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# Extraction of Recombinant Dog Gastric Lipase from Transgenic Corn Seed

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Several approaches were examined for extracting the relatively hydrophobic protein recombinant dog gastric lipase (rDGL) expressed in the endosperm of transgenic corn seed. The first approach used minimal processing of the seed before extraction (i.e. simple grinding of whole seed) followed by selective extraction to eliminate 72% of contaminant proteins without compromising rDGL recovery from the meal of whole grain. The second approach added defatting of the whole grain meal to reduce the amount of detergent in the subsequent step for extracting rDGL. The third approach incorporated dry-milling of the corn to recover an endosperm rich fraction, followed by extraction of this fraction. The dry milling strategy was most effective, resulting in recovery of 35 U rDGL/g of corn seed (50 U/g of endosperm) with a specific activity of 9 U/mg compared to 22 U and 3 U/mg for the first strategy and 36 U and 3.7 U/mg for the second. The reductions in host protein contamination and lower detergent levels of the endosperm route should simplify downstream purification steps.

**KEYWORDS: Lipase; extraction; detergent; defatting; upstream processing; seed fractionation; corn; maize; transgenic**

## INTRODUCTION

The feasibility of using plants as bioreactors to produce recombinant proteins has progressed since the first reports in 1983 (*1*, *2*). The most attractive feature of this technique is the low cost associated with production coupled with the expectation that purification will be no more costly. A recent study estimated a cost of $5.90/g for recombinant lactoferrin using rice as the host (*3*), while the estimated cost of $\beta$-glucuronidase was $43/g with transgenic corn as the host (*4*, *5*). The much lower cost for the lactoferrin case results from the higher expression level of 5 g of lactoferrin/kg of rice flour and the relatively easy ion exchange purification process (*3*). Although these costs may increase as growth and handling regulations become more stringent, they are still likely to compare favorably with $105 or $300−3000 for transgenic goats' milk or mammalian cell culture, respectively (*6*).

The protein of our interest is recombinant dog gastric lipase (rDGL) expressed in endosperm of corn seeds at a reported level of ca. 1 g/kg of corn (*7*); some information on the transformation of corn for this transgene has been reported (*8*). The molecular weight and isoelectric point of this rDGL are approximately 49 kDa and 6.7 measured according to SDS-PAGE and isoelectric focusing, respectively (*8*). DGL has a grand average hydropathicity (GRAVY), on the basis of the amino acid sequence and crystal structure, of −0.069 (*9*), which is relatively hydrophobic, but it is still water soluble (*8*, *10*). Gastric lipase has hydrophobic tips exposed in the open enzyme conformation that leads to binding of the hydrophobic substrate, i.e., lipids (*11*), and is effective in hydrolyzing lipids after adsorbing to an oil−water interface (*10*). Gastric lipase has been expressed and produced in insect cell culture (*12*, *13*), yeast (*14*), transgenic tobacco (*15*), and corn seed (*7*, *8*). Different host tissues require different conditions to extract lipases. The recovery of extracellular lipases from fermentation broths is simplified because lipases remain in the supernatant after centrifugation (*13*, *14*, *16*−*20*). Extraction of lipases from oilseeds requires detergents (polyoxyethylene (20) monooleate (Tween 80) (*21*), 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (*22*, *23*)) and/or a reducing agent such as dithiothreitol (*24*−*26*). In contrast, no detergent was used to extract rDGL from transgenic tobacco leaves (*15*).

For extraction, the host tissue matrix must also be considered to minimize contaminants in extracts. In our study, rDGL is expressed in corn seeds that consist mainly of endosperm (83%) and germ (11%). The endosperm is mostly starch (88%), while the germ is rich in oil (33%) and protein (18%) (*27*). Germ proteins have different solubility characteristics than endosperm proteins (*28*), so the separation task will change when expression and extraction are targeted to a particular fraction (*4*, *29*). Methods to separate germ and endosperm are well established in the grain milling industry (*28*, *30*). Further control of the purification burden can be obtained by manipulating extraction conditions.

For other recombinant proteins, reports are available regarding the effects of extraction conditions on total protein and recombinant proteins from corn seeds. Azzoni et al. (*31*) studied

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concentrations of recombinant aprotinin and total proteins in extracts with different combinations of pH and salt concentration. The concentration of total protein increased with pH and salt concentration (up to 200 mM), with pH being more significant. A lower pH is thus preferred for reducing contaminants. The concentration of extracted aprotinin was higher at a higher salt concentration and did not show a monotonic trend with pH. The optimum condition was concluded to be pH 3.0 and 200 mM NaCl to minimize contaminants and achieve satisfactory extraction of aprotinin. Farinas et al. (*32*) similarly studied corn proteins extracted from endosperm rich fraction only. The results were similar to those from whole grain: higher total protein concentration at a higher pH and salt concentration with pH being the dominant parameter. However, when recombinant human proinsulin is extracted from endosperm, pH 10.0 and 200 mM NaCl were needed to solubilize the proinsulin (*33*). The challenges associated with this selection included a high total protein and a low flux during filtration (*33*).

For the case of extracting rDGL from transgenic corn seeds, Roussel et al. (*8*) washed the ground corn meal twice with hexane to remove oil and then stirred the meal with an extraction buffer containing EDTA and Triton X-100. Although the authors did not report the quantitative outcome of the extraction step, it appeared defatting and detergent addition were required.

Because extraction consumes ca. 40% of the total cost when producing recombinant proteins from corn seeds (*4*), the objective of this work was to develop strategies that maximize the extraction of rDGL, minimize the material requirements of extraction, and facilitate downstream recovery. The first group of studies focused on the meal of whole grain, where variables during extraction included various combinations of detergent types and concentrations, pH values, and salt concentrations. The second group of studies was based on the information from the first group and was aimed to lower extraction cost by integrating upstream processing, which included (1) defatting the meal of whole grain and (2) extracting the rDGL-containing endosperm only after separating corn seeds into germ-rich and endosperm-rich fractions.

## MATERIALS AND METHODS

**Chemicals.** The 4-nitrophenyl butyrate (NPB), CHAPS, and L-histidine were purchased from Sigma (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA). Chemicals were used without further purification, and deionized water was used in all experiments.

**Transgenic Corn Seeds and the Meal of Whole Grain.** Transgenic corn seeds with expression of rDGL targeted to the endosperm were provided by Meristem Therapeutics (Clermont-Ferrand, France) and stored at 4 °C and low moisture. In preparation for extraction, seeds (30 g/batch) were ground in a coffee-grinder (Krups, Medford, MA) for 30 s followed by milling (Retsch mill DR 15/40, Retsch Inc., Haan, Germany) to a size that passed the outlet 18 mesh screen. The meal was stored at 4 °C until used.

**Extraction of rDGL from the Meal of Whole Grain.** *Buffer Systems.* Extraction buffers were prepared with 50 mM of the following buffer salt at a corresponding pH: citric acid (pH 2.5, 3.0, and 4.0); sodium acetate (pH 5.0); L-histidine (pH 5.5 and 6.0); sodium phosphate (pH 7.0); Tris (pH 8.0); sodium carbonate (pH 9.0 and 10.0). Detergents were then dissolved in the buffers to achieve different concentrations (w/w) of polyoxyethylene (20) sorbitan monolaurate (Tween 20), Tween 80, polyoxyethylene mono *p-tert*-octylphenyl ether (Triton X-100), or CHAPS. Buffers were also supplemented with various concentrations of sodium chloride in one set of experiments to evaluate the effects of salt concentration on lipase extraction.

*Extraction Kinetics.* The meal was stirred continuously at a ratio of 1 g of solids to 4 mL of 4% Tween 80 at pH 3.0, 5.0, or 5.5, and approximately 1.5 mL of slurry was sampled after predetermined periods at room temperature. The slurry was centrifuged immediately at 10 000*g* for 10 min (Sorvall RC5B Plus centrifuge, DuPont, Wilmington, DE), and the supernatant was filtered through a Corning $\mu$Star syringe tip filter (3 cm$^2$ effective filtration area, 0.45 $\mu$m pore size, cellulose acetate membrane, Corning Inc., Corning, NY). The samples were stored at −20 °C until analysis.

**Table 1.** Composition of Whole Corn and the Prepared Fractions (with 95% Confidence Intervals)

| material | amt of kernel (%)[a] | oil (%)[a] | protein (%)[a] |
|---|---|---|---|
| whole kernel | 100.0 | 4.8 ± 0.2 | 9.8 ± 0.5 |
| whole kernel washed with ice cold hexane | 100.0 | 2.8 ± 0.6[b] | |
| germ | 23.55 ± 1.1 | 13.8 ± 0.5 | 13.2 ± 0.4 |
| endosperm | 66.7 ± 1.1 | 1.63 ± 0.0 | 8.6 ± 0.6 |
| hull | 3.8 ± 0.5 | 2.7 ± 0.2 | 10.3 ± 0.3 |

[a] Dry basis. [b] Calculated after subtracting the oil amount extracted by ice cold hexane from the content of the whole kernel.

*General Extraction Protocol.* Extracts were prepared by stirring 1 g of corn meal in 4 mL of extraction buffer with a magnetic stir bar. The slurry was stirred for 14 h unless otherwise noted and then centrifuged at 10000*g* for 30 min. The supernatant was then syringe-filtered through the 0.45 $\mu$m cellulose acetate membrane. Extractions were performed at room temperature; cold extraction showed no improvement. The clarified samples were stored at −20 °C until analysis for enzyme activity and total protein concentrations.

**Extraction of rDGL from the Meal of Whole Grain after Washing with Cold Hexane (Defatting).** Similarly to Roussel et al. (*8*), defatting was done by mixing 10 g of whole grain meal with 100 mL of ice cold hexane for 1 h in an ice bath on a stir plate. The slurry was vacuum-filtered through no. 1 filter paper (Fisher) to remove hexane. After the sample was defatted for a second time, the filtered cake was washed with 10 mL of fresh ice cold hexane and air-dried overnight in a laboratory hood to remove residual hexane. Gravimetric analysis of the hexane extract showed the extraction to remove ca. 40% of the oil in the original corn meal (**Table 1**). The defatted meal was stirred at a ratio of 1 g of solids to 10 mL of 0.13% Triton X-100 with 50 mM sodium phosphate at pH 3.0. Roussel et al. (*8*) used an extraction time of 16 h for defatted meal, while we found 12 h was sufficient. The slurry was centrifuged (Sorvall RC5B Plus centrifuge, DuPont, Wilmington, DE) immediately at 10000*g* for 20 min at 4 °C, and the supernatant was filtered through a 0.45 $\mu$m cellulose acetate syringe membrane (Corning). The samples were analyzed for lipase activity and protein concentration immediately.

**Fractionation of Corn Seeds (Degerming).** Fractionation of corn seeds into endosperm rich and germ rich fractions was completed by following the optimal dry milling procedure developed in the Center for Crops Utilization Research at Iowa State University (*34*). The method passes the grain through a degermer, a mill, an aspirator, and a series of screens to recover an endosperm-rich fraction containing ca. 70% of the grain with an oil content of ca. 1.6% (dry base) from whole corn of 4.8% (dry base) (**Table 1**). This endosperm-rich fraction was ground further (Braun KSM2 Aromatic coffee-grinder, Braun, Woburn, MA) for 30 s in a cold room yielding the final endosperm meal for the extraction studies. The particle size distribution of the meal is compared to that of the whole grain meal in **Table 2**. The endosperm fraction is more finely ground than the whole grain meal.

**Extraction of rDGL from Endosperm Meal.** Extracts were prepared by stirring 1 g of endosperm meal in 10 mL of extraction buffer with a magnetic stir bar. Extraction buffers contained different concentrations of Triton X-100 and NaCl in 50 mM of sodium phosphate at pH 3.0 or 5.5. The slurry was stirred for 12 h unless otherwise noted and then centrifuged at 10000*g* for 30 min. The supernatant was then filtered through a 0.45 $\mu$m cellulose acetate membrane (Corning). Extractions were performed at room temperature. The clarified samples were assayed immediately for lipase activity.

After the pH, Triton, and NaCl concentrations were screened for the endosperm meal, extraction kinetics were determined on separate size fractions of the meal (sieved into standard screens of sizes nos.

**Table 2.** Size Distribution of Milled Endosperm Fraction and Whole Grain Meal

| screen mesh | particle size (*d*, mm) | wt % endosperm meal | whole grain meal |
|---|---|---|---|
| no. 18 | >1 | 5 | 14 |
| no. 35 | 1 > *d* > 0.5 | 15 | 37 |
| no. 40 | 0.5 > *d* > 0.425 | 20 | 7 |
| no. 50 | 0.425 > *d* > 0.300 | 20 | 10 |
| pan | <0.3 | 40 | 31 |

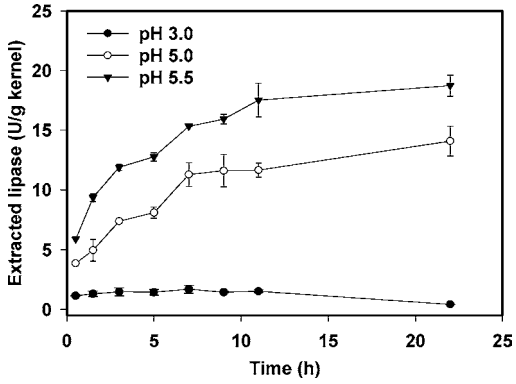


**Figure 1.** Extraction kinetics of rDGL from the meal of whole grain with 4% Tween 80 at pH 3.0, 5.0, and 5.5. Error bars are 95% confidence intervals.

18, 35, 45, and 50 from Fisher Scientific, Pittsburgh, PA), as well as the endosperm fraction directly from the dry milling (no final grinding step).

**Lipase Activity Assays.** The activity of lipase was determined based on the hydrolysis of substrate NPB into nitrophenol and butyric acid (*35*). Assays involved the emulsification of 6.7 $\mu$L of the oil phase (0.15 M NPB dissolved in *n*-heptane) in 1 mL of 0.01% (w/w) Tween 80 at pH 5.5, followed by the introduction of lipase samples. The absorbance of emulsions was monitored at 346 nm for 5 min at 25 °C (Ultraspec 4000 UV/visible spectrophotometer, Pharmacia Biotech, Piscataway, NJ). One unit of lipase activity corresponded to the production of 1 $\mu$mol of nitrophenol in 1 min.

**Protein Assay.** The presence of detergent interfered with the Bradford (*36*), Lowry (*37*), and biuret (*38*) methods. On the other hand, the bicinchoninic acid (BCA) method (*39*) had good tolerance for detergent. Reagent A, containing BCA and other chemicals, was purchased from Pierce (Rockford, IL), while reagent B, 4% cupric sulfate, was prepared from the salt. Assays followed the procedures of the Pierce manual and the incubation of samples was at 37 °C for 30 min in a water bath. Bovine serum albumin was used as the reference standard.

## RESULTS AND DISCUSSION

The development of the extraction strategy was initiated with the coarsely ground meal of whole grain as this required minimal upstream processing. Parameters studied included detergent types and concentrations, pH, salt concentrations, and kinetics. On the basis of the results from whole grain, three extraction strategies based on different starting materials were developed. To make direct comparisons of data from each starting material (whole grain, defatted whole grain, and endosperm), the lipase concentrations in extracts are presented per g of whole kernel.

**Extraction from the Meal of Whole Grain.** *Extraction Kinetics.* When the meal of whole grain was extracted with 4% Tween 80 at pH 5.0 and 5.5, there was an observable increase in lipase activity even after 22 h (**Figure 1**). Extended extraction also increased the amount of contaminant proteins (data not shown). Little lipase activity was observed in extracts of pH

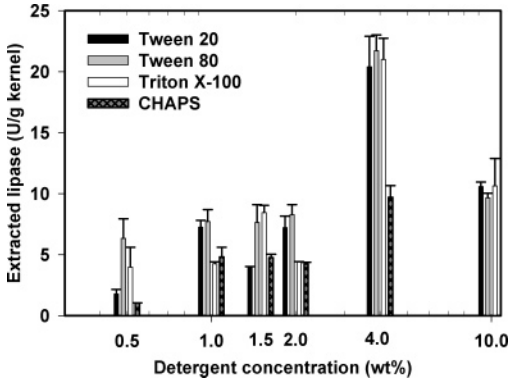


**Figure 2.** Lipase activities extracted from whole grain with different detergent concentrations at pH 5.5. Error bars are 95% confidence intervals.

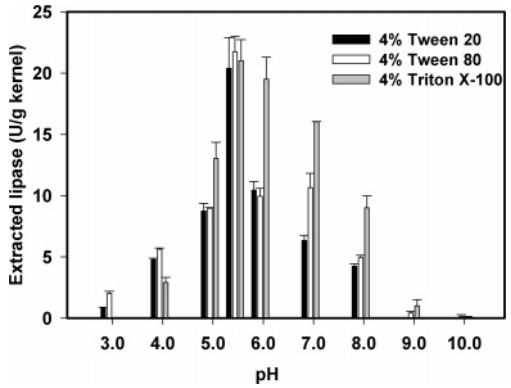


**Figure 3.** Lipase activity of extracts from whole grain with 4% detergent at different pH values of the extractants. Error bars are 95% confidence intervals.

3.0. Most lipase activity was observed after 12 h. An extraction time of 14 h was selected to conveniently incubate samples overnight when screening extraction parameters.

*Effects of Detergent Concentration.* No lipase activity was detected in the samples extracted without detergent at a pH ranging from 2.5 to 10.0. At a detergent concentration below 0.5%, extracts had lipase activities mostly lower than 1 U/mL for all detergent types and pH values. At pH 5.5, extracts showed increased lipase activity with an increase in detergent concentration up to 4% and then lowered activity at a detergent concentration of 10% (**Figure 2**). CHAPS, the most costly of the detergents, gave lower yields than the other three detergents at concentrations up to 4% and was not used further. The presence of detergent during extraction also extracted additional host corn proteins, and the specific activity of the sample with 4% Tween 80 at pH 5.5 was only 0.90 U/mg of protein.

Relatively hydrophilic recombinant $\beta$-glucuronidase (GRAVY = −0.383) (*9*) was extracted from corn seeds without detergent (*4*). However, this was also true of aprotinin (GRAVY = −0.106) (*9*), which is close in hydrophobicity to DGL (*29*, *31*). $\beta$-Glucuronidase may be the more appropriate comparison because it is much closer in molecular weight to DGL.

*Effects of Salt Concentrations and pH.* Extraction was secondarily affected by buffer pH (**Figure 3**). Very little activity was detected in extracts prepared at pH 3.0, 9.0, and 10.0. The maximum (22.1 U/g of kernel) was observed at an extraction pH of 5.5 where detergent type had only a small effect. For extractions with 4% detergent at pH 5.5, the addition of NaCl did not enhance lipase extraction (data not shown).

**Strategy I: Fractional Extraction.** Since detergent was required to extract rDGL from the meal of whole grain, a

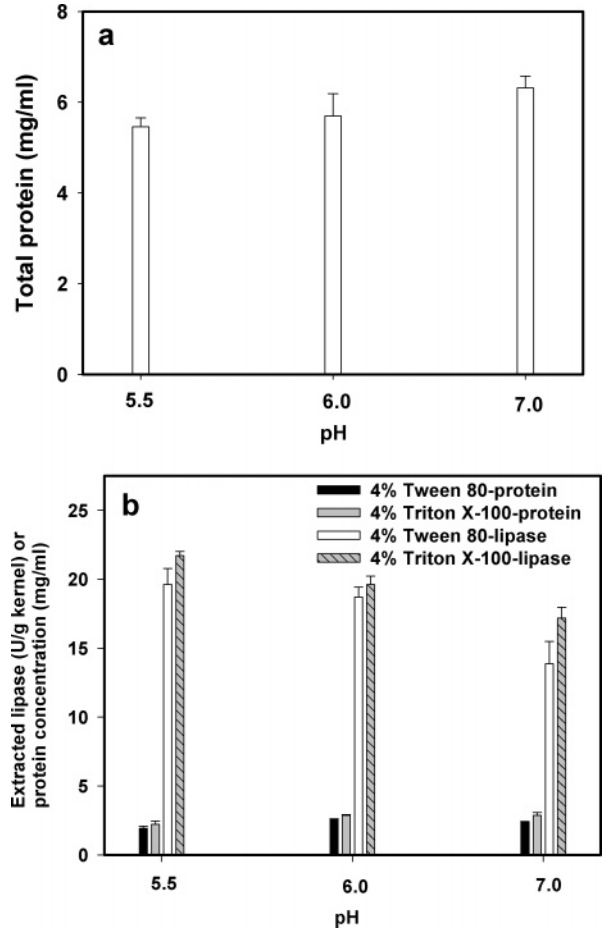


**Figure 4.** Total protein concentrations and lipase activities of extracts from whole grain after (**a**) first extraction (no surfactant) aimed at removal of native proteins (lipase activity was nondetectable and thus not plotted) and (**b**) second extraction aimed at recovery of lipase (surfactant at level shown). Error bars are 95% confidence intervals.

fractional extraction protocol was used to eliminate a portion of the soluble native proteins. During the first extraction step, the meal was treated at pH 5.5 without detergent for 2 h, during which most corn proteins were extracted by aqueous buffers. The slurry was then centrifuged at 10000*g* for 30 min, and the supernatant was decanted and filtered through a 0.45 $\mu$m membrane for analyses of protein concentration and lipase activity. The remaining solids were then resuspended with an extraction buffer containing 4% Tween 80 or Triton X-100 at pH 5.5, 6.0, or 7.0. The slurry was stirred for 22 h and then centrifuged and filtered as in the first extraction step.

The first extraction step removed 5.6, 5.9, and 6.4 mg/mL protein at pH 5.5, 6.0, and 7.0, respectively, with no loss of lipase (**Figure 4**). The protein concentrations of samples after the second extraction step were below 3 mg/mL (**Figure 4b**). For the case of extraction with 4% Triton X-100 at pH 5.5 at the second step, the amount of protein removed in the first step was approximately 72% of the total soluble protein. Approximately 22 U of lipase/g of kernel were extracted, similar to one step extraction (**Figure 3**), but specific activity (3 U/mg of protein) was improved more than three times from one step-extraction (0.9 U/mg of protein).

Extracts from different protocols behaved differently when filtered through the 0.45 $\mu$m cellulose acetate membrane. Membranes fouled severely after ca. 10 mL for all samples from one-step extraction, from the first step during fractional extraction, and from the second step fractional extraction with 4% Tween 80. However, a capacity of more than 100 mL of feed resulted for the extract from the second step during the fractional extraction with 4% Triton X-100, compared with ca. 10 mL when 4% Triton X-100 was used in one-step extraction. Identification of physicochemical differences leading to this observation was beyond the scope of this work. Nevertheless, this increased flux would be an advantage for downstream processing.

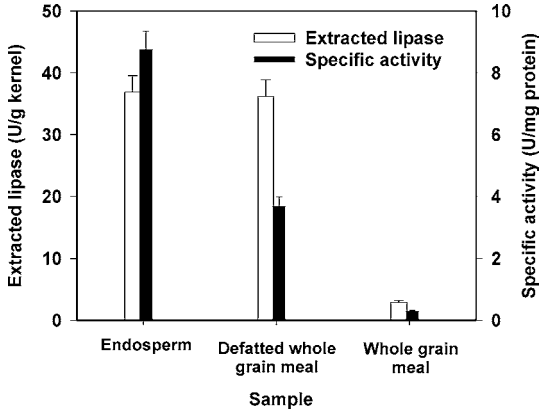


**Figure 5.** Comparison of rDGL extracted from the meal of whole grain, the meal of whole grain after defatting, and the endosperm fraction. The extraction buffer was 200 mM NaCl in 0.13% Triton X-100 at pH 3.0. Error bars are 95% confidence intervals.

**Strategy II: Defatting.** Roussel et al. (*8*) used a defatting procedure to extract rDGL from the meal of whole grain with a Triton concentration (0.0625%) significantly lower than the optimum (4%) detergent used in strategy I. The authors used pH 3.0 (other conditions unavailable in the paper), which would not be recommended according to our data from the meal of whole grain (**Figure 3**). To compare their strategy with the fractional extraction approach, we washed the meal with cold hexane twice and used a total of 0.13% Triton. The amount of rDGL extracted from the defatted meal was almost doubled from the optimum condition of strategy I (36 vs 22 U/g of kernel, **Figure 5** vs **Figure 4**), and extraction at pH 3.0 became effective with this procedure. Extract from the defatted meal also had higher specific activity (3.7 U/mg of protein) than that from strategy I (3 U/mg of protein). The comparison suggests that the removal of oil reduced the amount of detergent required and facilitated the extraction of rDGL, as would be expected if the oil were consuming detergent and adsorbing lipase.

**Strategy III: Fractionation of Seed Kernel.** Hexane extraction for oil removal may be replaced by the separation of germ from endosperm, an already mature process in the milling industry (*28*, *30*), based on the fact that germ is protein- and oil-rich (*4*, *28*) and rDGL was expressed in endosperm. **Table 1** lists the yield of endosperm in the degerming process and oil and protein contents in the whole kernel as well as in three fractions after degerming: germ; endosperm; hull. Detailed extraction studies were then performed for endosperm fraction, and the first parameter to be screened was pH because data shown in **Figure 3** demonstrated that pH was a dominant extraction factor at a fixed detergent concentration. Detergent type was not tested further because results from whole grain meal suggested that detergent type was not a sensitive parameter. Triton X-100 was used in the remaining studies.

*Effects of Extraction pH.* Two pH conditions were studied with 0.13% Triton X-100: optimum pH for the meal of whole grain (pH 5.5); pH 3.0 (promising in strategy II) (**Figure 6**). The extraction condition used in the defatting strategy was

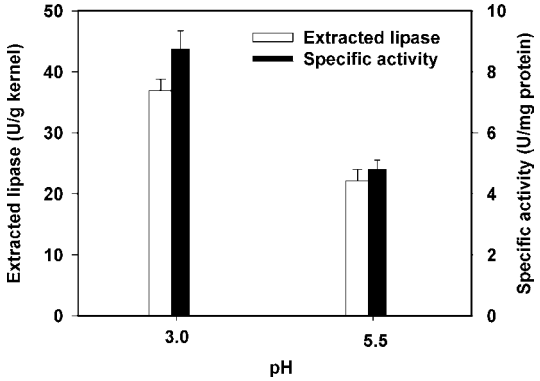


**Figure 6.** pH dependence of extraction from endosperm with 0.13% of Triton X-100 and 200 mM NaCl. Error bars are 95% confidence intervals.

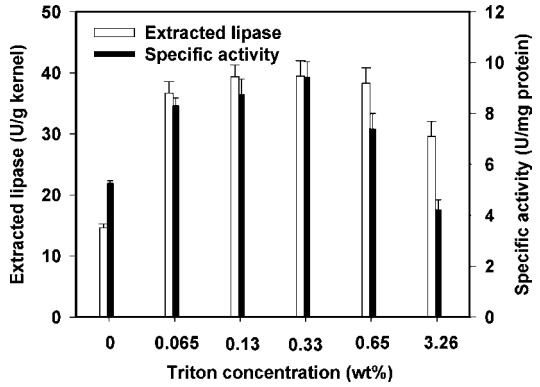


**Figure 7.** Effect of Triton X-100 concentration on the extractable rDGL from endosperm at pH 3.0 with 200 mM NaCl. Error bars are 95% confidence intervals.

equally effective for the lipase extraction from the endosperm fraction (**Figure 5**) confirming that rDGL was expressed in endosperm. Besides extracting more rDGL than from the meal of whole grain (**Figures 2**−**4**), samples extracted from endosperm at pH 3.0 had much higher lipase specific activity (9 U/mg of protein) than those from strategy I (3 U/mg of protein) and II (3.7 U/mg of protein). A specific activity of 9 U/mg corresponded to ∼14% purity according to specific activity of 65 U/mg of pure rDGL. The pH 3.0 was thus selected for remaining treatments using endosperm.

*Effects of Detergent Concentration.* When endosperm was extracted with different concentrations of Triton X-100, about 15 U of rDGL was extracted from 0.7 g of endosperm (1 g of kernel) even without detergent at pH 3.0, and the addition of Triton X-100 up to 0.33% facilitated the extraction of rDGL, followed by a decrease at higher Triton X-100 concentrations (**Figure 7**). The trend of surfactant concentration effects was similar to that from the meal of whole grain (**Figure 2**), but the corresponding behavior occurred at much lower detergent levels as a result of the lower oil content of the endosperm fraction. In addition, the removal of germ fraction reduced the extractable native protein concentration and as a result increased the specific activity of extracts (**Figure 7**). Further improvement was not significant when Triton X-100 was increased above 0.13%, and 0.13% Triton X-100 was chosen for further studies to reduce detergent use.

*Effects of Salt Concentration.* For extractions with 0.13% Triton X-100 at pH 3.0, the addition of NaCl enhanced rDGL extraction at low salt concentration (<200 mM) and then weakened the rDGL extraction (**Figure 8**). The observations were similar to those from the meal of whole grain at pH 3.0 (data not shown).

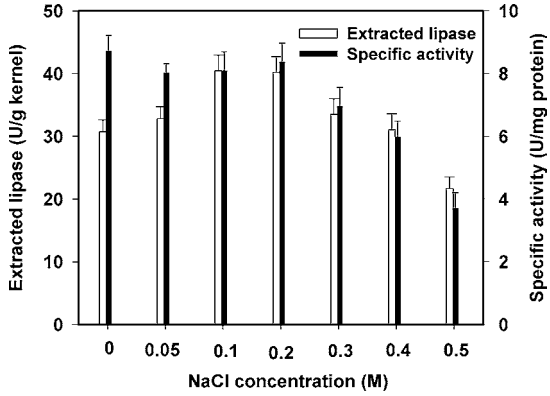


**Figure 8.** Effects of salt concentration on the extraction of rDGL from endosperm meal at pH 3.0 with 0.13% Triton X-100. Error bars are 95% confidence intervals.

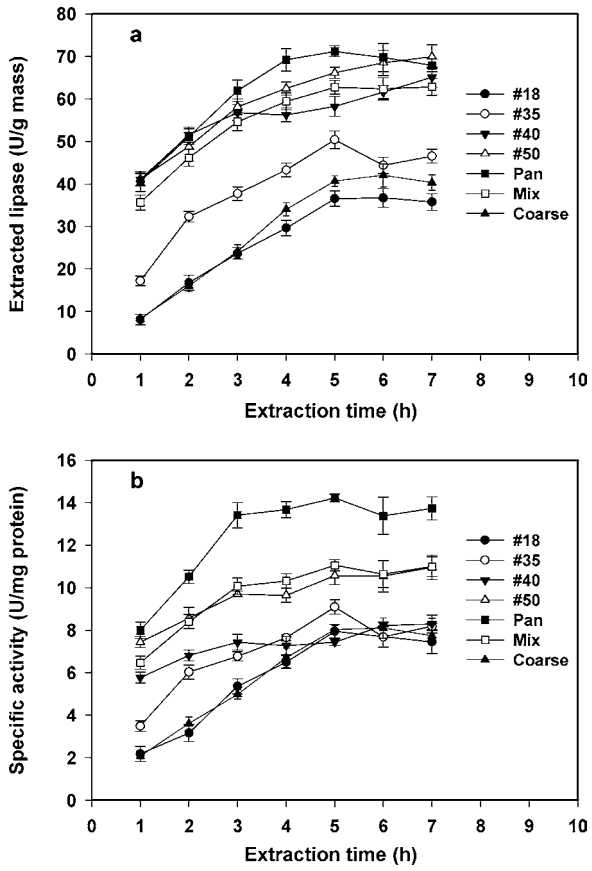


**Figure 9.** Extraction kinetics of rDGL from endosperm meal at pH 3.0 with 0.13% Triton X-100 and 200 mM NaCl: (**a**) lipase concentration; (**b**) lipase specific activity. The legend indicates endosperm subfraction used: unground (coarse); sieve fraction of ground; pooled sieve fractions (mix). Error bars are 95% confidence intervals.

The experimental design employed may have overlooked interactions that could have been detected by a factorial design. However, in this case, the primary effects of surfactant level and pH were quite large, and being able to examine a larger number of values of those parameters was a benefit. Where we did have a chance to observe interactions they were not apparent, as is seen in **Figures 3** and **4**, where pH trends did not change with surfactant type.

*Extraction Kinetics.* Since the extraction kinetics of a recombinant protein from transgenic plant seeds was a function of particle size (*40*), the ground endosperm-rich fraction was sieved to fractions of different fineness (**Table 2**). During

**Table 3.** Extraction of rDGL from Endosperm by Simple and Fractional Procedure[a]

| extractn | | extracted rDGL (U/g of kernel) | total protein (mg/mL) | specific activity (U/mg of protein) | yield of rDGL (%)[b] | yield of total protein (%)[b] |
|---|---|---|---|---|---|---|
| simple | | 39.1 ± 6.3 | 0.730 ± 0.1 | 8.5 ± 1 | 100 | 100 |
| fractional | first | 15.8 ± 1.6 | 0.711 ± 0.1 | 3.5 ± 0.4 | 40 ± 1 | 97 ± 2 |
| | second | 22.7 ± 1.6 | 0.265 ± 0.05 | 13.6 ± 1.5 | 64 ± 1 | 40 ± 3 |

[a] ± ranges are 95% confidence intervals. [b] Yields are relative to simple extraction.

extraction with 0.13% Triton X-100 and 200 mM NaCl at pH 3.0, there was an observed increase in lipase activity up to ca. 5 h for particles of various sizes (**Figure 9a**), with the finer fractions having a higher amount of extractable rDGL. The likely reason for the higher rDGL activity from finer fractions is that these fractions should be purer endosperm (where rDGL was expressed). The dry milling process relies on beaking the more brittle endosperm into smaller sizes than the germ (*41*); thus, the smallest fraction would be purest endosperm. In all cases, further extension of extraction time did not improve rDGL recovery, similar to the results from whole grain at pH 3.0 (**Figure 1**). The time to reach the maximum extractable lipase was shorter for the endosperm meal (5 h) than that for whole grain meal (7 h) possibly because whole grain meal was coarser (**Table 2**). Since most host proteins were extracted by 1 h, specific activity (**Figure 9b**) showed similar behavior.

*Fractional Extraction.* Surfactant-free buffer (10 mL of 50 mM sodium phosphate, 200 mM NaCl, at pH 3.0) was added to 1 g of endosperm, and the mixture was stirred for 1 h and centrifuged and filtered as above to provide a first-stage extract rich in readily soluble native proteins. The residual solids from centrifugation were resuspended in the above extract buffer supplemented to a level of 0.13% Triton X-100, stirred for 6 h, centrifuged, and filtered to prepare the coarse-clarified second-stage extract. Samples of each extract were assayed immediately for lipase activity and protein concentration.

Compared to the simple extraction (**Table 3**), the first fractional extract contained similar total protein, but unlike fractional extraction of whole grain meal this fraction contained approximately 40% of the rDGL activity, while the second fractional extract contained less native proteins and 60% of the rDGL activity. Hence, by fractional extraction, the final extract had 60% higher specific activity but unfavorably lower yield.

**Comparison of Three Strategies.** The amount of rDGL extracted from the meal of whole grain was affected by many variables including detergent concentration and type, buffer pH, and salt concentrations. The best extraction of rDGL was observed at pH 5.5 and 4% detergent (Tween 20, Tween 80, or Triton X-100). The oil present in the whole grain greatly increased the detergent requirement. The fractional extraction protocol eliminated 72% of contaminant proteins without compromising rDGL recovery from the meal of whole grain. A recovery of ca. 22 U of rDGL/g of kernel was enabled for whole grain meal, with a specific activity of 3 U/mg. The extract with 4% Triton X-100 from the second extraction step filtered much better than first stage or single-stage extracts. However, 0.16 g of detergent (1 g:4 mL of 4% Tween 80 or Triton) was required for the maximum recovery from 1 g of solids.

By first defatting the whole grain meal, it was possible to recover 36 U of rDGL/g of whole grain, with a specific activity of 3.7 U/mg and the amount of detergent needed was much less (0.013 g of detergent/g of solids). Thus defatting should reduce the cost associated with extraction and facilitate downstream purification but will add the cost of hexane extraction.

Dry milling and fractionation resulted in ca. 70% of seed mass being recovered as the endosperm fraction. The recovery of rDGL from this endosperm fraction was about 35 U/g of kernel (50 U/g of endosperm), with a specific activity of 9 U/mg. On the basis of the specific activity determined for purified rDGL (65 U/mg of protein), this recovery converted to 0.53 g/kg of corn. Since we only have an order of magnitude estimate of the level that could be produced in the seeds (1 g/kg of corn), we cannot report whether extraction was complete.

Additional advantages of using the endosperm fraction include reducing the solids going to extraction, reducing the native protein contamination of the extract going to purification, and reducing the amount of detergent (0.013 g of detergent/g of solids). Our preference on degerming prior to extraction was further supported by the fact that procedures for degerming are well-established in the grain-milling industry (*28*, *30*) and are more environmentally friendly than defatting with hexane.

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