Journal of the Science of Food and Agriculture
J Sci Food Agric **85**:609–614 (2005)
DOI: 10.1002/jsfa.2018

Transgenic corn seed for recombinant protein production: relevant aspects on the aqueous extraction of native components

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Abstract: Plants have been considered one of the most promising expression systems for the production of recombinant proteins. Foreseen advantages include high productivity, optimal processing and assembly, and the non-propagation of human or animal pathogens. A few successful examples of commercial proteins produced in plants have been reported in the literature, such us β-glucoronidase, avidin, hirudin and aprotinin. Although the purification scheme is always a challenge in downstream processing development, the extraction is the key point since the presence of impurities deleterious to the process efficiency and operational-life span of the equipment is determined at this step. This work reports the effect of pH and ionic strength in the extraction of proteins, phenolic compounds, lipids and sugars from transgenic corn seed. The phenolic compounds, lipids and reducing sugars were not significantly affected by changes in the ionic strength of the extracting solutions in the range 0–300 mM NaCl and pH 6.3. However, at high pH value (pH 10.0), high solubilization of proteins, phenolic compounds and lipids was achieved, whereas reducing sugars were not significantly extracted at this condition. This work is complementary to the studies reported in a previous paper and contributes to the development of recombinant protein recovery and purification process from transgenic corn.

Keywords: extraction; transgenic corn seed; proteins; phenolic compounds; sugars; lipids

INTRODUCTION

The expression of recombinant peptides and proteins, including enzymes, in plants is considered one of the most promising sources for the commercial production of pharmaceutical and food compounds. The main advantages of a plant system over other systems include low production and scale-up costs, natural storage stability of the recombinant proteins in tubers or seeds, well-established post-harvest handling and crop processing, and the fact that transgenic plants do not propagate human or animal pathogens.[1–3] Furthermore, plant cells tend to produce properly processed and assembled proteins, identical to those of the native source.[4]

In 2001, five major review articles were published on this subject.[5–9] Different plant species such as tobacco, potato, canola, corn, alfalfa, soybean and corn[5] have been studied as bioreactors in the synthesis of a variety of molecules. Among the recent successes in expressing proteins in plants are the production of human growth hormone,[10] human collagen,[11] vaccine antigens,[12] gastric lipase,[13] trypsin and aprotinin,[14] with the last three expressed in corn and purified at pilot plant or large-scale production level. Corn seeds have been widely studied as a host for recombinant protein expression, due to low costs involved in large-scale production.[6]

However, among the studies regarding the recovery and purification of proteins from plants[2,10,15–19], very few have addressed the extraction operation. Furthermore, few reports have dealt with the large-scale extraction of natural proteins from seeds.[20–22] The exceptions are the studies of zein extraction and purification from corn seed: Shukla and Cheryan[23] listed 30 methods to produce this protein.

Usually, the extraction of recombinant proteins from seeds or other plant tissues is done at bench scale by grinding the material at $-70\,^{\circ}C$ in a mortar with complex buffer solutions containing salts, detergents, reducing agents, and protease inhibitors. Studies aiming at large-scale process development report the use of a few specific buffers, usually phosphate buffer, at pH 7.5.[24–28] Kusnadi *et al*[16] investigated the effect of phosphate, borate and Tris buffers at different pH

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Contract/grant sponsor: CNPq
Contract/grant sponsor: FAPESP
(*Received 26 September 2003; revised version received 24 June 2004; accepted 24 August 2004*)
Published online 26 November 2004

values containing reducing agents (β-mercaptoethanol and DL-dithiothreitol), proteases inhibitor (EDTA) and salts (NaCl and $CaCl_2$) in the extraction of avidin and β-glucuronidase expressed in corn seeds. The optimum extraction buffers found were 20 mM Tris, 500 mM NaCl pH 7.9 for avidin, and 50 mM phosphate buffer pH 7.5 for β-glucuronidase. Bai *et al*[29] investigated the effects of the particle size and microstructures of the seed particles on the extraction kinetics of recombinant β-glucuronidase from transgenic canola using phosphate buffer. A higher extraction yield was achieved for ground flakes compared with ground flour with similar particle size. This was explained by the fact that ground flakes are more sheetlike and show more connections between broken cells, providing a path for the transfer of the solute to the surface. Azzoni *et al*[19] studied the extraction of recombinant aprotinin from transgenic corn seed. Recombinant aprotinin yield and mass fraction (in terms of total soluble protein) were evaluated as a function of the pH and ionic strength (in terms of NaCl concentration) of the extracting solution. An increase in NaCl concentration resulted in a higher yield and mass fraction of aprotinin in the extract, indicating higher selectivity for aprotinin in the extraction operation since the total protein yield did not increase accordingly. Conversely, aprotinin mass fraction (in terms of total soluble protein) in the extract significantly decreased as the extraction pH was raised. The work also showed that simple extraction using water maintained at pH 3.0 by adding HCl was efficient for extracting the recombinant protein. This fact corroborates the claim of Larrick *et al*[7] that low-cost salt buffers with one or two additives are efficient in protein extraction. Recently, Menkhaus *et al*[30] studied the suitability of transgenic peas as a host for recombinant protein production. The extraction efficiency of the target protein (β-glucuronidase) was evaluated and compared with native proteins and carbohydrates. The use of different recovery and purification operations was also addressed.

Since extraction is the first step in downstream processing of plant tissues containing recombinant proteins, a detailed knowledge of this step is essential for the development of an efficient process. Most related works reported in the literature are non-specific, and focus mainly on proteins (recombinant and native). In this work, we present relevant aspects on the extraction of recombinant proteins from transgenic corn seeds which could have impact in the downstream processing. We studied the effects of buffer ionic strength and pH on the level of carbohydrates, phenolic compounds and oil concentrations in the extracts, and on the native protein profile. Besides increasing the complexity of the extract and, consequently, the difficulty in the separation, these compounds could have other deleterious effects in the downstream processing of recombinant proteins. Oil may cause fouling of membrane filtration and chromatographic resins; phenolic compounds are known as protein deactivation and aggregation agents; and carbohydrates may result in lower filtration rates and microbial growth.

EXPERIMENTALS

Materials

Transgenic corn seed containing the recombinant aprotinin used in this work was the same as that used by Zhong *et al*.[2] D-Cathechin was purchased from Sigma (St Louis, MO, USA). The Coomassie Plus Protein Reagent was from Pierce (Pitsburg, PA, USA). The Soxhlet type extractor, model Q-308G22, was purchased from Quimis (Campinas, Brazil). All other chemicals used in this work were of at least reagent grade.

METHODS

Analytical assays

Protein analysis

Gel electrophoresis of proteins was carried out under denaturing conditions (SDS–PAGE) as described by Zhong *et al*.[2] The same volume (20 μL) of each extract was loaded into the gel for comparison.

Reducing sugar concentration

The concentrations of reducing sugars in aqueous extracts at different pH and ionic strength were determined by the dinitrosalicylic acid (DNS) method.[31] The concentration of total reducing sugars was also measured by the same method after acid hydrolysis of the sample. Standard curves for reducing and total reducing sugars were constructed using glucose and sucrose as references, respectively.

Determination of phenolic compounds

The concentration of phenolic compounds was determined by the Prussian Blue method described by Price and Butler.[32] Results were quantified according to a standard curve prepared daily from fresh solutions of commercial D-cathechin.

Determination of lipid concentration

The lipid concentration in solid samples (transgenic corn flour and cake after aqueous extraction) was determined using a Soxhlet type extractor. Lipids from 5 g samples of corn flour or dried cake were extracted with hexane according to the extractor manufacture's instructions. The results are presented as 'remaining lipids', calculated as the mass of lipids extracted by hexane divided by the initial mass of cake (dry cake after aqueous extraction). The control sample was corn flour not subjected to aqueous extraction. The amount of lipid removed by the aqueous extractions could be calculated by the difference between the lipid content in the cake and in the original corn flour.

Transgenic corn seed grinding

The seeds were grinded to a particle size smaller than 1.70 mm with an Intermediate Model laboratory-scale

mill from Thomas-Wiley, USA. The milling step was carried out at intermittent steps in order to avoid heating the sample.

Determination of the effect of pH and ionic strength on extraction of proteins, phenolic compounds, reducing sugars (RS), total reducing sugars (TRS) and lipids

Batches of corn meal (20 g) were suspended in 100 mL of extraction buffer of varying pH and ionic strengths. The buffers were the following: 200 mM glycine (pH 3.0), 200 mM sodium acetate (pH 4.0), 200 mM NaPi (pH 6.0 and 8.0) and 200 mM sodium carbonate/bicarbonate (pH 10.0). NaCl concentrations were 0, 50, 100, 200 and 300 mM. In the case of salt solutions, no buffer salts were added to the extracting solution: the pH of the extraction was 6.3 as a result of the natural buffering capacity of the corn-soluble molecules. The suspensions were stirred for 60 min (it was verified in previous experiments that no significant proteins or lipids are released to the solution after 30 min extraction) with a magnetic stirrer in a 250 ml beaker and the solid and liquid phases were separated by filtration on Whatman filter paper no 1. The filtrates were collected and kept at 4 °C until protein profile by SDS–PAGE, phenolic compounds, reducing sugars and total reducing sugars concentrations were analyzed. Initial and final pHs of all extracts were monitored. After drying at room temperature for 48 h, the lipid contents of the cakes (moisture content of approximately 14%) were determined.

RESULTS AND DISCUSSION

Soluble proteins

In a previous study, Azzoni *et al*[19] demonstrated that pH value in the range between pH 3.0 and 10.0 had a strong effect on the protein extraction yield from corn seed, whereas the effect of ionic strength was found to be less pronounced. In this work, we studied the quality of corn extracts obtained at pH 3.0, 4.0, 6.0, 8.0 and 10.0 in terms of the molecular mass profile of soluble proteins by SDS–PAGE analysis (Fig 1). The number of protein bands in the acrylamide gel increased as the extraction pH increased, specifically in the range of high molecular mass. These data suggest the use of the lowest possible pH for extraction to minimize the extraction of protein impurities and, thereby, maximize the target protein purity in the extract. This is also in agreement with the data of Azzoni *et al*[19] in the case of recombinant aprotinin produced in corn seeds: the mass fraction of aprotinin in the extract was almost 13-fold higher for extraction at pH 3.0 than at pH 10.0.

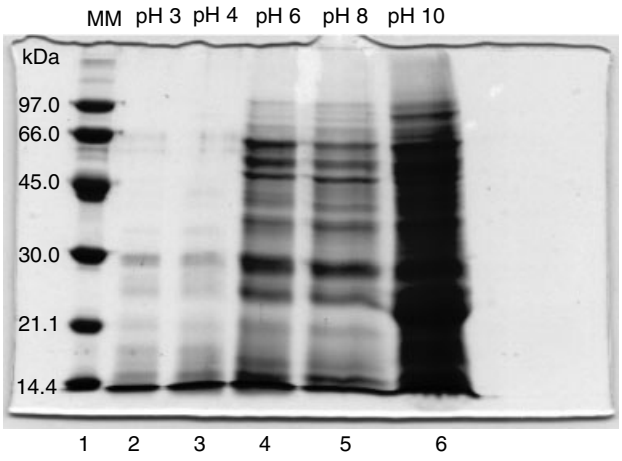

Figure 1. SDS–PAGE illustrating the differences on total protein extraction from transgenic corn seed flour at different pH. Lane 1, molecular mass markers; lane 2, extraction using 200 mM glycine pH 3.0; lane 3, extraction using 200 mM sodium acetate pH 4.0; lanes 4 and 5, extraction using 200 mM NaPi pH 6.0 and 8.0, respectively; and lane 6, extraction using 200 mM sodium carbonate/bicarbonate pH 10.0. Sample volume, 20 µl.

Phenolic compounds

Phenolic compounds are secondary plant metabolites known to interact with proteins, which may cause aggregation and sometimes deactivation of proteins. Despite their importance, information on the interactions of polyphenols with seed proteins is scarce and mainly limited to nutritional aspects.[33] We evaluated the amount of phenolic compounds extracted from corn flour with extracting solutions differing in ionic strength and pH. Ionic strength did not affect the solubility of these compounds for NaCl concentrations lower than 300 mM [Fig 2(a)]. The average concentration of phenolic compounds of the 15 measurements was 0.23 mM. However, extraction pH had a significant effect on the phenolic compounds extraction: The phenolic compound concentration extracted at pH 10.0 was almost three times higher than that extracted at pH 3.0 and 4.0 [Fig 2(b)]. Price and Butler[32] have also reported that high molecular mass phenolic compounds have low solubility at high salt concentration and that the solubility of low molecular mass phenols does not significantly depended on salt concentration. Maybe the salt concentration range used in this study (highest concentration of 300 mM) was not broad enough to observe this phenomenon, or high molecular mass phenols are present in low concentrations in corn seed in comparison with low molecular mass phenols. The average value of 0.23 mM (0.07 mg ml^{-1} in terms of D-cathechin) for phenolic compounds is low when compared with the concentration range of 1.5–15 mg ml^{-1} required to form aggregates with recombinant human growth hormone according to Maa and Hsu.[34] Since the concentration of total soluble proteins in the extract is around 4-fold higher than the protein concentration used for these authors, and aggregation should be a function of both phenolic and protein concentrations, we cannot say that the level of phenolic compounds is negligible. Therefore, extractions at high pH are not recommended at first.

Reducing sugars

Boyer and Shannon[35] report D-glucose and D-fructose to be the major reducing sugars present in corn

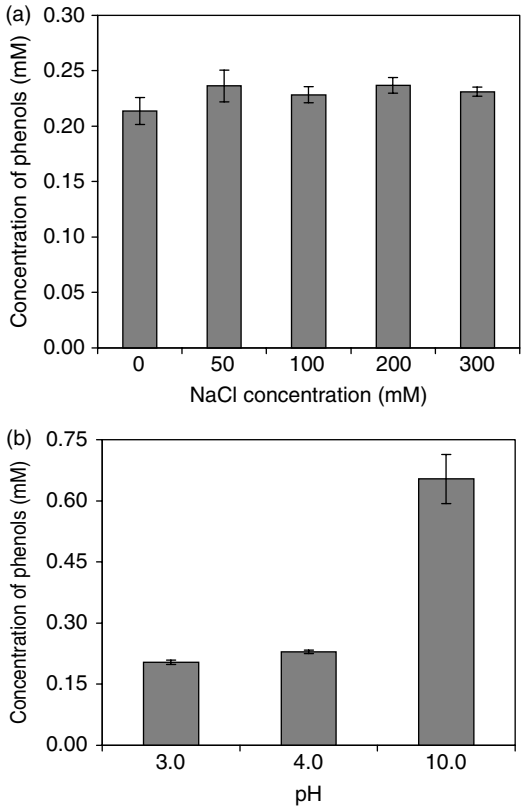

Figure 2. The effect of ionic strength and pH on the extraction of phenols (measured as D-catechin) from transgenic corn flour. (a) The effect of ionic strength. Extractions were carried out in aqueous NaCl solutions on a 1:5 solid to liquid ratio. In the experiments, the pH was kept at 6.3, taking advantage of the natural buffering capacity of the corn-soluble molecules. (b) The effect of pH. Extractions were carried out on a 1:5 solid to liquid ratio. The buffers used were 200 mM glycine pH 3.0; 200 mM acetate pH 4.0; 200 mM phosphate pH 6.0 and 8.0; and 200 mM carbonate pH 10.0. Error bars indicate standard deviation between triplicate.

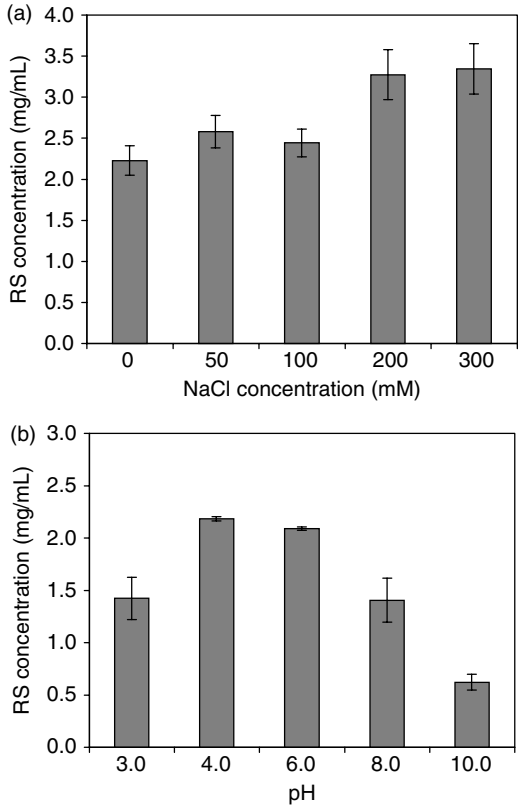

Figure 3. The effect of ionic strength and pH on reducing sugar (RS) extraction from transgenic corn flour. (a) The effect of ionic strength on RS extraction. Extractions were carried out in aqueous NaCl solutions. (b) The effect of pH on RS extraction. The buffers used were 200 mM glycine pH 3.0; 200 mM acetate pH 4.0; 200 mM phosphate pH 6.0 and 8.0; and 200 mM carbonate pH 10.0. Error bars indicate standard deviation between triplicate.

seeds and phytoglycogen (a branched polysaccharide formed with up to 30 glucose units and a structure similar to mammalian glycogen) as the most common polysaccharide. Sucrose is the disaccharide present in the seed at concentrations from 2 or 3 mg per grain, depending on the corn variety studied.

We evaluated the effect of ionic strength and pH on the extraction of carbohydrates by measuring the concentrations of reducing sugars and total reducing sugars in the extracts (Figs 3 and 4). The difference between the reducing and total reducing sugars concentrations correspond to the acid hydrolysable compounds such as polysaccharides and sucrose.

The ionic strength had some effect on the extraction of reducing sugars. The highest concentrations of carbohydrates were found in extracts prepared with high salt concentrations (200 and 300 mM). The extraction of reducing sugars had a maximum at pH 4.0 and had its lowest value (0.6 mg ml^{-1}) at pH 10.0, while the total reducing sugar levels remained almost constant for all conditions studied (Fig 4). Despite the differences found for the extraction of reducing sugars at some conditions, this study indicated that ionic strength and pH do not play an important role on the extraction of sugars from corn seed as it is used for the production of recombinant proteins. Similar results were recently presented by Menkhaus *et al*,[30] who found that extraction of soluble carbohydrates from transgenic pea was not significantly different when using dionized water or 50 mM sodium phosphate buffer at different pHs, with or without 1.0 M NaCl.

Lipids

Corn seeds have a relative low oil content (around 4%, mostly triacylglycerols) when compared with other seeds like soybean, which has a lipid content of around 20%.[36] The corn seed used in this work had a lipid content of 3.5% (control value, average four samples, standard deviation of 0.2%). The effect of pH and ionic strength of the extracting solution on the removal of lipids from corn seed flour was evaluated by measuring the remaining lipids in the filtration cake after extraction and filtration.

The most significant effect on the extraction was observed at high pH values [Fig 5(a)]. Oil removal by extraction increased as the pH increased. Three percent of the flour lipid content was removed at pH 3.0 whereas 80% was removed at pH 10.0.

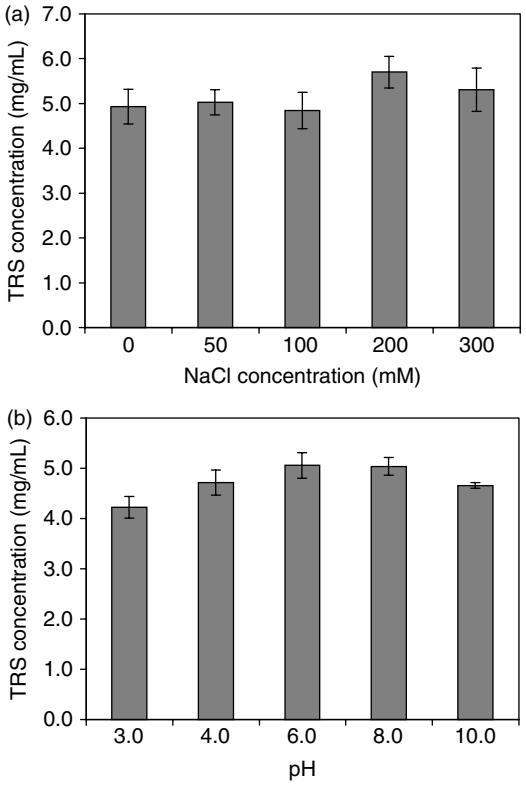

Figure 4. The effect of ionic strength and pH on total reducing sugar (TRS) extraction from transgenic corn flour. (a) The effect of ionic strength on TRS extraction. Extractions were carried out in aqueous NaCl solutions. (b) The effect of pH on TRS extraction. The buffers used were 200 mM glycine pH 3.0; 200 mM acetate pH 4.0; 200 mM phosphate pH 6.0 and 8.0; and 200 mM carbonate pH 10.0. Error bars indicate standard deviation between triplicate.

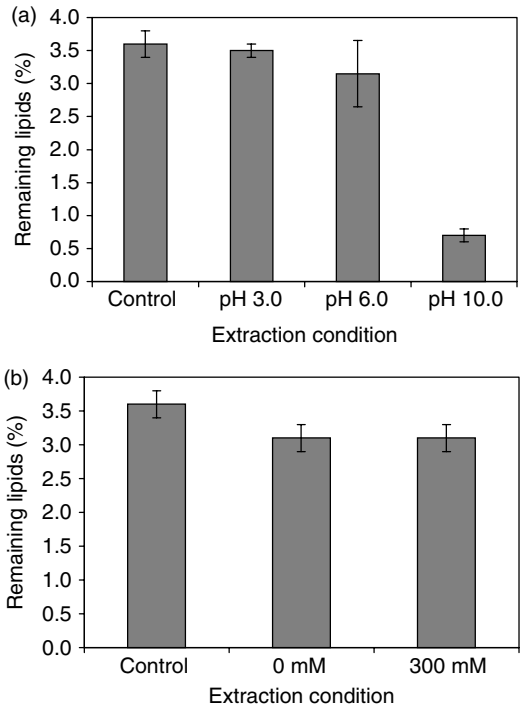

Figure 5. The effect of pH and salt concentration (NaCl) on lipid extraction from transgenic corn flour. (a) The effect of pH on lipid extraction. Values indicate the remaining lipids of the flour after extraction with 200 mM glicine buffer pH 3,0; 200 mM NaPi buffer pH 6,0; and 200 mM carbonate buffer pH 10.0. (b) The effect of NaCl concentration on lipid extraction. Values indicate the remaining lipids of the corn flour after extraction with deionized water (0 mM) and 300 mM NaCl solution at pH 6.3. Control indicates lipid content in the corn flour that was not extracted with any aqueous solution. Error bar for the control indicates standard deviation between four replicates. Other bars indicate deviation between duplicates.

Salt in the extracting solution did not affect removal of lipids from the flour [Fig 5(b)]. Deionized water and 300 mM NaCl solution had the same lipid extraction effect (3.1% of lipids equivalent to 11% reduction when compared with the control). Since the pH of these extraction solutions was close to 6.0 (6.3 due to the natural buffering capacity of the of the corn-soluble molecules), these results are in agreement with the data at pH 6.0 of Fig 5(a), where the average lipid content was also 3.1%. This results also suggest the use of the lowest possible extraction pH when determining the optimal extraction conditions of recombinant proteins, minimizing the extraction of lipids that are deleterious to downstream processing.

CONCLUSIONS

The results of this investigation indicated that solution pH could be manipulated to optimize yield or purity of extracted material since it substantially affects the extraction of native protein, carbohydrates, phenolic compounds and lipids. SDS–PAGE indicated that high molecular mass corn seed proteins are solubilized as the extraction pH increases. Phenolic compounds, lipids and reducing sugars are not significantly affected by changes in the ionic strength of the extracting buffer. However, extraction at pH 10.0 resulted in high solubilization of phenolic compounds and lipids. Therefore, if native proteins, phenolic compounds and lipids concentrations in the extract are to be minimized, a low pH value is desirable, as long it does not significantly affect the yield of the target recombinant protein. Thus, the low pH used in the work by Azzoni *et al*[19] to minimize native protein extraction and maximize recombinant aprotinin extraction was the right choice in the design of an optimized process condition since it also minimized solubilization of phenolic compounds and lipids.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil. They also acknowledge Dr Zivko L. Nikolov, Texas A&M University (USA), and Dr Rahoma S. Mohamed and Dr Gustavo Paim Valença, both from the State University of Campinas (UNICAMP, Brazil), for fruitful discussions and revision of the manuscript. The authors acknowledge ProdiGene Inc (ZN) from College Station, TX, USA, for the kindly donation of the transgenic corn flour used in this work.

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