz, C. E. Considerations for the Recovery of Recombinant Proteins from Plants. *Biotechnol. Prog*. **2004**, *20*, 1001−1014.

(9) Jervis, L.; Pierpoint, W. S. Purification Technologies for Plant Proteins. *J. Biotechnol*. **1989**, *11*, 161−198.

(10) De Lucca, P. C. Ph.D. Dissertation, Institute of Biology at the State University of Campinas, Campinas, SP, 2003.

(11) Bradford, M. M. A. Rapid and Sensitive Method for a Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(12) Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of the Bacteriophage T4. *Nature* **1970**, *227*, 680−685.

(13) Morryssey, J. H. Silver Strain for Proteins in Polyacrylamine Gels: A Modified Procedure with Enhanced Uniform Sensitivity. *Anal. Biochem.* **1981**, *117*, 307−310.

(14) Price, M. L.; Butler, L. G. Rapid Visual Estimation and Spectrophotometric Determination of Tannin Content of Sorghum Grain. *J. Agric. Food Chem.* **1977**, *25*, 1268−1273.

(15) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31*, 426−428.

(16) Bailey, M. R.; Woodard, S. L.; Callaway, E.; Beifuss, K.; Magallanes-Lundback, M.; Lane, J. R.; Horn, M. E.; Delaney, D. D.; Ward, M.; Van Gastel, F.; Howard, J. A.; Hood, E. E. Improved Recovery of Active Recombinant Laccase from Maize Seed. *Appl. Microbiol. Biotechnol*. **2004**, *63*, 390−397.

(17) Rees, E. D.; Singer, S. J. A Preliminary Study of the Properties of Proteins in Some Nonaqueous Solvents. *Arch. Biochem. Biophys*. **1956**, *63*, 144−159.

(18) Zaman, F.; Kusnadi, A. R.; Glatz, C. E. Strategies for Recombinant Protein Recovery from Canola by Precipitation. *Biotechnol. Prog*. **1999**, *15*, 488−492.

(19) Zhang, C.; Glatz, C. E. Process Engineering Strategy for Recombinant Protein Recovery from Canola by Cation Exchange Chromatography, *Biotechnol. Prog*. **1999**, *15*, 12−18.

(20) Menkhaus, T. J.; Eriksson, S. U.; Whitson, P. B.; Glatz, C. E. Host Selection as a Downstream Strategy: Polyelectrolyte Precipitation of β-glucuronidase from Plant Extracts. *Biotechnol. Bioeng*. **2002**, *77*, 148−154.

(21) Balasubramaniam, D.; Wilkinson, C.; Van Cott, K.; Zhang, C. Tobacco Protein Separation by Aqueous Two-phase Extraction. *J. Chromatogr. A* **2003**, *989*, 119−129.

(22) Azzoni, A. R.; Kusnadi, A. R.; Miranda, E. A.; Nikolov, Z. L. Recombinant Aprotinin Produced in Transgenic Corn Seed: Extraction and Purification Studies. *Biotechnol. Bioeng*. **2002**, *80*, 268−276.

(23) Boyer, C. D.; Shannon, J. C. Carbohydrates of the Kernel. In *Corn Chemistry and Technology*; Watson, S. A., Ramstad, P. E., Eds.; American Association of Cereal Chemists Inc: St. Paul, 1987; pp 253−272.

(24) Sosulski, F.; Krygier, K.; Hogge, L. Free, Esterified, and Insoluble-Bound Phenolic Acids. 3. Composition of Phenolic Acids in Cereal and Potato Flours. *J. Agric. Food Chem*. **1982**, *30*, 337−340.

(25) Maa, Y. F.; Hsu, C. C. Aggregation of Recombinant Human Growth Hormone Induced by Phenolic Compounds. *Int. J. Pharm.* **1996**, *140*, 155−168.

Accepted for publication July 20, 2005.

BP050103R