PCR Amplification of Wheat Sequences from DNA Extracted During Milling and Baking

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ABSTRACT

DNA-based analyses are highly sensitive and specific. Because processing steps can have profound effects on the proteins and DNA present in foods, this project examined the effects of breadmaking on wheat DNA size and polymerase chain reaction (PCR)-based detection of sequences. DNA was extracted from wheat kernels, milling fractions, and flour, and from samples taken at various steps during and after the baking process. Kernels contained primarily high molecular weight DNA (>12,000 base pairs [bp]), whereas flour DNA exhibited a broad range of molecular weights from >12,000 bp to <300 bp. A marked reduction in DNA yield and size occurred after the first 5 min of baking. PCR successfully amplified products of both high and low copy number genes, even from DNA extracted from bread loaves five days after baking. However, successful amplification required that the maximum product size be no more than the average molecular weight of the DNA recovered from the source. The data also demonstrate that PCR can be used to detect the presence of yeast (*Saccharomyces cerevisiae*), a minor ingredient.

Before breadmaking, wheat is milled into flour, which is then mixed with additional ingredients and subjected to fermentation and baked. The processing steps involve changes in pH, shear forces, and increases in temperature that have a significant effect on the biochemical components; proteins are denatured and starch is gelatinized. Substantial information exists on the effects of baking on carbohydrates and proteins, whereas little information exists on the effects on wheat DNA. DNA-based analyses of food products have increased as means for compositional analysis including cultivar identification, product adulteration, presence of food-borne pathogens, and detection of ingredients derived from genetically modified sources. DNA is useful for analytical tests due to the fact that DNA is highly thermostable and even partially degraded DNA is suitable for detection systems. Another advantage of DNA-based analysis is that DNA can be extracted from any plant tissue at any developmental stage and is not affected by environmental influences. Food proteins are heat-labile and, depending on the degree of product processing, may be denatured and unrecognizable by an antibody in a processed product (Dien et al 2002). Additionally, the synthesis of proteins may not be present at equal amounts in all tissues of the plant.

The polymerase chain reaction (PCR) is a powerful method for amplification of specific DNA sequences. Applications to the food industry include source authentication and determination of product adulteration (Bryan et al 1998; Matsunaga et al 1999; Dahinden et al 2001), detection of microbial contamination (Agarwal et al 2002), as well as the presence of low-level ingredients including allergens (Holzhauser et al 2000). For detection of genetically modified organisms (GMO) and ingredients derived from them in grain and grain-based products, PCR-based analyses provide high sensitivity and specificity (Meyer 1999). With the development of efficient and reproducible methods for the genetic transformation of wheat (Vasil et al 1992; Weeks et al 1993; Cheng et al 1997) the release of genetically modified wheat is a possibility.

In this study, the effects of milling and baking on DNA integrity were analyzed. Of particular interest was the ability to amplify target DNA sequences of varying nucleotide lengths and copy numbers within the wheat genome using PCR.

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MATERIALS AND METHODS

Milling Samples

Wheat kernels (cv. Karl 92) were tempered to 12% moisture and milled using a Quadramat Senior Mill. Bran, shorts, and flour samples were collected, placed in sealed containers, and stored at 4°C.

Baking Samples

Wheat flour (Regional Bake Standard 99RBS, 11.8% protein 13.9% moisture) that serves as a control in the evaluation of test lines by the USDA-ARS Hard Winter Wheat Quality Laboratory was used for breadmaking. Approved Method 10-10B (AACC 2000) was slightly modified according to Finney (1984) to be more representative of industrial baking. The formula consisted of 100 g of flour, 6 g of sugar, 3 g of shortening (Bunge Foods, Bradley, IL), 1.5 g of salt, 1 g of dry active yeast (Fleischmann's Yeast, LaSalle, QC, Canada), and 5 mg (50 ppm) of ascorbic acid (Fisher Scientific, Fair Lawn, NJ). After mixing, the dough was fermented for 120 min at 30°C followed by two punch (molding) steps at 51 and 86 min, passage through a sheeter (pan stage) at 103 min, and placed into the fermentation cabinet at 30°C. The bread was baked for 18 min at 218°C. Samples were taken of the premixed flour, dough immediately after mixing, dough after 1st punch, dough at 2nd punch, molding, and immediately before oven stage. Bread samples were taken from the center of separate loaves baked for 5, 10, and 15 min. After the bake was completed, loaves were cut in half and a sample taken from the internal area of the loaf. The remaining halves were stored in plastic bags at room temperature (≈22°C) and sliced after 24 hr. Bread slices were sampled at 1, 3, and 5 days by cutting an internal section of the bread with a razor blade. All of the baking samples were performed in duplicate with a total of three measurements on each sample. A nonyeast containing dough sample was prepared by combining flour and water and mixed to minimum mobility in a 35-g mixograph. All samples were frozen at –70°C until extraction.

DNA Extraction and Evaluation

DNA from kernels and milling fractions were extracted using the method of Edwards et al (1991) and for bake samples using the Wizard Magnetic DNA Purification System for Food (Promega, Madison WI). Samples were ground to a fine powder in liquid nitrogen in a precooled mortar and pestle and DNA was extracted. For most samples, 100–200 mg of material was used, although the Wizard system allowed for a sample size up to 500 mg

TABLE I
Oligonucleotide Primers for PCR Amplification

Target	Oligonucleotide Sequence	Product Size	Reference
Dgas44	5' CTTCTACGGGTCAGGGCAC 3' (forward) 5' CTAATGCCCCTGCGGCTTAA 3'(reverse)	286 bp	Bryan et al (1998)
Thionin	5' CTGCCAGCCATGGGAAGCAA 3' (forward) 5' CTTGCCCTGTGAAATCTCAGAC 3' (reverse)	900 bp	Van Campenhout et al (1998)
Thionin	5' CTGCCAGCCATGGGAAGCAA 3' (forward) 5' ACTCAAGGGCCAATTTAGGG 3' (reverse)	238 bp	This study
Yeast AP	5' GATGGTTTAGGTGACCAATT 3'(forward) 5' GGTCAAGTTCAAACCCTTGT 3' (reverse)	201 bp	Greiner et al (1997)

TABLE II
DNA Yield (ng DNA/mg) from Samples Taken at Different Stages of Milling and Breadmaking[a]

A. Results from Karl Milling Samples		B. Results from 99 RBS Flour Bake Samples	
Kernel	228 ± 17	Flour	69 ± 27
Bran	718 ± 47	Dough	57 ± 11
Shorts	335 ± 31	Punch	58 ± 7
Flour	73 ± 4	Proof	63 ± 3
		Pan	60 ± 5
		5 min	51 ± 5
		10 min	22 ± 11
		15 min	23 ± 4
		Bread	22 ± 10
		1 day	22 ± 8
		3 day	23 ± 10
		5 day	22 ± 9

[a] Values represent mean ± standard deviation for three replicates.

from dough and bread samples according to the manufacturers instructions. DNA concentration was determined using an UV-spectrophotometer (Ultospec 3000, Amersham Biosciences, Piscataway NJ) (Sambrook and Russell 2001) and quality was assessed by agarose gel electrophoresis. The gels were composed of 0.5% agarose (Invitrogen, Carlsbad, CA) prepared and run with 1X Tris-borate-EDTA buffer (TBE) (Sambrook and Russell 1999). DNA (1.0 µg) was loaded into wells and gels were run at 25V overnight (≈18 hr) at 4°C with DNA molecular size markers, 1–12 Kb DNA Ladder (Invitrogen, Carlsbad, CA) run in adjacent wells.

Polymerase Chain Reaction (PCR) Amplification

Oligonucleotide primers (Integrated DNA Technologies Coralville, IA) were designed to amplify wheat DNA sequences represented in the wheat genomes at both low and high copy numbers. Primer sequences and the expected amplicon size are shown in Table I.

PCR was performed in 50 µL volumes using a GeneAmp 2400 thermal cycler (Perkin-Elmer, Foster City, CA). Each reaction consisted of 1X PCR buffer, 100 ng of DNA, 50 µM dNTPs, 2.5 units HOTSTARTaq polymerase (Qiagen, Valencia, CA), and 2.5 µL of 20-fµm primer stocks. The amplification protocol consisted of an initial 15-min denaturation step at 95°C, necessary to activate the Taq polymerase, followed by 30 cycles of denaturation at 94°C for 1 min, annealing for 45 sec (65°C for Dgas44, 58°C for thionin, and 52.5°C for yeast acid phosphatase [AP]), and extension at 72°C for 30 sec. A final extension step was performed at 72°C for 5 min. After PCR, samples were analyzed by electrophoresis through a 1.5% agarose gel and photographed using a digital camera (DC120, Kodak, Rochester NY).

RESULTS AND DISCUSSION

DNA Extraction and Analysis from Milled Kernels

The concentration of DNA (ng of DNA/mg of sample) extracted from kernels and the milling fractions is shown in Table IIA. The greatest yield of DNA was obtained from the bran fraction followed by the shorts, kernel, and flour, respectively. The ratio of flour to kernel to shorts to bran was ≈1:3:5:10, similar to the ratios reported by Bourdet and Autran (1974).

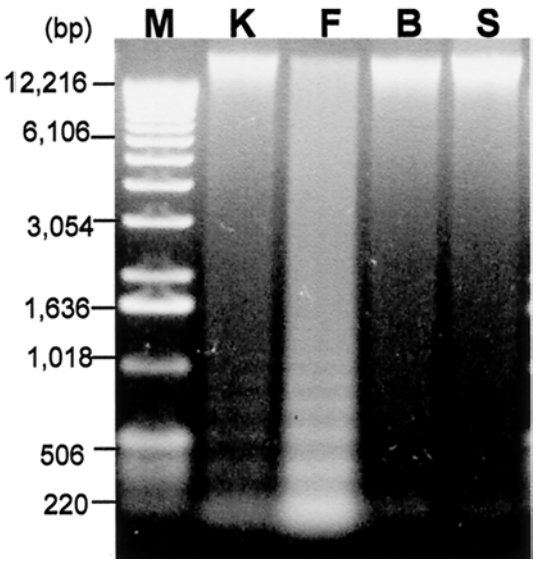

Fig. 1. Agarose gel electrophoresis of total DNA isolated from intact wheat kernels and milling fractions from Karl 92. M = Markers in base pairs (bp), K = kernel, F = flour, B = bran, S = shorts.

Agarose gel analysis of the milling fractions is shown in Fig. 1. DNA isolated from wheat kernels was predominantly composed of high molecular weight (>12,000 bp) and a smear of lower molecular weight degraded DNA. Flour contained a broad smear of degraded DNA ranging from >12,000 bp to <300 bp. A ladder of DNA fragments of ≈190 bp was observed in the flour and to a lesser degree in the kernel preparations. This observation is consistent with the well-documented developmental process, apoptosis (programmed cell death) that occurs in the cereal endosperm as part of kernel maturation (Young and Gallie 1999). As the kernel matures, the endosperm cells undergo this process resulting in degraded DNA of defined fragment size. The extracted DNA from bran and shorts was composed of intact high molecular weight DNA due to the embryo (germ) and aleurone layers, the regions of the kernel containing intact and living cells (Fath et al 2000), present in those fractions. Thus, the physical separation of bran and endosperm during milling also serves to separate the DNA into high and low molecular sizes.

DNA Extraction and Analysis from Baked Samples

The DNA yield (ng of DNA/mg of sample) from baking samples (99 RBS flour) is shown in Table IIB. The data demonstrate a general decrease in DNA yield with concurrent processing of flour into dough and bread. The yield of DNA is slightly higher in fermentation stages, perhaps due to the multiplication of yeast during the proofing stages. The most notable effects occur during baking at 5–10 min, when the DNA yield decreases to ≈45% of that of flour. Further baking and storage have no additional effects on DNA yield as demonstrated in the quantification of the DNA baked for 15 min and up to five days postbake. This data

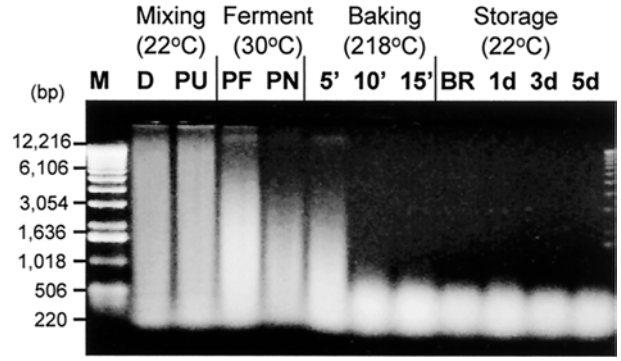

Fig. 2. Agarose gel electrophoresis of DNA extracted from mixing and baking samples using 99 RBS flour. M = Markers in base pairs (bp), D = dough, PU = punch, PF = proof, PN = pan, 5' = 5 min into the bake, 10' = 10 min into the bake, 15' = 15 min into the bake, BR = bread, 1d = bread stored for one day at room temperature, 3d = bread stored for three days at room temperature, 5d = bread stored for five days at room temperature.

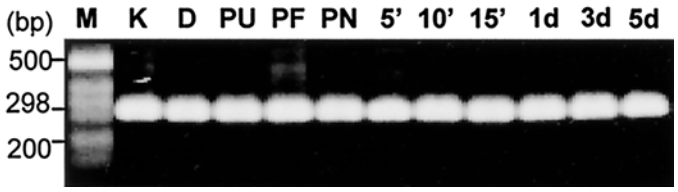

Fig. 3. Agarose gel electrophoresis of PCR products generated after amplification of Dgas44 from Karl 92 kernels and mixing and baking samples using 99 RBS flour. M = Markers in base pairs (bp), K = Karl 92 kernel, D = dough, PU = punch, PF = proof, PN = pan, 5' = 5 min into the bake, 10 = 10 min into the bake, 15' = 15 min into the bake, 1d = bread stored for one day at room temperature, 3d = bread stored for three days at room temperature, 5d = bread stored for five days at room temperature.

correlates well with the visual depiction of changes in the molecular weight distribution of the DNA due to degradation shown in Fig. 2.

During processing and breadbaking, DNA is further degraded into lower molecular weight fragments. The mixing and punching stages have a discernable effect on DNA size, indicated by a slight decrease in molecular weight that is observed after proofing and pan stages. However, the effect of DNA yield at these stages is not significant. The most notable changes occur at 5–10 min of baking, resulting in DNA with an average molecular size of ≈300 bp (Fig. 2). The effects that occur during this period are concomitant with the rise in temperature and changes in physical state of the dough from that of flowable dough to that of the foam structure of bread. The molecular weight distribution of the DNA extracted from dough at 10 min of baking is similar to that from fully baked bread and bread that had been stored at room temp for five days, indicating that further heat and storage did not alter the average molecular weight of DNA or ability to perform in the PCR assays. The average molecular weight of DNA from bread observed in this study is similar to that reported by other investigators (Allman et al 1992; Straub et al 1999).

Amplification of Target Sequences by PCR

PCR amplification of the Dgas44 target sequence resulted in a band at the expected molecular size of 286 bp from all samples examined (Fig. 3). Dgas44 is a repetitive sequence that occurs at a high (200–300) copy number within the D genome of bread wheat (McNeil et al 1994). This target was useful in the detection of adulteration of pasta with bread wheat flour (Bryan et al 1998; Alary et al 2002). Titration experiments of input DNA from bread indicated that significant amplification was possible from five-day-old bread with as little as 0.1 ng (100 pg) template DNA (data not shown), similar to the sensitivity of Dgas44 amplification reported by Bryan et al (1998) in pasta.

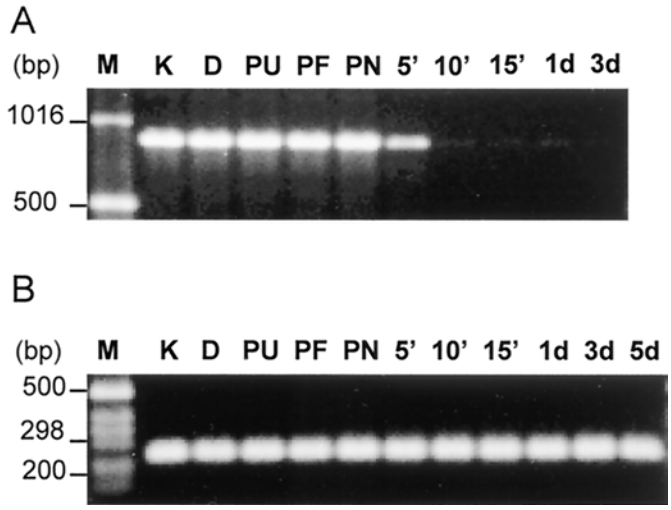

Fig. 4. Agarose gel electrophoresis of PCR products generated after amplification of Type I thionin from Karl 92 kernels and mixing and baking samples using 99 RBS flour. Markers in base pairs (bp), K = Karl 92 kernel, D = dough, PU = punch, PF = proof, PN = pan, 5' = 5 min into the bake, 10'= 10 min into the bake, 15' = 15 min into the bake, 1d = bread stored for one day at room temperature, 3d = bread stored for three days at room temperature. **A,** Using primers for 900 bp product. **B,** Using primers for 238 bp product.

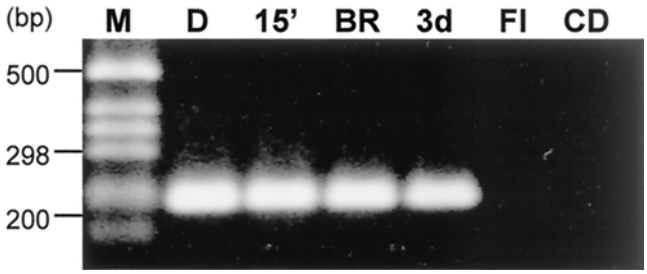

Fig. 5. Agarose gel electrophoresis of PCR products generated after amplification of yeast acid phosphatase from mixing and baking samples using 99 RBS flour. M = Markers in base pairs (bp), D = dough, 15' = 15 min into the bake, Br = bread, 3d = bread stored for three days at room temperature, F = flour, CD = control dough (flour + water mix).

To demonstrate the sensitivity and limitations of PCR detection a low copy number target, type-I thionin, which is represented by a single copy in each of the three (A, B, D) genomes of bread wheat (Van Campenhout et al 1998) was performed. Initially, a 900-bp amplicon was targeted for amplification. The product was successfully amplified from DNA extracted from the 99 RBS flour, dough, punch, proof, and pan samples (Fig. 4A). A 900-bp band of lesser intensity (≈1/3) was amplified from DNA taken from bread at 5 min into the baking process, and an extremely faint band that is not visible in the reproduction, was observed in samples taken after further baking as well as in bread samples. A novel reverse primer was designed to amplify a 238-bp region of the same sequence. The results of this (Fig. 4B) demonstrate the successful amplification of the 238-bp fragment from all stages, including late-stage baking and fully baked bread. A titration experiment determined that the lower limit of detection in five-day-old bread is 1–5 ng of input DNA (data not shown). These results demonstrate that, under these conditions, a longer amplicon size may lead to false negative result.

Detection of minor ingredients in the baking formula is increasingly necessary. This may be required due to product mislabeling, cross-contamination, or adulteration during processing, which can result in the presence of potential food allergens in a consumer product. An additional concern is the presence of ingredients that may be derived from genetically modified sources such as Roundup

Ready soybeans. Stram et al (2000) demonstrated the presence of soy as a common contaminant in wheat flour.

To determine whether a low copy number sequence from foreign DNA contributed by a minor ingredient could be detected, a low copy number sequence from the yeast acid phosphatase (AP) gene (Bajwa et al 1984; Greiner et al 1997) was investigated. Amplification from control yeast DNA revealed an intense band at 201 bp, whereas no amplification product was observed in 99 RBS flour or control dough (flour and water only), even with the increase of template DNA to 500 µg. A band of expected size was amplified from mixing and baking samples (containing 1% yeast based on flour weight), as well as bread stored for three days (Fig. 5). Konietzny and Greiner (1997) used this primer set in an attempt to detect yeast DNA in beer without success due to difficulty in the DNA extraction. Both Arlorio et al (1999) and Corsetti et al (2001) demonstrated the use of PCR for detection of microorganisms responsible for fermentation from breads using the 16S rDNA and 16S/23S rRNA spacer region for specific detection and identification of yeast and bacteria species in sourdough breads.

These data confirm both the effect of processing (milling and baking) on DNA integrity and demonstrate that low copy number genes from minor constituents can be detected from DNA purified from food matrices. The DNA extraction procedure requires optimization for accurate detection in processed food samples. Different processing and extraction conditions can greatly affect the quantity and quality of DNA recovered from products. Additionally, complex matrices such as food systems may contain inhibitory substances that adversely influence PCR amplification. All of these factors must be considered when developing DNA-based detection methods.

CONCLUSIONS

DNA in wheat kernels is comprised of high and low molecular weight molecules. Milling physically separates the bran and germ, which contain high molecular weight DNA, from endosperm (flour), which contains a wide range of DNA sizes. PCR amplification of high copy number (Dgas44) and single copy (thionin) wheat genes is possible from five-day-old bread, provided the amplified product size is 200–300 bp in length. A low copy number yeast gene was amplified from samples taken after yeast addition, indicating that DNA from minor ingredients can also be detected with appropriate primers.

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