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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 β-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) monoooleate (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...
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 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 (35). 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/v) of polyoxyethylene (20) sorbitan monolaurate (Tween 20), Tween 80, polyoxyethylene mono p-tetra-olpheylen 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 10,000g for 10 min (Sorvall RC5B Plus centrifuge, DuPont, Wilmington, DE), and the supernatant was filtered through a Corning uStar syringe tip filter (3 cm² effective filtration area, 0.45 μm pore size, cellulose acetate membrane, Corning Inc., Corning, NY). The samples were stored at −20 °C until analysis.

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 10,000g for 30 min. The supernatant was then syringe-filtered through the 0.45 μm 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 10,000g for 20 min at 4 °C, and the supernatant was filtered through a 0.45 μm 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 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 10,000g for 30 min. The supernatant was then filtered through a 0.45 μm 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.
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 because it is much closer in molecular weight to DGL.

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 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 β-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). β-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
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 μm 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 μm 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.

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
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).

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
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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