Wet-Milling Transgenic Maize Seed for Fraction Enrichment of Recombinant Subunit Vaccine

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The production of recombinant proteins in plants continues to be of great interest for prospective large-scale manufacturing of industrial enzymes, nutrition products, and vaccines. This work describes fractionation by wet-milling of transgenic maize expressing the B subunit of the heat-labile enterotoxin of Escherichia coli *(LT-B), a potent immunogen and candidate for oral vaccine and vaccine components. The LT-B gene was directed to express in seed by an endosperm specific promoter. Two steeping treatments, traditional steeping (TS, 0.2% SO_2 + 0.5% lactic acid) and water steeping (WS, water only), were evaluated to determine effects on recovery of functional LT-B in wet-milled fractions. The overall recovery of the LT-B protein from WS treatment was 1.5-fold greater than that from TS treatment. In both steeping types, LT-B was distributed similarly among the fractions, resulting in enrichment of functional LT-B in fine fiber, coarse fiber and pericarp fractions by concentration factors of 1.5 to 8 relative to the whole kernels on a per-mass basis. Combined with endosperm-specific expression and secretory pathway targeting, wet-milling enables enrichment of high-value recombinant proteins in low-value fractions, such as the fine fiber, and co-utilization of remaining fractions in alternative industrial applications.* © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 26: 458–465, 2010
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Introduction

Transgenic plants offer a highly attractive platform for the production of therapeutic proteins, enzymes and value-added compounds, and the topic has been extensively reviewed.[1–4] The use of maize as a biofactory for producing recombinant proteins has been studied for nearly a decade now[5–7] and continues to be of great interest because of the numerous advantages it presents compared to bacterial, yeast, or mammalian counterparts.[5] The infrastructure for producing, harvesting, transporting, storing, and downstream processing of maize is well established. As one of the most cultivated crops of the world,[8] maize production is also less costly than alternative bioreactors. As a non-bacterial and non-mammalian system, the use of maize for producing recombinant proteins greatly reduces the possibility of mammalian pathogen contamination. As a higher eukaryote, maize is also equipped with the metabolic machinery to carry out post-translational modifications that influence the activity and nature of the recombinant protein of interest.[9]

The maize kernel is composed of discrete tissues that include the embryo or germ, pericarp, bran, and

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endosperm.[10] Successful use of maize as a biofactory has been reported for industrial enzymes and proteins,[11–13] alternative plastics,[14] and edible vaccines[15–17]. Researchers have utilized a wide selection of promoters to drive expression of genes of interest in particular tissues, organs, or developmental stages. The two most widely used tissues for recombinant protein expression are the germ[12,13,18] and the endosperm.[19,20] The smaller amount of germ per kernel (11%) on mass basis compared to endosperm (82.9%) could be a potential advantage for achieving greater recombinant protein concentration. The endosperm, however, is a natural storage tissue for proteins (primarily water-insoluble zeins) and starch represents up to 83% of the total kernel mass, which also makes it an attractive target for foreign protein accumulation.

Maize fractionation is traditionally accomplished by using dry- or wet-milling procedures. Dry-milling with degerming allows recovery of bran-rich, germ-rich, and endosperm-rich (of different grit sizes) fractions by physical and mechanical means. On the other hand, wet-milling can result in recovery of six solid fractions (germ, gluten meal, pericarp, coarse fiber, fine fiber, starch) and steep water.[10,21] While dry-milling procedures are suitable for separating the germ and recovering endosperm with low oil and fiber contents, wet-milling offers the advantage of recovering other fractions, such as highly purified starch and maize gluten meal, which can be used for other applications. Traditional wet-milling involves steeping with 0.2% SO_2, which is key in breaking the cross-linking disulfide bonds in the endosperm protein matrix, producing acidic conditions that favor *Lactobacillus* growth (lactic acid softens the grain for grinding) and eliminating putrefying bacterial growth.[22,23] The presence of SO_2 and lactic acid in the steep water increases release of proteinaceus material from the maize kernels[22] and enhances starch recovery.[24] Although efforts have been focused on optimizing wet-milling conditions for maize fractionation, much less has been done to study the effects of wet-milling on recombinant protein recovery from transgenic maize. Fractionation studies for recovery of recombinant proteins have been carried out for germ-targeted[12,25–27] and endosperm-targeted proteins[20,25,28] mainly using dry-milling or other non-wet-milling procedures.

Transgenic maize seed expressing recombinant avidin in germ was fractionated using a custom-made dehuller/degermer; combined sieving and aspirating allowed recovery of a germ-rich fraction, which was compared to whole grain for its stability at elevated temperatures that are used during grain processing operations.[12] Transgenic maize expressing β-glucuronidase (GUS) and avidin in germ was also fractionated either by hand or dry-milling, with majority of extractable recombinant proteins recovered in the germ fractions.[26] The fractions enriched in GUS and avidin, were further processed for purification of the recombinant proteins. Preliminary fractionation of GUS produced in maize by using the traditional wet-milling process adversely affected recombinant GUS activity.[27] Therefore, dry fractionation and germ flotation processes were used for fractionating transgenic maize expressing GUS enzyme. Comparison of dry fractionation with germ flotation separation revealed that the flotation method yielded higher enzyme recovery with up to 80% of the recombinant GUS activity accounted for.[27] Dry-milling and hand fractionation of transgenic maize expressing the green fluorescent protein under control of an embryo- or endosperm-specific promoter were used to study the efficiency of the fractionation procedures.[25] Standard dry-milling procedures were also used to study the benefit of fractionation in purification of a recombinant dog lipase targeted to be expressed in the endosperm of transgenic maize.[20] In this study, it was reported that recombinant lipase can be extracted from both endosperm and germ fractions, but utilization of endosperm fraction can eliminate the need for germ defatting and its effects on recombinant protein purification.

The heat labile enterotoxin (LT) of *Escherichia coli* is the causative agent of the common disease called traveler's diarrhea, a disease similar to but less severe than the sometimes deadly cholera disease caused by *Vibrium cholerae*. LT is a hetero-hexameric protein of about 84 kDa in size, composed of A and B subunits joined by a polypeptide linker. The B subunit (LT-B) is a homopentameric ring that binds specifically to monosialoganglioside G_{M1} receptor in the membranes of the intestinal cells. Binding of LT-B to G_{M1} receptor mediates internalization of the A subunit into the cytosol where it is responsible for the biochemistry that leads to toxicity-associated symptoms.[29] The separate subunits, however, are not cytotoxic. Because of its potential properties as mucosal adjuvant and its lack of toxicity, LT-B has been produced in several plant systems for possible utilization as oral vaccines. LT-B has been expressed in potato,[30] tobacco,[31] maize[17,32] and soybean.[33]

Maize-derived LT-B has been shown to have potential as a vaccine or vaccine component when administered orally to mice due to its potent antigenic nature.[17,32] Fractionation studies of LT-B maize have been conducted to determine whether orally delivered antigens can withstand the common commercial fractionation processes.[15,34] Constitutively expressed LT-B maize was used for dry-milling fractionation, and the LT-B-rich germ fractions were further processed for utilization in feeding studies showing that LT-B is able to survive the dry-milling process while maintaining its antigenicity[15,34].

The objective of the current study is to establish a fractionation procedure that will allow one to obtain fractions enriched in functional LT-B protein and remaining fractions suitable for other industrial applications such as biofuel (ethanol) production. We chose to evaluate the wet-milling process because it is a well established procedure that can recover six different fractions instead of three fractions compared to the dry-milling process; we hypothesized that fractionation by wet-milling would enable recovering fractions with greater LT-B concentrations appropriate for direct dosage or which would make downstream purification easier and less costly, if needed.

Because of the unknown effects of elevated steeping temperatures and SO_2/lactic acid used in traditional steeping on maize derived LT-B, we performed wet-milling fractionation using traditional steeping (TS; 0.2% SO_2 + 0.5% lactic acid) and water steeping (WS; water without SO_2 or lactic acid). Mass balance data for both steep types show that there are significant differences in the recovery of coarse fiber, fine fiber, gluten meal and steep water fractions. Water steeping yielded a better recovery of functional LT-B overall than did traditional steeping. Both steep treatments result in enrichment of the fine fiber fraction with LT-B, a highly desirable output for currently a low value fraction. Because the starch fractions obtained from these steep treatments have very low levels of LT-B, it may also be ideal for traditional uses of starch, such as biorefinery or fermentation for ethanol

production. Altogether, these results show that wet-milling of LT-B maize is an adequate fractionation method for recovery of functional LT-B and has potential for co-production of starch and germ fractions for industrial purposes.

Materials and Methods

Transgenic maize

Transgenic maize expressing LT-B was generated at the Center for Plant Transformation at Iowa State University (ISU) and designated as transgenic line P77 (LT-B maize;[19]). This line expresses LT-B in the endosperm. The material used in this study was grown in Colorado under a regulated field release (U.S. Department of Agriculture, Animal and Plant Health Inspection Service field release permit # 04-131-01r) during the summer of 2004.

Fractionation of LT-B maize

Wet-milling of transgenic maize was performed at the Center for Crops Utilization Research Unit at ISU using a modified procedure of that described for 100 g wet-milling of maize[23] and detailed further in Ref. 35. Steeping was carried out using 0.2% SO_2 and 0.5% lactic acid for traditional steeping (TS) and water only for water steeping (WS). Two random 100-g samples were used for each steep condition, generating two replicates for each fraction recovered. All solid fractions recovered were dried, ground using a coffee grinder and sieved using a stainless-steel 40 μm mesh screen to achieve homogeneity before analysis. Each fraction recovered was analyzed for LT-B content in quadruplicate.

Protein extraction

For assaying LT-B, sieved solid samples were incubated with 10 μL buffer per mg of dry matter containing 25 mM sodium phosphate (pH 6.6), 100 mM NaCl, 0.1% Triton X-100 (v/v), 1 mM ethylene-diamine-tetra-acetic acid (EDTA), 10 μg/mL of leupeptin, 0.1 mM serine protease inhibitor Perfabloc SC, for 2 h at 37°C. Total aqueous extractable protein (TAEP) was determined by using the Bradford assay.[36] Protein extraction for Western blots was carried out at 50°C using the same buffer.

LT-B detection by G_{M1} capture ELISA

Quantification of LT-B in the samples was carried out using a modification of the monosialoganglioside (G_{M1}) dependent enzyme-linked immunosorbent assay (ELISA) described previously.[37] The protocol was modified as follows. Monosialoganglioside G_{M1} from bovine brain (G7641, Sigma, St Louis, MO) was used at a 10 μg/mL concentration, 50 μL per well. Streptavidin-horseradish peroxidase conjugate (554066, BD Biosciences, San Jose, CA) was used at a dilution of 1:1000 in 1% dry milk (DM) (w/v) in phosphate buffered saline [PBS; 0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl, (pH 7.2)]. Horseradish peroxidase substrate [ABTS; 0.5 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 0.1 M citric acid, pH 4.35] was activated prior to use, by adding 5.5 μL 30% H_2O_2 to 5.5 mL ABTS solution. Activated horseradish peroxidase substrate was added to the plate and incubated in the dark at room temperature for 30 min. Absorbance was measured spectrophotometrically at 405 nm at the end of the reaction. Sample wells were blanked against non-transgenic maize protein extracts and all measurements were performed in quadruplicate. Raw ELISA data were converted using an ELISA standard curve constructed using purified bacterial LT-B (kindly provided by Dr. John Clements, Tulane University, LA). LT-B content was expressed as μg LT-B/g solids or % LT-B/ TAEP.

LT-B detection by Western blotting

Equal amounts of TAEP were diluted in 6× Laemmli SDS-sample buffer[38] and boiled for 5 min. Samples were separated on 18% polyacrylamide SDS-PAGE gels as described.[38] The separated proteins were transferred to a 0.45 μm nitrocellulose membrane using the BioRad Semidry Transblot apparatus according to the manufacturer's instructions. Unless otherwise specified, all incubations were carried out at 25°C for 1 h. Membranes were blocked with 5% DM in PBST (PBS, 0.05% Tween-20 (v/v)). Rabbit anti-LT-B (RECO-55G, Immunology Consultants Laboratory, Newberg, OR) was used as primary antibody at a 1:1000 dilution in 1% DM in PBST. A horseradish peroxidase conjugated goat anti-rabbit (A0545, Sigma, St Louis, MO) was used as secondary antibody at a 1:2000 dilution in PBST. Colored bands were revealed by incubation with horseradish peroxidase substrate, 3,3′,5,5′-Tetramethylbenzidine (T0565, Sigma, St. Louis, MO) and dried membranes used for imaging.

Data analysis

The experimental design was a split plot. The main plot treatment was the milling condition (TS or WS, 2 replicates each in a completely randomized design) and the split treatment was the fraction (six levels for solid fractions, completely randomized design within main plots). The data were analyzed using SAS proc mixed with a complete factorial treatment structure and a random effect for the main plot error (replicate with milling condition). Variance components were estimated using method=Type3. Mass balance data were analyzed using t-tests for mean comparisons.

Results and Discussion

The expression of LT-B in maize has been accomplished by using different types of promoters to regulate the protein production in different maize tissues. The endosperm-specific promoter drives LT-B expression and accumulation specifically in the endosperm tissue[19] while the embryo-specific or constitutive promoter results in embryo (or germ) enriched LT-B expression.[16] Maize-derived LT-B from both expression strategies has been shown to be effective in mice and humans protecting against traveler's diarrhea caused by enterotoxigenic strains of *E. coli*.[15,17,34] The LT-B in these studies, however, was either processed as whole maize product[17] or as germ-enriched product.[15,34] No wet-milling process for recovery of edible vaccines or vaccine components from maize has been reported. Most grain fractionation processes for recovery of recombinant proteins produced in maize have been based mainly on hand separation or dry-milling procedures.[12,15,20,26,27,34] On the other hand, an early preliminary study on fractionation of transgenic maize producing β-glucuronidase (GUS) by wet-milling showed detrimental effects on recombinant GUS activity.[27] In order to study the effects of the steeping composition on LT-B

recovery from wet-milled fractions, we carried out the milling process using traditional (SO_2 + lactic acid, TS) and water steeping (WS).

Comparison of mass recovery from wet-milling of LT-B maize using traditional and water steeping

Table 1 presents the mass balance data for TS and WS wet-milled fractions of LT-B maize. The values are the means of the two replicates for each treatment. Yields of starch, germ and pericarp fractions were not significantly different between the two steep treatments. On the other hand, the yields of gluten meal, coarse and fine fiber, and steep water fractions were significantly different between traditional and water steeping. Gluten meal and steep water recovery were higher using traditional steeping, while recovery of coarse and fine fiber were higher using the water steep. Addition of SO_2 + lactic acid to the steeping process prevents colonization of undesirable microorganisms but allows growth of *Lactobacillus* for lactic acid synthesis, which helps in kernel hydration and swelling, promotes kernel enzyme activation, and aids in deconstructing the protein matrix by reduction of disulfide bonds and increased protein solubility. Traditional steeping also reduces protein interactions with starch, which results in fractions with higher purity, a desired characteristic for downstream processing.[10,21] The use of SO_2 + lactic acid during steeping likely explains the differences observed in mass yields for both treatments.

Starch and germ fractions are usually composed of 0.3 and 12% protein on dry basis (db), respectively.[10,21] SO_2 + lactic acid affect mostly the endosperm of the maize kernel, from which the other fractions are derived. In the commercial wet-milling process, gluten meal and steep water fractions typically contain 60 and 46% protein db, respectively. Coarse and fine fiber collectively are composed of 12% protein db, and higher recovery of these fractions in water steeping may be due to less accessibility and protein deconstruction of the endosperm material in the absence of SO_2 + lactic acid.[10,21] Our data indicates that the yield recoveries from both steeping treatments are in line with that reported for the wet-milling processes.[10,21]

Table 1. Mass Balance Data for Recovered Fractions Derived from Wet Milling LT-B Transgenic Corn*

Fraction	Traditional Steep Yield (%, db)† ± Standard Error	Water Steep Yield (%, db) ± Standard Error
Coarse fiber	$3.87^a \pm 0.15$	$8.63^b \pm 0.09$
Fine fiber	$2.48^a \pm 0.09$	$7.06^b \pm 0.29$
Germ	$2.76^a \pm 0.09$	$3.12^a \pm 0.31$
Gluten meal	$20.52^a \pm 0.32$	$16.71^b \pm 0.22$
Pericarp	$5.66^a \pm 0.06$	$5.57^a \pm 0.08$
Starch	$59.98^a \pm 0.03$	$56.78^a \pm 0.36$
Steep liquids	$4.57^a \pm 0.07$	$2.22^b \pm 0.02$
Total solids	99.84 ± 0.30	100.07 ± 0.02

* Values followed by the same letter within a fraction are not significantly different ($P<0.05$). † Dry basis.

LT-B is enriched in the fiber fraction of wet-milling products

We evaluated the recovery of functional LT-B in each fraction collected from both steeping treatments. LT-B is synthesized as a monomer protein (11.6 kD) that assembles into a homopentamer given the proper environment in the native bacterial periplasm,[39] or in the heterologous plant compartment where it accumulates.[33,37] The pentameric form is recognized specifically by the host intestine's monosialoganglioside (G_{M1}) receptor, to internalize the toxin and mediate the immune response.[29] Therefore, as a potential subunit vaccine or vaccine component, LT-B's functionality depends on its successful assembly into a functional pentamer. While LT-B protein can be detected in different forms using a sandwich ELISA with LT-B specific antibodies,[40] in this study, we focused on the recovery of the functional pentameric LT-B protein.

Table 2 presents the distribution of functional LT-B in each of the recovered wet-milling fractions for both steeping methods. The values reported for coarse fiber, fine fiber, germ, gluten meal, pericarp, and starch correspond to the least squares means for each fraction and steep method combined, while steep liquids and whole kernel data represent the averages of four readings.

The field-grown LT-B maize seed has LT-B level measured at 28.44 μg LT-B per gram of seed when analyzed as whole kernel ground meal sieved in the same manner as the solid fractions recovered from wet-milling. The same batch of maize used for fractionation was used as a sample for whole kernel processing. As can be seen in Table 2, the traditional steeping and water steeping gave similar distributions of LT-B in the different fractions. The functional LT-B is concentrated in the fine fiber fraction obtained from both treatments. Detectable levels of functional LT-B were observed in all fractions, with the germ and starch being the

Table 2. LT-B Recoveries in Wet-Milled Fractions of Transgenic Corn

	Traditional Steep				Water Steep			
	LT-B Content	LT-B Recovery			LT-B Content	LT-B Recovery		
Fraction	(μg LT-B/g solids)	(μg LT-B/g corn)*	% WK LT-B†	% Total LT-B‡	(μg LT-B/g solids)	(μg LT-B/g corn)*	% WK LT-B†	% Total LT-B‡
Coarse fiber	39.85	1.54	5.42	12.15	41.40	3.57	12.56	17.65
Fine fiber§	234.53	5.82	20.45	45.81	115.14	8.13	28.58	40.16
Germ	9.56	0.26	0.93	2.08	15.01	0.47	1.65	2.31
Gluten meal	10.18	2.09	7.35	16.46	27.51	4.60	16.16	22.71
Pericarp	48.98	2.77	9.75	21.84	59.35	3.31	11.62	16.33
Starch	0.05	0.03	0.03	0.08	0.06	0.04	0.04	0.05
Steep liquids	4.40	0.20	0.71	1.58	7.13	0.16	0.56	0.78
Total		12.71	44.64	100.00		20.27	71.17	100.00

* μg LT-B/g corn is calculated as following: (fraction LT-B content × fraction mass yield)/100. † % of LT-B per mass is calculated as following: (fraction LT-B content × fraction mass yield / 28.44). ‡ % of LT-B per mass is calculated as following: (fraction LT-B content × fraction mass yield / total recovered LT-B). § LT-B content significantly different (α=0.05) for traditional and water steeping.

lowest LT-B containing fractions. No significant differences were observed between steep treatments for LT-B contents in wet-milled fractions for any of the fractions, except for fine fiber levels expressed in per mass basis.

Table 2 also shows the functional LT-B recovery in each of the wet-milled fractions. In general, the trend observed for LT-B content is conserved, with the fine fiber, coarse fiber, gluten meal and pericarp having the highest recoveries of LT-B per gram of maize fractionated. A total of 12.71 μg LT-B were recovered per gram of maize fractionated using traditional steeping, representing 44.64% of whole kernel LT-B (28.44 μg/g). On the other hand, 20.27 μg LT-B were recovered per gram of maize fractionated using water steeping, accounting for 71.17% of the total LT-B in whole kernel.

This result suggests that TS may have negative effects on recombinant protein recovery, as was observed for GUS activity[27] and LT-B.[41] Addition of SO_2 and lactic acid enables breakage of cross-linking disulfide bonds in the endosperm protein matrix,[22,23] and possibly of the recombinant protein as well. In the case of LT-B and of most proteins, correct folding and functionality depends highly on disulfide bond formation. It is likely that SO_2 and lactic acid directly affect LT-B protein stability, folding and assembly into functional pentamers. The high recovery of functional LT-B observed from wet-milling using water steeping suggests this method is more adequate than TS for high recovery of functional LT-B in wet-milled fractions.

Table 2 shows that for LT-B yield, however, both steeping treatments result in 20–30% recovery in fine fiber in terms of whole kernel LT-B (28.44 μg LT-B/g) and 40–45% recovery in terms of total recovered LT-B (12.70 μg LT-B/g for TS, and 20.24 μg LT-B/g for WS). This result highlights the importance of yield and concentration for downstream utilization of the wet-milling fractions. From a practical and technical standpoint it is possible to envision the combination of all LT-B-enriched fractions (coarse fiber, fine fiber, gluten meal, and pericarp) for use as an edible vaccine, while allowing use of germ and starch for alternative uses. Because this product is designed as a potential edible vaccine (without need for recombinant protein extraction) it is possible to enhance the LT-B content at the expense of purity and concentration by achieving this combination of fractions. However, it is also necessary to consider the target organisms and their normal feeding habits. For example, a combination feed could be certainly applied for livestock, but a highly concentrated dose might be desirable for human vaccination; in the latter case, wet-milling with traditional steeping conditions might be recommended for higher LT-B concentration recovery in fine fiber.

LT-B can be concentrated in the fine fiber fraction recovered from wet-milling by a factor of 4 to 8, depending on the steeping treatment. Fine fiber represents 2.48 and 7.06% of kernel mass for TS and WS (Table 1), respectively. LT-B is therefore highly concentrated in a relatively small amount of mass, which is favorable for direct utilization or further processing of the enriched fraction and enhanced vaccine potency, as has been shown to be the case of dry-milling LT-B-germ-enriched fractions in previous studies.[15] Coarse fiber, gluten meal, pericarp and steep liquor also contain LT-B and represent a combined 34.62 and 33.13% kernel mass for TS and WS, respectively. There are no significant differences in LT-B recovery for these fractions using TS or WS steeping treatments. Maize gluten meal, fiber and steep liquid are low-value fractions usually sold as maize gluten feed for the livestock industry.[10,21]

It is commonly thought that the fine fiber fraction contains mostly cell wall remnants from endosperm cells. Our previous studies[42] have shown that LT-B accumulates in the secretory system of endosperm cells when carrying its native signal peptide. It is our hypothesis that the association of LT-B to the fine fiber fraction obtained from wet-milling fractionation is the result of tissue specific expression and sub-cellular compartmentalization in the secretory system of endosperm cells.

Smaller amounts of LT-B were detected in germ and starch fractions recovered from both TS and WS in wet-milling. LT-B levels in the germ and starch are lowest compared to the other wet-milled fractions. The very low accumulation of LT-B in germ is due to the preferential expression of the protein in the endosperm tissue of seed.[19] The fate of LT-B in germ fraction from wet-milling fractionation remains to be tested to establish whether it is utilizable for traditional oil recovery. Because of safety concerns, it is likely that germ meal and unfermentable solids from starch conversion containing LT-B would not be used as food or feed, but used for other industrial purposes such as biodiesel, paper coatings, or adhesives, etc. Similarly, the lack of enrichment of LT-B in the starch fraction would allow the utilization of starch in downstream processes such as fermentation to produce biofuel and industrial chemicals. This result is highly desirable considering that starch represents 59.98 and 56.78% of kernel mass in TS and WS wet-milling, respectively (Table 1).

Our data indicate that WS treatment results in higher levels of extractable LT-B across all milling fractions combined when compared to TS treatment. However, in a per mass basis, TS seems to provide the highest concentration factor for the fine fiber fraction only (8.2 vs. 4.0), at expense of significant loss of LT-B from the other fractions (hence the 44.64% recovery in TS vs. 71.17% recovery in WS treatments presented in Table 2). This is consistent with previous findings[27] where lactic acid and sulfite treatment result in loss of biological activity of proteins, probably due to denaturing.[22] In this sense, the data are useful for future studies in which specific objectives want to be met regarding total LT-B yield or high concentration factors in particular fractions.

Total aqueous extractable proteins and monomeric LT-B in wet-milled fractions

To assess whether the total aqueous extractable proteins (TAEP) in transgenic maize kernels distribute similarly as the recombinant protein, we compared the maize TAEP and LT-B in the different fractions recovered from fractionation by wet-milling (Figures 1A,B). The TAEP includes native corn proteins and recombinant LT-B. All solid fractions contain between 0.2 and 1.2 mg TAEP per mL, with the germ fraction being the richest of all in TAEP content. Figure 1B shows the LT-B content as a percent of the TAEP in each fraction. Figure 1B confirms that the fine fiber fraction contains the highest concentration of LT-B in terms of mass and TAEP. Contrary to what is shown in Figure 1A, the germ fraction richest in TAEP contains the second to last lowest levels of % LT-B per TAEP, confirming low accumulation of LT-B in germ as described above.

Figures 1A,B are presented to show that the distinct distribution of LT-B in the different fractions is independent of

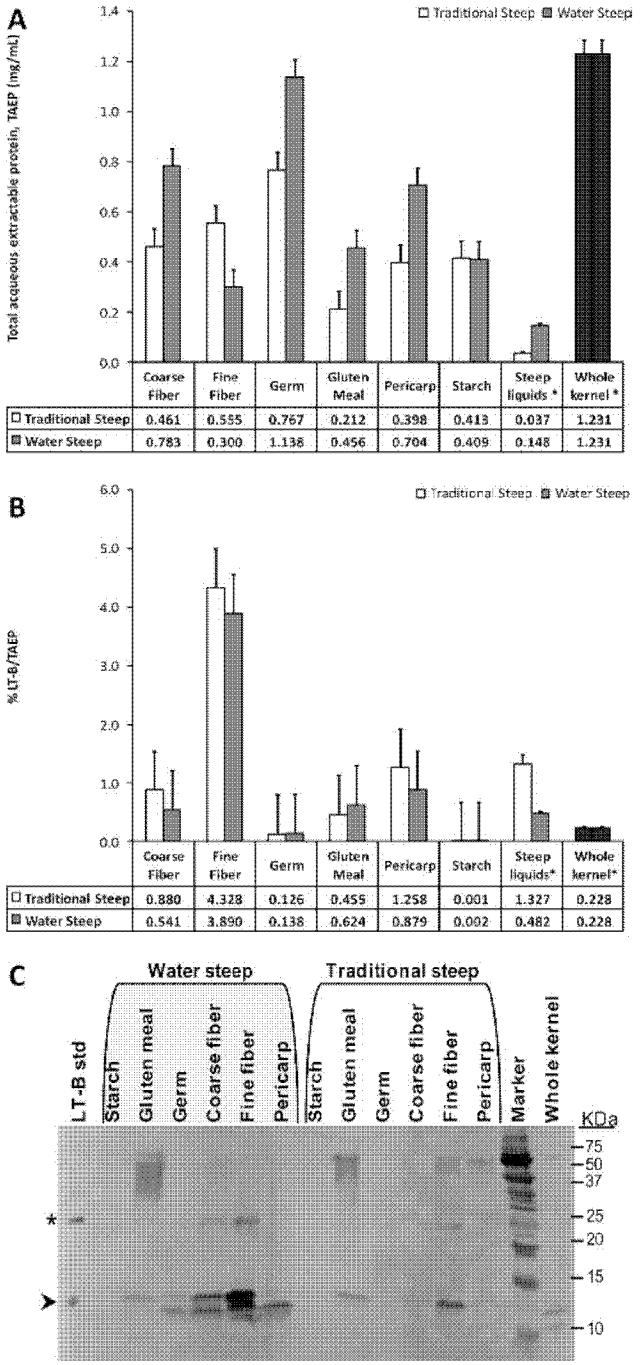

Figure 1. Total aqueous extractable protein (TAEP) and LT-B distribution in fractionation products of LT-B maize using two types of steep conditions.

(A) TAEP content. (B) Percent LT-B per TAEP. (C) Western blotting analysis using anti-LT-B antibody. Error bars denote standard error of the means. Asterisk in A and B denotes fractions not included in the statistical model. Asterisk in C denotes LT-B multimer. Arrowhead in C denotes LT-B monomer.

the native corn protein distribution. Similar to what has been presented in Table 2, Figure 1B also shows the highest concentration factor obtained by fractionation of the transgenic material, when compared to whole kernel content, is in the fine fiber fraction. While in whole kernel LT-B can be detected at a level of 0.228% LT-B per TAEP, the fine fiber fraction levels are detected at 3.89–4.33% LT-B per TAEP, a concentration factor of 17–19%.

Figure 1C presents a Western blot of TAEP from fractions obtained from wet-milling of TS and WS treated transgenic LT-B maize. Because G_{M1}-specific ELISA can only capture functional LT-B when it is in pentameric form, the Western blot is used here to detect all forms of LT-B proteins, typically the monomeric LT-B in a denaturing SDS gel. Figure 1C shows that in general, the levels of functional LT-B observed using G_{M1} capture ELISA in all fractions correspond with the detection of monomeric LT-B by Western blotting. It also shows that at equal TAEP levels across fractions, WS fractions have higher levels of LT-B. As mentioned previously, it is possible that LT-B exists in a variety of forms from monomers to pentamers. It has been reported that the level of functional pentameric LT-B detected by G_{M1} ELISA is within 40% of the level detected using a sandwich ELISA that detects total LT-B protein.[16] In the present work, we focused on assessing pentameric LT-B, as this is the functional form of the protein for G_{M1} receptor – mediated internalization of the protein for immune response elicitation.

Conclusions

Fractionation of transgenic LT-B maize by traditional wet-milling (steeping in 0.2% SO_2 + 0.5% lactic acid) and water steeping results in significantly different mass recoveries for coarse fiber, fine fiber, gluten meal and steep water fractions (fractions associated with high protein content). No effect of steeping treatment was observed for recovery of germ, pericarp and starch fractions.

Analysis of functional LT-B content in wet-milled fractions showed that functional LT-B was detected in all fractions regardless of steeping conditions; however, greatest enrichment of functional LT-B was in the fine fiber fraction for both steeping treatments. Fine fiber fractions from wet-milled transgenic LT-B maize were enriched up to a factor of 8.2 when compared to the level in whole kernels and present great potential for further utilization as a vaccine or vaccine component. With a relatively low protein content of 12%, LT-B enrichment in the fine fiber fraction represents potentially reduced maize protein impurities for utilization as oral vaccine or downstream processing or purification of LT-B. One of the remarkable properties of maize-derived LT-B is its potent immunogenicity when delivered orally.[43] Therefore, the enriched fine fiber fraction, a low-value fraction, shows enhanced added value due to its potential use as a direct delivery system for LT-B.

Previous human trials for plant-derived LT-B have used dosage corresponding to 0.75–1 mg LT-B per dose delivered as an enriched germ product of maize[34] or as potato tuber.[44] Even with the moderate LT-B expression level in the line used for this work, this dosage can be easily achieved from the fraction enrichment process employed. The concentration factor achieved by the wet-milling process presented here would result in a 4 g dose of enriched fine fiber fraction instead of 35 g dose of ground whole corn. This is eight times less material to be eaten or resuspended in water for consumption. Furthermore, expression levels can further be increased through breeding and selection to achieve an even higher concentration. The potential of the proposed approach also resides in being able to concentrate (if using combined LT-B enriched fractions) about 97% of the total LT-B in the grain, in about 37% of the grain's mass, allowing for

utilization of the reminder ~63% solids for alternative uses instead of incurring in waste disposal costs.

Wet-milling is traditionally optimized for starch purification and not for recovery of proteins from particular fractions, and has come to use traditional steeping treatments to achieve such purpose. We have expressed the LT-B in maize endosperm tissue by using an endosperm specific promoter (27 kD γ-zein promoter). Therefore, it is anticipated that it can be enriched in one of the fractions derived from the endosperm tissue (eg starch, fine fiber, coarse fiber or gluten meal). On the other hand, we did not add specific sub-cellular targeting sequences in our construct, but rather conserved the native bacterial signal peptide, which results in targeting to the secretory pathway of maize.[42] This is the first report of a recombinant protein under the control and direction of the endosperm specific promoter and bacterial signal peptide, respectively, being enriched in the fiber fraction. We anticipate that recombinant proteins controlled and directed using similar regulatory elements and targeting sequences will have similar fates in the wet-milling fractionation process.

Traditional steeping has a negative effect on total recovery of functional LT-B. The applicability of water steeping for recovery of other recombinant proteins, however, needs to be addressed individually, as the effect might be reduced or enhanced depending on the protein's properties. It is also conceivable that the recovery of LT-B may change when different extraction conditions are used. Yet, it is unlikely the overall LT-B distribution in corn fractions described here will be affected as we predict this is the result of combination of regulatory and targeting sequences used (endosperm-specific promoter and signal peptide for secretory pathway localization). Because the LT-B corn is intended for direct oral delivery purpose, no further extensive protein extraction procedures were intended.

When compared to dry-milling fractionation, the value of wet-milling is that the bulk of the grain (starch) is highly purified, the recombinant protein can be concentrated in low mass fraction, and the amount of waste matter and associated costs are reduced by utilizing all fractions of the kernel. As discussed above, the wet-milling process would allow use of starch and germ for other purposes, while dry-milling (that results in recovery of bran-rich, germ-rich and endosperm-rich fractions without recovery of highly purified starch) would not. If there is no interest for utilization of pharma crop for more than one application, dry-milling could be an attractive alternative that allows on-farm-site processing to prevent accidental pharma corn seed spillage during transportation. Though dry-milling has been used as a fractionation method for recovery of recombinant proteins,[20,25,26] we believe that results from using dry-milling would not be the same as those presented here, even when combining high fiber fractions.

The information presented in our work can help shape strategies for designing specialty crops of tomorrow. The recovery of functional LT-B in fractions by using conventional wet-milling procedures enhances the potential use of maize as a bioreactor for vaccine or vaccine components. The enrichment of LT-B in the fine fiber fraction reduces significantly the quantity of material necessary to achieve a particular dose of antigen. It is also possible that some LT-B-rich fractions (coarse fiber, fine fiber, gluten meal, and pericarp) from wet-milling can be combined to achieve greater yield for LT-B, since these fractions possess very little value anyway. Wet-milling is a well established operation that can be applied to processing of added-value specialty crops.

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