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RECOMBINANT HUMAN PROINSULIN FROM TRANSGENIC CORN ENDOSPERM: SOLVENT SCREENING AND EXTRACTION STUDIES

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Abstract - Recombinant pharmaceutical proteins are being produced in different systems such as bacteria and mammalian cell cultures. The use of transgenic plants as bioreactors has recently arisen as an alternative system offering many practical and economic advantages. However, finding an optimum strategy for the downstream processing (DSP) of recombinant proteins from plants still remains a challenge. In this work, we studied the extraction of recombinant human proinsulin (rhProinsulin) produced in the endosperm of transgenic corn seeds. An efficient extraction solvent was selected and the effects of temperature, solvent-to-solid ratio, time, and impeller rotational speed on the extraction were evaluated using an experimental design. After an extraction kinetics study, temperature was further evaluated to maximize rhProinsulin concentration in the extracts and to minimize the native corn components carbohydrates, phenolic compounds, and proteins. A high efficiency condition for extracting rhProinsulin with the selected solvent – 50 mM sodium bicarbonate buffer pH 10.0 and 5 mM DTT – was an extraction time of 2 h at a solvent-to-solid ratio of 10:1 and 25°C. The maximum rhProinsulin concentration in the extracts at that condition was 18.87 mg l^{-1} or 0.42% of the total soluble protein. These values are within the range in which the production of pharmaceutical proteins in plants can be competitive with other expression systems. The results presented provide information for the development of an additional production platform for the hormone insulin.
Keywords: Transgenic corn; Human proinsulin; Recombinant protein; Molecular farming; Extraction.

INTRODUCTION

Plants have been considered as a potential system for the production of recombinant pharmaceutical proteins. The advantages of plants over traditional systems include the low cost of production, the simple scale-up, the absence of human pathogens, and the ability to accurately fold and assemble complex proteins (Ma et al., 2003). More than 100 recombinant proteins – including antibodies, vaccines, human blood products, hormones, and growth regulators – have been expressed in a range of different plant species (Twyman et al., 2003). Although there are several studies focusing on improving the expression level of the recombinant protein in plants, there have been few related downstream processing (DSP) studies, and finding an optimum strategy for DSP remains a challenge. The cost of DSP is important in determining the economic viability of the plant system selected and must be addressed when planning scale-up and commercialization. The cost breakdown for a highly

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purified pharmaceutical protein from transgenic corn showed that the transgenic raw material constitutes only 5-10% of the total manufacturing cost, whereas extraction and purification comprise as much as 90% (Nikolov and Hammes, 2002).

One of the DSP steps in the production of recombinant proteins from transgenic plants, extraction is a key unit operation since it defines the feed composition. In spite of this, there are only a few reports that address extraction. Larrick et al. (2001) studied the production of antibodies in tobacco and found that grinding transgenic tobacco in water instead of using a complex buffer was an efficient strategy since this operation released up to 70% of the immunoglobulin. Kusnadi et al. (1998) investigated the efficiency of the extraction of rGUS produced in transgenic corn. They found that the presence of salt (0.5 M NaCl), a detergent (0.05% Tween-20), or a cocktail of protease inhibitors in the extraction buffer did not increase the extraction yield of rGUS. Azzoni et al. (2002) studied the extraction and purification of recombinant aprotinin from transgenic corn. They showed that using a buffer at pH 3.0 with 200 mM NaCl it was possible to maximize aprotinin concentration and its fraction of the total soluble protein in the extract, thus reducing the amount of impurities in subsequent unit operations. Robic et al. (2006) showed that pH and not NaCl concentration is the a variable that strongly affects the extraction of rGUS from transgenic soybean seeds.

In this work we studied the extraction of recombinant human proinsulin (rhProinsulin) produced in transgenic corn seeds. rhProinsulin is the precursor to insulin production and it can be enzymatically converted into the biologically active insulin used in the treatment of diabetes mellitus (Winter et al., 2002). Human proinsulin is an α-helical protein with a molecular mass of 9,500 Da and an isoelectric point of 5.5. Currently insulin production is primarily based on the expression of a proinsulin fusion protein in *Escherichia coli* or *Saccharomyces cerevisiae*. Even though recombinant insulin production by fermentation is a well-established process, the cost of insulin production is still high. The transgenic plant used in this work is an additional platform for the production of this important hormone.

Previous studies (Farinas et al., 2005) showed that rhProinsulin was not efficiently extracted with aqueous solutions of NaCl at different concentrations and pH values, probably due to interaction with native maize components. Here we report on a study of 30 extraction solvents (mono and multicomponent) aiming to find an efficient extraction solvent for rhProinsulin that offers simplicity and low cost. Once an extraction solvent was selected, the effects of operational variables (temperature, solvent-to-solid ratio, time, and impeller rotational speed) on rhProinsulin extraction were analyzed using a complete 2^4 factorial design. As a result, a specific set of experimental conditions was selected for rhProinsulin extraction. The concentration of the native corn components, proteins, carbohydrates, and phenolics, were also analysed. The study of these native corn components is relevant due to their deleterious effects on DSP unit operations.

MATERIALS AND METHODS

Materials

Transgenic corn seeds expressing the human proinsulin gene were provided by the Plant Laboratory at CBMEG (Centro de Biologia Molecular e Engenharia Genética, Unicamp, Brazil). Expression of the heterologous protein was directed towards the endosperm of the maize seed using a γ-kafirin gene promoter from sorghum and a signal peptide for α-coixin (De Lucca, 2003). Recombinant human proinsulin produced in *Escherichia coli* used as the positive control was kindly donated by BIOMM, Brazil. All solutions used for solvent preparation and extract analysis were prepared with deionized water (Milli-Q System, Millipore, USA). All chemicals used were of at least analytical grade. A DU 650 spectrophotometer (Beckman, USA) was used for spectrophotometric measurements and a microplate reader (Multiskan II MS, Finland) was used for the ELISA assay measurements at 492 nm.

METHODS

Seed Degerming and Grinding

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. The flour was stored at 4°C until it was used in the extraction experiments.

Extraction for Solvent Selection

Small-scale rhProinsulin extractions were used for solvent screening experiments. Endosperm-rich flour (50.0 mg) was vigorously mixed with 0.50 ml of a specific extraction solvent at room temperature (Table 1) for 5 min using a high-speed homogenizer. Samples were then stirred for 3h in an orbital mixer (Marconi, Brazil) at a speed just high enough to have the solids suspended. After that suspensions were centrifuged at 13,000 *g* for 20 min and supernatants were analyzed with SDS-PAGE and Western blot techniques.

Table 1: Solutions evaluated for rhProinsulin extraction from transgenic corn endosperm.

Solvent #	Solvent
1	4 M urea
2	4 M urea with 5 mM dithiothreitol (DTT)
3	4 M urea with 0.2% Triton X-100
4	0.2% Triton X-100
5	5 mM DTT
6	0.2% Triton X-100 with 5 mM DTT
7	50 mM sodium bicarbonate buffer at pH 10.0
8	50 mM sodium bicarbonate buffer at pH 10.0 with 4 M urea
9	50 mM sodium bicarbonate buffer at pH 10.0 with 0.2%Triton X-100
10	50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT
11	50 mM sodium bicarbonate buffer at pH 9.0 with 5 mM DTT
12	50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT and 4 M urea
13	50 mM Tris-glycine buffer at pH 3.0 with 200 mM NaCl
14	300 mM NaCl at pH10.0 with 0.6% 2-mercaptoethanol
15	70% ethanol
16	70% ethanol with 5 mM DTT
17	70% ethanol - 30% 50 mM Tris-glycine buffer at pH 3.0
18	70% ethanol - 30% 50 mM Tris-glycine buffer at pH 3.0 with 5 mM DTT
19	70% ethanol - 30% 50 mM sodium bicarbonate buffer at pH 10.0
20	70% ethanol - 30% 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT
21	70% isopropanol
22	70% isopropanol - 30% 50 mM Tris-glycine buffer at pH 3.0
23	70% isopropanol - 30% 50 mM sodium bicarbonate buffer at pH 10.0
24	30% isopropanol - 70% 30 mM sodium acetate buffer at pH 7.0
25	70% isopropanol - 30% 50 mM Tris-glycine buffer at pH 3.0 with 5 mM DTT
26	80% isopropanol with 5 mM DTT
27	60% isopropanol with 5 mM DTT
28	45% isopropanol with 5 mM DTT
29	30% isopropanol with 5 mM DTT
30	Control solvent (50 mM Tris-HCl buffer at pH 8.5 with 2 mM EDTA, 5 mM benzamidine, 5 mM DTT and with 0.2% Triton X-100)

Extraction for Studies of Operational Variables

A study of the effects of operational variables on the extraction of rhProinsulin and selection of high-efficiency conditions was conducted in a glass reactor (5.5 cm diameter) equipped with a waterjacket for temperature control. Suspensions were stirred using a stirrer (Q-251D, IKA Labortechnik, Germany) equipped with an axial-flow impeller (pitched-blade turbine with four blades 4 cm in diameter at 45 degree angles, positioned 1 cm from the bottom). Endosperm-rich flour (at different solvent-to-solid ratios) was mixed with 50 ml of the selected extraction solvent (50 mM sodium bicarbonate buffer pH 10.0 with 5 mM DTT). After extraction, the suspension was centrifuged at 13,000 *g* for 20 min to separate the extract (clarified supernatant) from spent solids. The extract was analyzed for rhProinsulin, total soluble protein, phenolics, and carbohydrates.

Analysis of the Extracts

Total soluble protein (TSP) concentration in aqueous extracts was determined with the method of Bradford (1976) using bovine serum albumin (Sigma, USA) as standard. Molecular mass profiles for proteins in the extracts were evaluated with SDS-

PAGE conducted under denaturing conditions as described by Laemmli (1970). Gels (15%) were stained with Coomassie Brilliant Blue G 250. rhProinsulin concentration was determined as total proinsulin with an enzyme-linked immunosorbent assay (ELISA) kit from DakoCytomation, UK. Western blotting of the extracts was done as described by De Lucca (2003). Soluble carbohydrates were quantified as reducing sugars (RS) and total reducing sugars (TRS, which include acid hydrolysable compounds such as polysaccharides and sucrose) using the dinitrosalicylic acid method proposed by Miller (1959) with glucose and sucrose as standards, respectively (Synth, Brazil). Phenolics were quantified as described by Price and Butler (1977) using D-catechin (Sigma, USA) as the standard.

Experimental Design Methodology

A full 2^4 factorial design was used to evaluate the effect of four independent process variables – temperature, solvent-to-solid ratio (v:m ratio), extraction time, and impeller rotational speed – on rhProinsulin extraction. The experimental design had 19 runs including the three central points. Experiments were carried out in a random order. The factors and levels investigated are shown in Table 2. The dependent variables (responses) were rhProinsulin concentration and rhProinsulin mass extracted per mass of flour. The software Statistica (Statsoft, version 5.0) was used to analyze the experimental data and generate the ANOVA data (analysis of variance).

Table 2: Levels of variables for the complete 2^4 factorial design.

Coded variable levels	-1	0	+1
Impeller rotational speed (rpm)	300	500	700
Solvent-to solids ratio (ml g^{-1})	5:1	7.5:1	10:1
Temperature (°C)	4	22	40
Time (min)	60	120	180

RESULTS AND DISCUSSION

Selection of Solvent

The effects of different compounds on rhProinsulin extraction were investigated individually and in different combinations (Table 1). The efficiency of extraction was evaluated qualitatively with SDS-PAGE and Western blot analysis.

The rhProinsulin extractions with aqueous solutions of a single component such as urea (a chaotropic agent), Triton X-100 (a nonionic detergent), DTT (a reducing agent), sodium bicarbonate buffer and alcohol-based solutions (with ethanol or isopropanol) (solvents # 1, 4, 5, 7, 15, and 21) did not result in any significant extraction, since there was no visible band for rhProinsulin in the Western blot analysis (data not shown). Higher extraction efficiency was obtained by combining some of the components (solvents # 8, 11, and 12), nevertheless extracts had a high complex protein profile in terms of molecular mass. The best recovery was achieved with 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT (solvent # 10; Figure 1, lane 4). Solvents # 2, 3, 6, 9, 13, and 14 were not efficient in proinsulin recovery. As the control solvent (Figure 1, lane 7), we used a complex solution (solvent #30) comprising 50 mM Tris-HCl at pH 8.5 with 2 mM EDTA, 5 mM benzamidine, 5 mM DTT and 0.2% Triton X-100 (De Lucca, 2003), which was less effective than 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT. The band for rhProinsulin (MM of 9,500 Da) in the SDS-PAGE images (Figures 1 and 2) is indicated by an arrow (lane C+ with control proinsulin sample). However, the bands corresponding to that molecular mass in the other lanes of Figure 1 most probably do not correspond to proinsulin, since there was no visible band in the corresponding Western blot for lanes 2, 3, 5 and 6.

Since there was no condition that resulted in an extract with high rhProinsulin extraction and low protein complexity, we further investigated the use of alcohol-based solutions (solvents #15-29) as (Figure 2). Even though the total protein complexity obtained in these extracts was relatively low when comparing the fewer number of bands visualized in the SDS-PAGE gels of Figure 2 relative to Figure 1, the extraction of rhProinsulin was not efficient, since only weak bands were detected with the Western blot analysis for solvents #15 to #29. Based on these results, the solvent 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT was selected for subsequent studies.

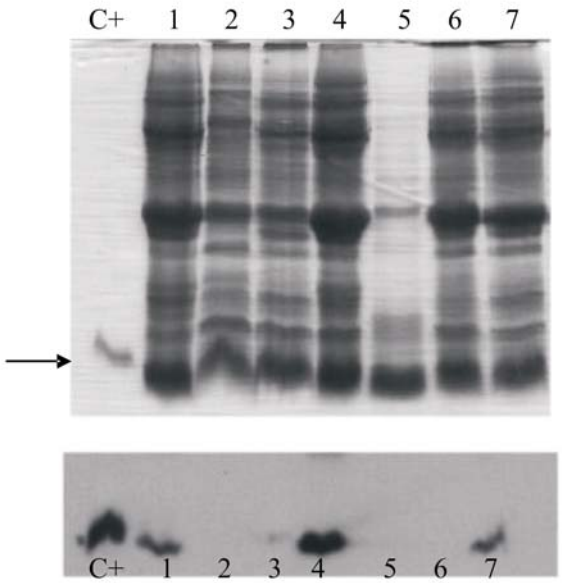

Figure 1: Selection of water-based solvents: SDS-PAGE (15% gel) (top) and Western Blot (bottom) of the extracts produced with different solvents. **C+:** positive control (300 ng of proinsulin); **lane 1:** 50 mM sodium bicarbonate buffer at pH 10.0 with 4 M urea; **lane 2:** 0.2% Triton X-100 with 5 mM DTT; **lane 3:** 50 mM sodium bicarbonate buffer at pH 10.0 with 0.2% Triton X-100; **lane 4:** 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT; **lane 5:** 50 mM Tris-glycine buffer at pH 3.0 with 200 mM NaCl; **lane 6:** 300 mM NaCl at pH 10.0 with 0.6% 2-mercaptoethanol; **lane 7:** control solvent (50 mM Tris-HCl buffer at pH 8.5 with 2 mM EDTA, 5 mM benzamidine, 5 mM DTT, and 0.2% Triton X-100).

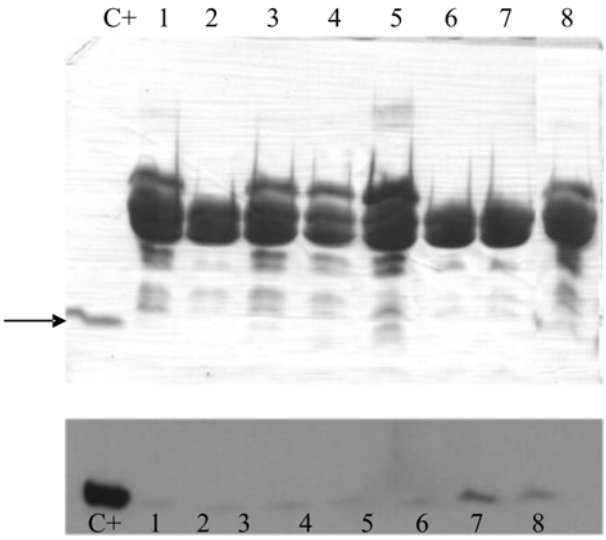

Figure 2: Selection of alcohol-based solvents: SDS-PAGE (15% gel) (top) and Western Blot (bottom) of the extracts produced by different solvents. **C+:** positive control (300 ng of proinsulin); **lane 1:** 70% ethanol with 5 mM DTT; **lane 2:** 70% ethanol with 30% 50 mM Tris-glycine buffer at pH 3.0; **lane 3:** 70% ethanol with 30% 50 mM Tris-glycine buffer at pH 3.0 with 5 mM DTT; **lane 4:** 70% ethanol with 30% 50 mM sodium bicarbonate buffer at pH 10.0; **lane 5:** 70% ethanol with 30% 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT; **lane 6:** 70% isopropanol; **lane 7:** 70% isopropanol with 30% 50 mM Tris-glycine buffer at pH 3.0; **lane 8:** 70% isopropanol with 30% 50 mM sodium bicarbonate buffer at pH 10.0.

Effects of Operational Variables on rhProinsulin Extraction

As in any solid-liquid extraction, the extraction of recombinant proteins from plants involves a combination of different operational variables and optimization of these process conditions is required to achieve maximum efficiency. Therefore, a complete 2^4 factorial design for the extraction of rhProinsulin from transgenic corn endosperm was used (Table 3). Of the variables analyzed – temperature, time, solvent-to-solid ratio, and impeller rotational speed – only temperature had a statistically significant effect on rhProinsulin extraction at a confidence level of 90% (p<0.10) (Table 4). This effect was positive, meaning that an increase in temperature led to an increase in rhProinsulin extraction. However, the temperature should not be increased indefinitely since higher temperatures cause protein denaturation. Shukla et al. (2000) also studied the effect of temperature on the extraction of proteins (zeins) from corn. They found that zein solubilization in the range of 20 to 60°C was highest at 50°C. They credited the positive effect of temperature on protein extraction to the fact that zein transfer from corn grits is rate-limited by the transport of zein out of the endosperm and that the diffusion coefficient of protein increases 3.0 to 3.4% per Celsius degree increase in temperature. However, after a given temperature, heat denaturation of the protein would overcome the positive effect of temperature on the diffusion coefficient.

Table 3: Matrix of the experimental design for the extraction of rhProinsulin from endosperm of transgenic corn seeds using 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT.

Run	Rotational speed	v:m ratio	Temperature	Time	rhProinsulin concentration (mg l^{-1})	rhProinsulin mass per flour mass (mg kg^{-1})
1	-1	-1	-1	-1	1.28	6.41
2	+1	-1	-1	-1	1.24	6.18
3	-1	+1	-1	-1	0.27	2.74
4	+1	+1	-1	-1	0.16	1.59
5	-1	-1	+1	-1	4.56	22.80
6	+1	-1	+1	-1	3.90	19.48
7	-1	+1	+1	-1	1.30	12.98
8	+1	+1	+1	-1	2.06	20.62
9	-1	-1	-1	+1	2.57	12.87
10	+1	-1	-1	+1	4.11	20.55
11	-1	+1	-1	+1	0.19	1.87
12	+1	+1	-1	+1	0.20	1.97
13	-1	-1	+1	+1	1.40	6.99
14	+1	-1	+1	+1	2.04	10.18
15	-1	+1	+1	+1	2.17	21.72
16	+1	+1	+1	+1	4.37	43.72
17	0	0	0	0	3.50	26.22
18	0	0	0	0	3.40	25.47
19	0	0	0	0	3.42	25.64

Table 4: Results of the effect, standard deviation, t-value, and p-value from the 2^4 experimental design of the extraction of rhProinsulin from endosperm of transgenic corn seeds with 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT.

	rhProinsulin concentration				rhProinsulin mass			
	Effect	Standard deviation	t-value (8)	p-value	Effect	Standard deviation	t-value (8)	p-value
Average*	2.22	0.33	6.71	0.00	15.26	2.42	6.31	0.00
Rotation (1)	0.54	0.72	0.75	0.47	4.49	5.27	0.85	0.42
v:m ratio (2)	-1.29	0.72	-1.80	0.11	0.22	5.27	0.04	0.97
Temperature (3)*	1.47	0.72	2.04	0.08	13.04	5.27	2.47	0.04
Time (4)	0.29	0.72	0.40	0.70	3.38	5.27	0.64	0.54
1x2	0.17	0.72	0.24	0.81	2.66	5.27	0.50	0.63
1x3	0.19	0.72	0.27	0.79	2.89	5.27	0.55	0.60
1x4	0.56	0.72	0.77	0.46	3.75	5.27	0.71	0.50
2x3	0.80	0.72	1.11	0.30	9.68	5.27	1.83	0.10
2x4	0.50	0.72	0.69	0.51	4.45	5.27	0.84	0.42
3x4	-0.74	0.72	-1.03	0.33	-1.70	5.27	-0.32	0.76

*factor statistically significant at p<0.10 (90% level of confidence).

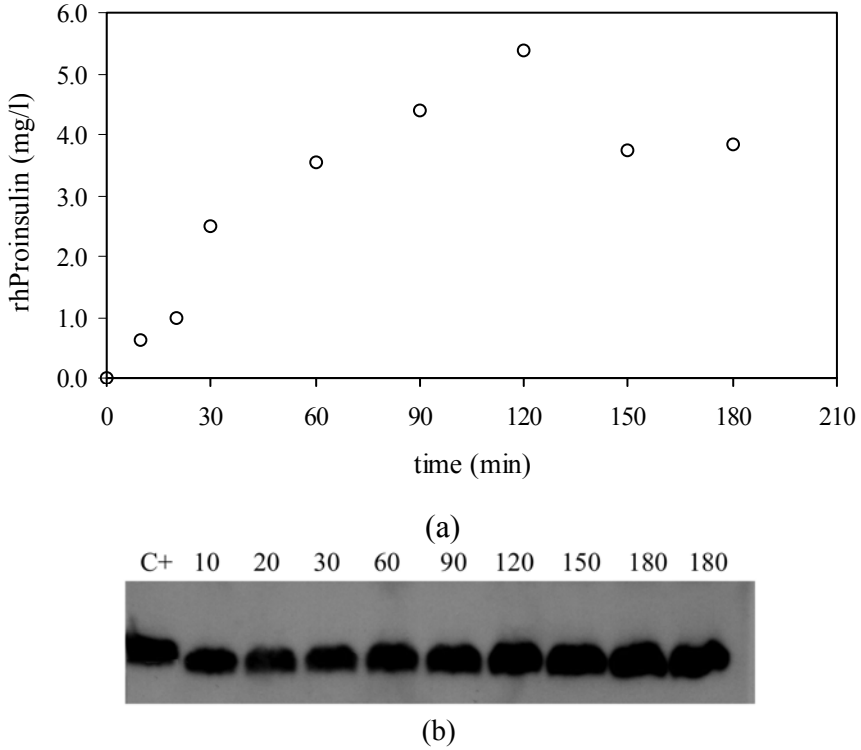

Figure 3: Kinetics of the extraction of rhProinsulin from transgenic corn endosperm. Conditions: extraction solution, 50 mM sodium bicarbonate buffer pH 10.0 with 5 mM DTT; solvent-to-solid ratio, 7.5:1; temperature, 25 °C; and impeller rotational speed, 500 rpm. a) Samples analyzed with ELISA method. b) Western Blot of the samples collected after 10, 20, 30, 60, 90, 120, 150, and 180 (duplicated) min. C+ is 300 ng of the positive control. Sample volume: 15 μl.

The solvent-to-solid ratio was statistically significant at a confidence level of 89% (*p*-value of 0.11), the second most important variable in rhProinsulin extraction (Table 4). Its effect was negative, meaning that a change in the solvent-to-solid ratio from 5:1 to 10:1 resulted in a decrease in the rhProinsulin concentration in the extract, which could be interpreted as a dilution effect. However, the solvent-to-solid ratio showed a positive effect in terms of mass of rhProinsulin extracted (Table 4). The impeller rotational speed did not show a significant effect on the process, since it had a *p*-value equal to or higher than 0.47. Similarly, the extraction time did not have a significant effect on rhProinsulin extraction for the range studied, being significantly only for extraction periods shorter than 60 min (Figure 3). A similar result in terms of the effect of stirring on protein extraction from corn endosperm was verified by Russel and Tsao (1982), the rate of extraction of zein was independent of the stirring speed.

The concentration of rhProinsulin in the extracts was lower (maximum concentration was 4.37 mg l^{-1}) than that in the semi-quantitative Western Blot results obtained by De Lucca (2003) (around 20 mg l^{-1}, almost five times higher). However, De Lucca carried out extractions under high-shear-rate mixing – a condition not applicable to large-scale processes – 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 DTT), the same solvent as that used as the control in this study.

The search for a high efficiency condition for rhProinsulin extraction was continued with a set of experiments carried out at a 10:1 solvent-to-solid ratio and an impeller rotational speed of 500 rpm at two different temperatures, 40 and 25°C (Table 5) with a 2h extraction time. This extraction time was chosen based on the results of the experimental design and the kinetics study (Figure 3). The temperature of 40°C was chosen because of the positive effect of this variable on rhProinsulin extraction; on the other hand, 25°C is a convenient temperature in terms of process operation and cost of energy. The 10:1 solvent-to-solid ratio was selected because of its positive effect on the mass of rhProinsulin extracted. Speed of rotation was set at the central point (500 rpm), since this was not a statistically significant variable for rhProinsulin extraction. The concomitant analysis of native corn components (proteins, carbohydrates, and phenolics) was also carried out (Table 5).

Table 5: rhProinsulin, TSP, RS, TRS, and phenolics concentrations in the extracts from the endosperm of transgenic corn seeds prepared with the solvent 50 mM sodium bicarbonate buffer at pH 10.0 with 5 mM DTT, a solvent-to-solid ratio (ml g^{-1}) of 10:1, an impeller rotational speed of 500 rpm, and a 2h extraction.

Temperature (°C)	Concentration Values*					
	rhProinsulin (mg l^{-1})	rhProinsulin mass per flour mass (mg kg^{-1})	TSP (mg ml^{-1})	RS (mg ml^{-1})	TRS (mg ml^{-1})	Phenolics (mmol l^{-1})
40	20.72±1.06	207.17±10.63	4.66±0.03	0.65±0.04	2.18±0.15	1.04±0.05
25	18.87±0.48	188.67±4.80	4.45±0.36	0.71±0.01	2.19±0.05	1.53±0.04

*Values represent average of triplicate experiments with its corresponding standard deviation.

The same trend as in Table 3 was verified- the positive effect of temperature on rhProinsulin extraction. There was some variation in the level of rhProinsulin concentration since the latter experiments were done with a batch of seeds with higher level of expression. rhProinsulin concentrations obtained by extraction carried out at 40°C were no more than 10% higher than at 25°C (Table 5). These results supported the selection of ambient temperature, which is more advantageous in terms of process engineering. Thus, the most favorable experimental condition to maximize solubilization of rhProinsulin from corn endosperm at a solvent-to-solid ratio of 10:1 and at an impeller rotation speed of 500 rpm is an extraction at 25°C for 2h with a 50 mM sodium bicarbonate buffer pH 10.0 with 5 mM DTT solution as the solvent.

A simple and efficient extraction procedure can significantly reduce the overall costs and can affect process viability. Here we obtained a maximum rhProinsulin concentration of 20.72 mg l^{-1} or 0.45% TSP using a process that is potentially scaleable. According to Hood et al. (2002), production of pharmaceutical proteins in plants at levels of 0.1 to 1.0% TSP is sufficiently competitive with other expression systems, making plants economically viable as bioreactors. Even though the expression level of rhProinsulin has not yet been fully optimized (De Lucca, 2003), this study is the first process engineering study showing the potential of producing insulin from a transgenic plant host. In a recent study, concentrations of rhProinsulin of no more than 97.33 μg l^{-1} were achieved (Farinas et al., 2005). By further evaluating the extraction step we increased the rhProinsulin concentration 200 times. Moreover, the level of concentration of rhProinsulin in the extracts is relatively high compared to other recombinant human proteins in transgenic plants, as described by Daniell et al. (2001).

Co-Extraction of Corn Native Components: Proteins, Carbohydrates, and Phenolic Compounds

The study of extraction of the native proteins, carbohydrates, and phenolic compounds is relevant due to their effects on DSP unit operations: a) native proteins are the key contaminants in the design of the unit operation separation train employed for purification of the target recombinant protein (Menkhaus et al., 2004); b) soluble carbohydrates may cause fouling of filtration membranes and chromatographic resins and can also contribute to microbial growth; and c) phenolic compounds are one of the most chemically active secondary metabolites and they can interact with proteins, triggering aggregation (Jervis and Perpoint, 1989).

The results of the extraction carried out at 25 and 40°C in terms of TSP, RS, TRS and phenolics are shown in Table 5. The results show that the studied temperatures did not have a significant effect on TSP and RS extraction. The extraction of phenolic compounds was negatively affected by temperature, showing a 50% higher value at 25°C. However, even the higher phenolic concentration found (1.5 mM or 0.4 mg ml^{-1}) is considered too low to affect protein stability. This analysis showed that in order to improve the extraction of rhProinsulin, we should focus mainly on the extraction of the recombinant protein, since variations in the concentration of these native compounds had a minor effect on the process.

CONCLUSIONS

In this study we addressed the extraction step in the DSP of rhProinsulin produced in the endosperm of transgenic corn seeds. A systematic study was carried out to determine the most favorable

conditions for rhProinsulin recovery. Besides finding a high efficiency condition for the extraction of recombinant proinsulin, here we also report results on the solubilization of some native components. The results indicate that the condition to maximize rhProinsulin extraction with the 50 mM sodium bicarbonate buffer pH 10.0 with 5 mM DTT as the selected extraction buffer is a solvent-to-solid ratio of 10:1, a temperature of 25°C, and an extraction time of 2h. The maximum rhProinsulin concentration in the extracts at this condition was 18.87 mg l^{-1} or 0.42% of the total soluble protein. This value falls within the range required for the production of pharmaceutical proteins in plants to be competitive with other expression systems. Moreover, this study confirms the potential of producing recombinant human proteins having pharmaceutical applications using transgenic plants.

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