

Fate of Maize DNA During Steeping, Wet-Milling, and Processing

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ABSTRACT

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The fate of DNA during steeping, wet-milling, and subsequent processing of maize was examined using a sensitive polymerase chain reaction (PCR-based) detection system. The system used specific amplification of maize DNA sequences by primers generated toward plant nuclear- and chloroplast-encoded genes. The PCR method facilitated analysis of DNA content in food products, which is an important issue in use of genetically modified organisms. In a conventional laboratory wet-milling countercurrent steep system, DNA was detected in maize kernels throughout the process but was not found in steepwater. After kernels were wet-milled, DNA was detected in the starch, germ, coarse fiber, and wet gluten fractions but not in the fine fiber fraction. When dried by heating at

135°C for 2 hr, DNA was degraded to undetectable levels in the wet-milled gluten fraction and hydrated kernels. DNA was not detected in feed pellets, starch, dextrose, sorbitol, or high-fructose maize syrup made from industrial wet-milled samples. Although DNA could be detected in laboratory wet-milled fractions, some degree of degradation occurred after extended exposure to steepwater. Countercurrent steepwater samples from the later stages of the steeping process were able to degrade DNA. The level of DNA degradation appeared to correspond to the presence of sulfur dioxide and may represent a physiochemical rather than an enzyme-mediated process. Our results indicate that some steps in the steeping and wet-milling process can degrade maize genomic and plastid DNA.

Wet-milling is a significant use of maize in the United States and is exceeded only by maize use in the animal feed industry (Eckhoff and Paulsen 1996, Yang et al 1998). Wet-milling is performed primarily to isolate and recover starch for use in production of food-grade modified and unmodified starches, dextrose and fructose syrups, ethanol, and other chemicals by fermentation (Eckhoff and Paulsen 1996).

The initial and most important step in maize wet-milling is steeping, which involves soaking maize kernels for 20–40 hr in process water containing 0.1–0.2% sulfur dioxide and 1–2% lactic acid produced by fermentation during steeping (Eckhoff and Paulsen 1996, Yang et al 1998). Steeping induces chemical and biochemical reactions inside the kernel that lead to partial dissolution of endosperm protein and release of starch granules (Hull et al 1996a,b; Yang et al 1998). Although there is substantial information available on carbohydrate and protein distribution during maize steeping, wet-milling, and processing (Eckhoff et al 1993; Eckhoff et al 1996; Eckhoff and Paulsen 1996; Hull et al 1996a,b), the fate of maize DNA during this process has not been characterized.

Genetically engineered crops have been introduced successfully into production but concerns remain about the use of genetically modified organisms. The presence of genetically modified DNA has caused various groups and political organizations to restrict marketing of products containing genetically modified organisms (Williams 1998). It is necessary to determine which maize products contain DNA, because DNA may be degraded during different processing stages. The objective of our study was to examine the fate of DNA during steeping, wet-milling, and subsequent processing of maize, using a sensitive polymerase chain reaction (PCR-based) detection system. The detection system, which is used for higher plant DNA sequences, uses PCR primers for specific amplification of plant chloroplast- and nuclear-encoded genes. Our results indicate that some steps in the steeping and wet-milling process can degrade maize genomic and plastid DNA.

MATERIALS AND METHODS

Maize samples were obtained from three sources: a laboratory-scale steep system with a continuous countercurrent steep in lactic

acid and sodium meta-bisulfite (Eckhoff and Paulsen 1996), a laboratory wet-milling process (Eckhoff et al 1996), and an industrial-scale wet-milling process. The laboratory-scale countercurrent steep system contained 16 tanks, of which 12 were active and four were used for loading and unloading (Yang et al 1998). Maize kernels were steeped in 12 tanks in 2,000 ppm of sulfur dioxide (initially added as sodium meta-bisulfite) at 50°C for 36 hr. Samples of steepwater and maize kernels were taken from each of the steep tanks for analysis once the system achieved a steady state. Analyses of SO₂ and lactic acid were conducted according to Yang et al (1998). In the laboratory system, tank 1 contained new steepwater from maize steeped 36 hr, and each subsequent tank contained maize at a 3-hr lag from the previous tank, with steepwater aging 3 hr in a countercurrent arrangement (Yang et al 1998).

In the laboratory wet-milling process, after batch steeping for 24 hr in 0.55% lactic acid and 2,000 ppm of sodium meta-bisulfite, maize products were separated by germ isolation, and fiber, protein, and starch were recovered (Eckhoff et al 1996). Additional maize products resulting from industrial-scale steeping and wet-milling were provided by Archer Daniels Midland (Decatur, IL).

DNA was extracted from samples with a QIAmp tissue kit (Qiagen, Chatsworth, CA) and a Nucleon Phytopure kit (Amersham Life Science, Buckinghamshire, England) with modifications. Maize product samples were frozen initially with liquid nitrogen and ground to a fine powder with a mortar and pestle. Powdered samples (0.1 g) were treated with extraction buffer according to kit instructions. Dehydrated samples were incubated with an initial 2× volume of DNA extraction buffer to accommodate hydration and swelling of material. During the QIAmp procedure, a centrifugation step was added to eliminate cell wall or starch gel material before addition of the sample to the column. Control samples were spiked with 10 ng of DNA to verify the extractability of DNA from each sample.

Four higher plant-specific sequences were selected as the basis of the DNA detection procedure, and PCR primer pairs were designed to amplify these regions of the DNA. The sequences included the large (chloroplast-encoded) and small (nuclear-encoded) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), ribosomal subunit protein 4 (rsp4; chloroplast-encoded), transfer RNA (trnS; chloroplast-encoded and adjacent to rsp4) and ATPase β-subunit (atpB; chloroplast-encoded and adjacent to RUBISCO large subunit).

Primers generated for amplification of the sequences were:

- 1) RUBISCO small subunit (rbcS) (Lebrun et al 1987)
CCAATTCTGTAGATCCAACAC (forward)
GGAAAGCAAAGGAACCATG (reverse)

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- 2) *rsp4* and *trnS* (Souza-Chies et al 1997)
ATGTCCCGTTATCGAGGACCT (forward)
TACCGAGGGTTCGAATC (reverse)
- 3) RUBISCO large subunit (*rbcL*) (Doebley et al 1990)
ATGTCACCACAAACAGAGAGACTAAAGC (forward)
AAAGTTATTTTCGCGTTCCCCTTCTAACT (reverse)
- 4) *rbcL* and *atpB* (Hoot et al 1995)
GAATCCAACACTTGCTTTAGTCTCT (forward)
TAACATCTCGGAAATATTCCGCCAT (reverse)

For detection by PCR amplification, 5 μ L of extracted maize DNA products was added to a Ready-To-Go PCR bead (Pharmacia Biotech, Piscataway, NJ) that included all buffers and enzymes required for the PCR. The PCR included 25 pmol of each primer and was performed in a T100 thermocycler (MJ Research, Watertown, MA) under the following conditions: 60 cycles of 30 sec at 92°C, 30 sec at 50°C, and 90 sec at 75°C, followed by a terminal extension of 5 min at 75°C. Each DNA isolation was subjected to PCR amplification with four primer pairs (separate tubes for each primer pair). The PCR amplification products were size-separated by electrophoresis on 1% agarose gel in Tris-borate-EDTA (TBE) buffer (Sambrook et al 1989). PCR amplification products ranged in size from 0.45 to 1.5 kb. Maize genomic DNA also was size-separated by gel electrophoresis under the same system. The appearance of small DNA products of \approx 50 bp indicated degradation of DNA. Degradation was quantitated by computer image analysis of degraded DNA at a low molecular weight (50 bp) with a camera (Ultra-Lum, Carson, CA) and image analysis software (Gel-Pro Image Analyzer, Media Cybernetics, Silver Spring, MD).

A methyl-green/DNA complex agar diffusion method also was used to analyze DNA degradation. (Holt 1994). An agar medium containing a methyl-green dye attached to DNA was poured into petri plates. Wells were produced in the agar with a cork borer. Samples (5 μ L) were applied to the wells, and DNA degradation was indicated by the extent of clearing (disaggregation) of the methyl-green/DNA complex around the well (Atlas 1993).

RESULTS AND DISCUSSION

To detect DNA associated with various maize fractions generated by the wet-milling process, a sensitive PCR-based assay method was developed. The method was based on amplification of specific plant genes associated with nuclear (*rbcS*) and plastid (*rbcL*,

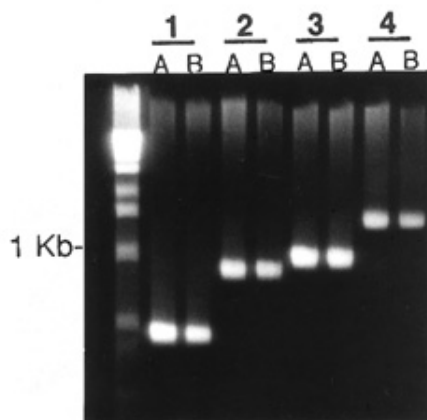


Fig. 1. Polymerase chain reaction (PCR) products generated after amplification of maize genomic DNA. Four primer pairs were designed to amplify chloroplast- and nuclear-encoded genes in higher plants: 1, *rbcS* primer pair amplification of 445-bp product; 2, *rsp4* + *trnS* primer pair amplification of 900-bp product; 3, *rbcL* + *atpB* primer pair amplification of 1-kb product; 4, *rbcL* + *atpB* primer pair amplification of 1.66-kb product. A = 14 ng of DNA; B = 1.4 ng of DNA.

rsp4, transfer RNA, and ATPase β -subunit) DNA. For PCR-based DNA detection, it was necessary to consistently extract DNA from the various fractions generated by maize wet-milling and processing. Although DNA isolation from plant materials often has been conducted with extraction buffer containing cetyltrimethylammoniumbromide (CTAB) detergent (Porebski et al 1997), this approach did not provide adequate DNA extraction when used with the maize wet-milling and processing products. More consistent and complete extraction of DNA was obtained with commercial kits (QIAmp and Phytopure) for DNA extraction and purification. The kits provided consistent extraction of DNA from spiked samples.

As shown in Fig. 1, when PCR was conducted with primer pairs generated from sequence information, specific amplification of maize DNA occurred. After 30 cycles of PCR amplification at an annealing temperature of 50°C, the smallest amount of maize DNA that could be detected was 1.4 ng of DNA in a 25- μ L reaction. When the number of PCR amplification cycles was increased to 60, the detection limit was lowered 1,000-fold to 1.4 pg of DNA in a 25- μ L reaction. PCR amplification involving the four primer pairs appeared to be specific for plant DNA, because no amplification was observed when PCR was conducted with the primers in the presence of DNA from yeast (*Saccharomyces cerevisiae*), gram-positive bacteria (*Corynebacterium glutamicum*) and gram-negative bacteria (*Escherichia coli*) (data not shown). When considered as a whole, the results demonstrate that the PCR-based amplification method was highly sensitive and specific for plant DNA detection and was useful for detection of genetic material in various maize products that may be of concern to marketers of genetically modified organisms.

The PCR-based assay method was used on samples at various stages of steeping, wet-milling, and processing of maize to detect the presence of DNA (Table I). In the initial steeping process, DNA was associated with maize kernels at all stages of hydration but not with steepwater (Table I). Although steeping results in the release of protein from maize kernels into steepwater (Eckhoff and Paulsen 1996), the release of DNA into steepwater was not detectable in a 10-mL sample. It is possible that DNA was released from the kernel and degraded. Similar to countercurrent steeping, DNA was not detectable in 10 mL of steepwater when steeping was conducted as a batch steep for 24 hr.

When batch-steeped maize was subjected to wet-milling, DNA was found in the starch, germ, coarse fiber, and wet gluten fractions separated by wet-milling. DNA was not found in the fine fiber fraction. Heating associated with drying also appeared to destroy

TABLE I
Polymerase Chain Reaction (PCR) Detection of DNA in Samples from Steeping, Wet-Milling, and Processing of Maize

Sample Type	DNA Present ^a
Countercurrent steep bank	
Maize from tanks 1–12	+
Steepwater from tanks 1–12	–
Batch steep or wet-milled	
Starch	+
Germ	+
Coarse fiber	+
Fine fiber	–
Wet gluten	+
Dried gluten	–
Dried kernel	–
Steepwater	–
Industrial-scale process	
Gluten prior to feed pellet production	+
Feed pellets	–
Wet gluten	+
Starch	–
Dextrose	–
Sorbitol	–
High-fructose corn syrup	–
Corn meal (dry-milled)	+

^a Presence (+) or absence (–) of DNA detected after 60 cycles of PCR.

DNA associated with wet-milling products. As shown in Table I, when the gluten fraction was dried by heating at 135°C for 2 hr, DNA that was detectable by PCR was destroyed. Drying kernels in this manner also led to complete destruction of detectable intact DNA.

The presence of DNA in maize products generated by industrial-scale processing of maize after wet-milling also was examined using the PCR-based method (Table I). Again, DNA was detected in wet gluten and gluten fractions used for the production of feed pellets. However, the heating process involved in feed pellet production appeared to destroy intact DNA. DNA was not detected in starch, dextrose, sorbitol, and high-fructose maize syrup products generated by industrial-scale processing. However, DNA was detectable in a maize meal product produced by industrial-scale dry-milling.

Although DNA could be detected in various fractions generated by steeping, wet-milling, and processing of maize, we found that the steeping process itself promoted DNA degradation. When DNA associated with countercurrently steeped maize was isolated and

size-separated by agarose gel electrophoresis, it was apparent that as steeping progressed over 36 hr degradation of maize genomic DNA into smaller fragments increased (Fig. 2). Moreover, the degradation of DNA in hydrating kernels induced by steepwater occurred to the greatest extent during the later stages of the steeping process, when steepwater was freshly added and contained the highest concentration of sulfur dioxide (tank 1). Degradation was evident when data for sulfur dioxide and lactic acid levels found in previous studies on the laboratory-scale steeping system (Yang et al, 1998) were compared with data on the extent of maize DNA degradation during steeping (Fig. 3). Degradation of DNA was quantitated by measuring DNA amounts at a low molecular weight (≈ 50 bp). Degradation coincided with high SO_2 concentrations.

Degradation of DNA also was evident in samples of steepwater from a countercurrent steep system applied to an agar plate in a diffusion assay used to measure DNA nuclease (degradation) activity (Fig. 4). In the assay, the extent of clearing (dissociation) of a methyl-green/DNA complex adjacent to an agar well corresponded to DNA degradation activity. As shown in Fig. 3, increased degradation of DNA was associated with freshly added steepwater, which contained the largest amount of sulfur dioxide.

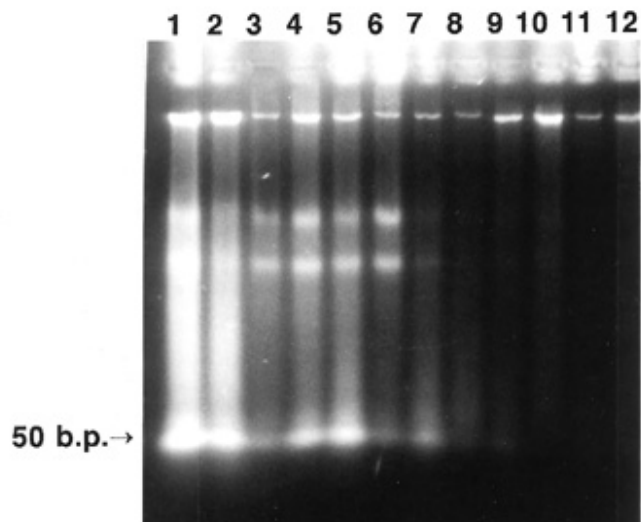


Fig. 2. Degradation of DNA from maize kernels during countercurrent steeping. Maize DNA was isolated from kernels in 12 steep tanks (lanes 1–12, respectively) and size-separated by agarose gel electrophoresis. Appearance of lower molecular weight fragments (≈ 50 bp) indicates degradation of DNA; fragments are most evident in tanks 1–6, in which sulfur dioxide levels were highest.

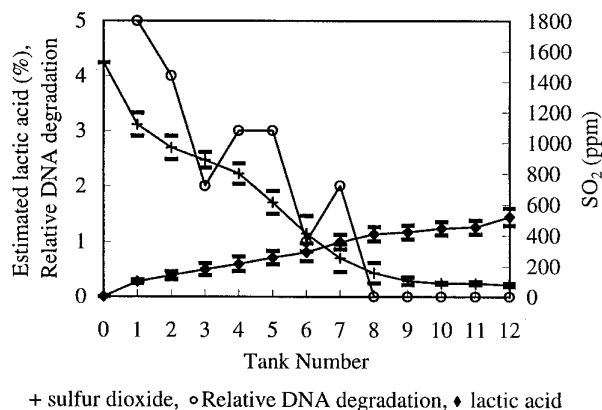


Fig. 3. Contents of continuous countercurrent steep tanks and DNA degradation during a 36-hr steep. Lactic acid, sulfur dioxide (data from Yang et al 1998), and DNA degradation were quantitated in each tank during the steep. To quantitate DNA degradation, a relative pixel value scale was set between 0 and 5 (5 = maximal level of DNA degradation to 50-bp size). Maize kernels were added fresh to tank 12; steepwater was added fresh to tank 1.

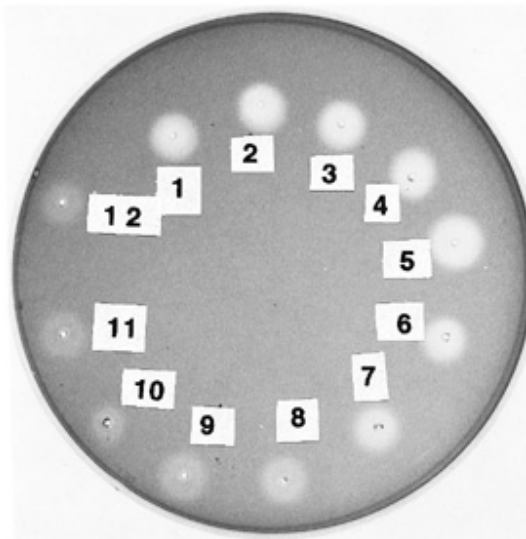


Fig. 4. Detection of DNA degradation in countercurrent steepwater by methyl-green agar plate assay. Steepwater (5 μL each) from 12 steep tanks was added to wells on a methyl-green/DNA agar plate. Well numbers corresponded to tank numbers in the countercurrent steep system. Sulfur dioxide levels were highest in tank 1 and decreased as shown in Fig. 3. Color clearing indicates extent of DNA degradation.

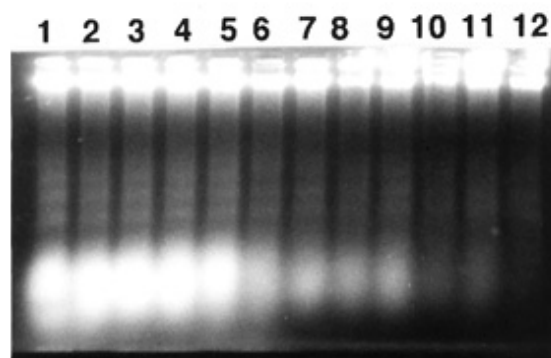


Fig. 5. Degradation of maize leaf DNA with addition of steepwater. DNA (1 μg) was incubated for 1 hr in 10 μL of steepwater from one of 12 countercurrent steep tanks (lanes 1–12, respectively). Each DNA sample was size-separated by agarose gel electrophoresis. Sulfur dioxide levels were highest in tank 1 and decreased as shown in Fig. 3. Appearance of lower molecular weight fragments indicates DNA degradation.

A similar steepwater effect on degradation of DNA also was observed when purified maize DNA was treated with steepwater containing a range of sulfur dioxide concentrations (Fig. 5). When purified maize DNA was incubated with steepwater from various stages of a countercurrent steeping process, an increased ability to degrade DNA into smaller fragments corresponded to increased sulfur dioxide levels in steepwater. As a result, it appears that the ability to degrade DNA coincides with longer steep times and higher sulfur dioxide concentrations.

Degradation of DNA by steepwater did not appear to be due to the presence of nuclease released from either maize or microorganisms present in the steep solution. The ability of steepwater to degrade DNA was unaffected by treatments known to destroy enzyme activity such as boiling for 3 min, chloroform treatment, or treatment with denaturing detergents such as SDS (data not shown). On the other hand, the increased ability to degrade DNA did appear to correspond to the addition of sulfur dioxide (initially added as sodium meta-bisulfite), which may reflect a physiochemical process driven by elevated sulfur dioxide levels present in steepwater at the later stages of the steeping process. Nevertheless, equal molar concentrations of other acids (lactic and sulfuric) did not induce degradation of DNA (data not shown). Addition of sodium bisulfite to DNA causes chemical changes in the cytosine component of DNA (Hayatsu 1976) by forming a sulfonated cytosine reaction intermediate that is susceptible to deamination. During the development of genomic methylation pattern mapping with use of bisulfite, prolonged exposure of DNA to 2.5M sodium bisulfite and 1.5M sodium meta-bisulfite causes DNA degradation that is detected by gel electrophoresis (Raizis et al 1995). Degradation of DNA after addition of sodium meta-bisulfite and subsequent conversion to sulfur dioxide in steepwater may proceed in the same manner as DNA degradation by sodium bisulfite.

CONCLUSIONS

The use of a sensitive PCR-based assay method that uses primers generated to plant-specific DNA sequences allowed analysis of the fate of maize DNA during wet-milling and subsequent processing. Although maize DNA can be detected in some fractions and products generated by wet-milling and processing, it is likely to show some degree of degradation due to the steeping process itself. The DNA present in certain wet-milling fractions and processed maize products exposed to heat treatment was fully degraded to levels not detected by our PCR protocol.

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