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Polymerase chain reaction (PCR): a possible alternative to immunochemical methods assuring safety and quality of food

Detection of wheat contamination in non-wheat food products

Michael Allmann, Urs Candrian, Christiane Höfelein, and Jürg Lüthy

Department of Food Chemistry, Institute of Biochemistry, University of Berne, CH-3012 Berne, Switzerland

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Polymerase chain reaction (PCR): Alternative zu immunochemischen Methoden in der Qualitätssicherung von Lebensmitteln. Nachweis von Weizenverunreinigungen in Nicht-Weizenprodukten

Zusammenfassung. Eine schnelle, sensitive Nachweismethode, basierend auf der Polymerase-Kettenreaktion(PCR)-Technologie, wurde entwickelt, um Weizenverunreinigungen in dietätischen Nicht-Weizenprodukten zu bestimmen. Das PCR-System wurde so ausgearbeitet, daß zunächst mit einer Vervielfältigung spezifisch für Eukaryonten DNA die Substratqualität bestimmt wurde, um falsch-negative Resultate auszuschließen. Die nachfolgende PCR, ein weizentypisches, hochrepetetives Weizen-DNA-Segment vervielfältigend, gab Auskunft über eine mögliche Weizenverunreinigung. Getestet wurden 35 verschiedene, verarbeitete Nahrungsmittel, Mehle, Stärken und Additive. Diese 35 Proben wurden auch mit einer immunochemischen Methode untersucht, welche die Weizenkomponente Gliadin maß. Die Resultate beider Methoden erlaubten eine weitergehende Beurteilung der untersuchten Proben als eine Methode allein. So konnten Weizenverunreinigungen von als Trägerstoff für Gewürze und Aromen zugesetztem Gliadin unterschieden und Weizenstärkezusatz als solcher erkannt werden.

Abstract. A rapid, sensitive and specific anlysis of food samples determining wheat contamination was established using polymerase chain reaction (PCR) technology. First, primers specific for highly conserved eukaryote DNA sequences were used to prove isolated nucleic acid substrate accessibility to PCR amplification. Subsequently, a highly repetitive and specific genomic wheat DNA segment was amplified by PCR for wheat detection. This assay was tested with 35 different food samples ranging from bakery additives to heated and processed food samples. In addition, the PCR method was compared to an immunochemical assay that detected the wheat protein component gliadin. Combination of both assays allowed a detailed characterization of wheat contamination. Hence, wheat flour contamination could be distinguished from gliadin used as a carrier for spices as well as from wheat starch addition.

Correspondence to: M. Allmann

Introduction

Since 1985, when the polymerase chain reaction (PCR) was discovered the field of deoxyribonucleic acid (DNA) analysis dramatically changed in molecular biological, clinical and food microbiological laboratories. This method allows rapid in vitro amplification and analysis of small amounts of specific DNA fragments that are part of a complex sample matrix [1]. Applications include forensic and diagnostic settings, detecting tumour viruses or oncogene DNA sequences. Other DNA analysis applications include microbiological assays that detect and type pathogenic strains of microorganism in clinical and in food samples. In the work presented here, we have investigated the accessibility of DNA analysis by PCR in characterization and quality assurance in complex and processed food products. The primary condition for DNA analysis is the natural occurrence of nucleic acids in investigated food samples, in its constituent components or undesired contaminations. A PCR method based on specific detection of wheat DNA was compared with a commercial immunochemical method [2] measuring the wheat protein gliadin in food samples. The significance of unexpected and undeclared wheat addition to cereal food products ranged from unfair trade practices to inaccurate food processing. However, unexpected wheat addition can pose a serious hazard to people suffering from different wheat allergies. A common wheat intolerance is coeliac disease (CD) [3]. Clinical symptoms of CD are triggered by a daily intake of 10 mg wheat gliadin [4]. Recently, we described a PCR DNA analysis system based on a single copy gene determining wheat DNA

in cereal food samples [5]. However, two problems arse with this preliminary assay:

1. Insufficient sensitivity due to use of a single copy gene as the PCR target.
2. False negative results caused by non-amplifiable, degraded DNA of cooked and processed food samples.

Hence, we have solved these problems in the following DNA analysis system by a double PCR consisting of a "eukaryote PCR" determining the appropriateness of the nucleic acid substrate for a PCR reaction and of a "wheat PCR" analysing the eventual wheat DNA content. The eukaryote PCR extensively detects eukaryote DNA by targeting a 137 base-pair (bp) fragment of a highly conserved region within wheat, maize, yeast, frog and human genomic DNA [6–8]. Using the wheat PCR, we amplified a 109-bp segment in the major repeat unit of the intergenic region between the 25 S and 18 S wheat ribosomal RNA genes [8]. This repeat unit was chosen because of its heterology to repeat units in other cereal intergenic 25–18 S regions; homology to the rye unit is 51% [9] and to the maize unit less than 30% [10]. More than 450,000 repeat units per hexaploid wheat genome DNA [8, 11] allow exceedingly sensitive detection of wheat DNA, equal to theoretically 10^{-4}–10^{-5} wheat cells. Taken together, we can expect to obtain a positive amplification signal with the eukaryote PCR when the degraded nucleic acid fragments are longer than 137 bp and, consequently, longer than the wheat DNA-specific 109-bp PCR segment, so excluding false negative results.

Materials and methods

Cereal samples. Cereal samples were obtained from the Eidgenössische Getreideverwaltung in Berne, Switzerland: the wheat varieties Arina, CWRS, Eiger, Forno, and Zenith, the rye variety Eho, the oat variety Hockey, the barley variety Image and the maize variety Linth. The semiprocessed rice variety Carnaroli and a brand millet product were supplied by Riseria Taverne (Taverne, Switzerland).

Food samples. Nineteen food ingredients and additives were provided by a manufacturer of gliadin-free and wheat-free dietary bakery products (X-01 to X-19, see Table 1). Ten processed "gliadin-free" food samples, soups, herb mixes and sauces, were obtained from a instant food company (Y-01 to Y-10). A further six food samples, suspected to be contaminated with wheat or gliadin, were provided by people suffering from coeliac disease (Z-01 to Z-06). All food samples are further described in Table 1.

Nucleic acids extraction. Where necessary, samples were ground to a fine powder and 100 mg powder was transferred to a 2 ml Eppendorf tube containing 1 ml extraction buffer [100 mmol TRIS-HCl (pH 7.5), 100 mmol NaCl, 10 mmol ethylendiaminetetraacetat (EDTA), 2% (w/v) sodium dodecylsulphate (SDS). Proteinase K was added to give 0.5 mg/ml. Samples were digested for 4 h at 37° C on a Eppendorf Thermomixer 5437 (500 rpm). After digestion, a one-half volume (vol.) of saturated NaCl was added to each tube, vortexed for 30 s and centrifuged at 13,000 *g* for 15 min at 4° C. The DNA-containing supernatant was mixed with 2 vol., of absolute ethanol, and a one-tenth vol. of 4 mol LiCl and left at −70° C for at least 30 min. The precipitated nucleic acids were collected by centrifugation at 13,000 g for 3 min at 4° C. The pellet was air dried, washed with ethanol 70% (v/v) and resuspended in 200 µl TE buffer [10 mmol TRIS-HCl (pH 7.5), 1 mmol EDTA]. Nucleic acids isolated from intact cereal grains were digested with RNase, added to give 50 µg/ml, and incubated at 37° C for 4 h. After extraction with chloroform/phenol, the aqueous phase was removed and the DNA precipitated with ethanol (see above). The DNA was redissolved in TE buffer.

Synthesis of PCR primers. Oligonucleotide primers were synthesized on an Applied Biosystems 381 synthesizer using phosphoramidite chemistry. The primer sequences were: TR01 5′-GCGGCGTGTGCCACGTACGTGGTTT and
TR02 5′-GAACGGGCGTTACGTGGACACGGGA used for the wheat PCR and
TR03 5′-TCTGCCCTATCAACTTTCGATGGTA and
TR04 5′-AATTTGCGCGCCTGCTGCCTTCCTT used for the eukaryote PCR.

PCR reactions. Nucleic acid extracts from food samples were added in 10-µl aliquots to 90 µl PCR reaction mix in a 0.5-ml-Sarstedt reaction tube. This mix contained PCR buffer (Promega), 200 µmol nucleotide triphosphate mix (dATP, dCTP, dGTP and dUTP), 0.5 µmol primers, 2 units *Tag* polymerase (Promega) and 0.5 units *N*-uracyl-glycosylase (Promega). Reaction mixture (90 µl) and 10 µl substrate DNA were mixed and overlaid with a few drops of mineral oil. Amplification was accomplished with a Thermal Cycler (Hybaid, Middx., UK) by using the tube-controlled temperature mode and wheat PCR step-cycle programme: preincubation at 37° C for 30 min; 25 to 35 cycles consisting of denaturation at 95° C for 1 s, annealing at 68° C for 30 s and extension at 72° C for 10 s; followed by a final extension at 72° C for 2 min. The step-cycle programme of the eukaryote PCR differed only in the annealing temperature, 64° C, and cycle number, 45. After the reaction was completed, each reaction mix was analysed by standard agarose gel electrophoresis: a total of 20 µl of each PCR reaction mix was electrophoresed on a agarose gel in TRIS-borate buffer, stained with ethidium bromide and photographed at UV (254 nm). PCR fragments were identified by restriction enzyme digestion and dot blot analysis [12].

Gliadin analysis. Gliadin extraction and analysis were carried out as recommended in the distributors' manual of the Gluten Lab Kit, immunoenzymatic detection of gliadin (Transia, Lyon, France). According to the manual, the test is a direct sandwich enzyme immunoassay based on monoclonal antibodies, that recognize wheat gliadin and related prolamins from triticale, rye, and barley but not from maize, rice, and oat.

Results

First, the double PCR system was tested and optimized with DNAs isolated from wheat, rye, oat, barley, maize, rice, and millet. All these DNAs yielded a positive amplification signal of the expected size with the eukaryote PCR. With the wheat PCR all tested wheat DNAs were positive; a weak positive signal could be also obtained with rye DNA (equal to less than 0.01 ng genomic wheat DNA), which may not be visible on the photographic reproduction; all other DNAs were negative (Fig. 1). The sensitivity of this wheat-specific DNA analysis was 1 pg genomic wheat DNA, equal to 50 ng wheat. The same detection limit of 1 pg DNA could still be achieved by addition of 1 µg non-target yeast DNA as background. Under these conditions, artificial wheat DNA contaminations of yeast DNA of about 1 mg/kg could be detected.

Second, we investigated the potential of DNA isolation from different and complex food products, ingredients as well as additives. As shown in Table 1, the thickening agent dextrin, ingredients with desired high absorption capacity such as guar flour or carob starch

Table 1. Food samples, results of gliadin and genomic wheat DNA analysis (for further details see text)

Nr	Products	Gliadin mg/kg	Eukaryote DNA	Wheat DNA	Commentary
X-01	Carboxymethylcellulose	–	–	–	No gliadin or wheat flour contamination
X-02	Carob flour	–	Not applicable		
X-03	Product based on carob flour	–	Not applicable		
X-04	Manioc starch pasted	–	–	–	No gliadin or wheat flour contamination
X-05	Guar starch pasted	–	Not applicable		
X-06	Isolated amylopectin pasted	–	Not applicable		
X-07	Emulgator [b]	–	+	–	No wheat flour contamination
X-08	Leaven [b]	–	Not applicable		
X-09	Crude ground rice	–	+	–	No wheat flour contamination
X-10	Dried soya extract A	–	+	–	No wheat flour contamination
X-11	Dried soya extract B	–	+	–	No wheat flour contamination
X-12	Dried milk extract	±[c]	+	+	Wheat flour contamination <0.03%
X-13	Maize starch	–	+	–	No wheat flour contamination
X-14	Rice starch	±[c]	–	–	No gliadin or wheat flour contamination
X-15	Wheat starch	±[c]	+	+++	Gliadin content <15 ppm
X-16	Self raising flour [b]	±[c]	+	+	Wheat flour contamination <0.03%
X-17	Rice flour A	40	+	++	Wheat flour contamination <0.10%
X-18	Rice flour B	24	+	++	Wheat flour contamination <0.06%
X-19	Rice flour C	24	+	++	Wheat flour contamination <0.06%
Y-01	Condiment with herbs A	40	–	–	[d]
Y-02	Clear instant soup A	±[c]	+	–	No wheat flour contamination
Y-03	Clear instant soup B	80	+	–	[d]
Y-04	Clear instant soup C	50	+	–	[d]
Y-05	Instant soup with peas and bacon	36	+	++	Wheat flour contamination <0.09%
Y-06	Instant soup with spicies	100	+	+	Wheat flour contamination <0.25%
Y-07	Instant soup with peas	110	+	++	Wheat flour contamination >0.25%
Y-08	Instant sauce, hollandaise	120	+	++	Wheat flour contamination >0.25%
Y-09	Instant sauce, gravy	120	+	+++	Wheat flour contamination >0.25%
Y-10	Malt extract	±[c]	–	–	No gliadin or wheat flour contamination
Z-01	Condiment with herbs B	±[c]	+	–	No wheat flour contamination
Z-02	Instant soup with bacon	20	+	+++	Wheat flour contamination <0.05%
Z-03	Maize product, polenta	33	+	+++	Wheat flour contamination <0.08%
Z-04	Curry	60	–	–	[d]
Z-05	Rice based fried cereal product	±[c]	+	–	No wheat flour contamination
Z-06	Corn based fried cereal product	40	+	–	[d]

[a] 1 ppm means 0.1 mg gliadin/100 g food
[b] Based on maize starch
[c] Gliadin content cannot be reliably detected
[d] Gliadin contamination, gliadin as spice carrier protein, or contamination with another immunoactive cross-reacting protein

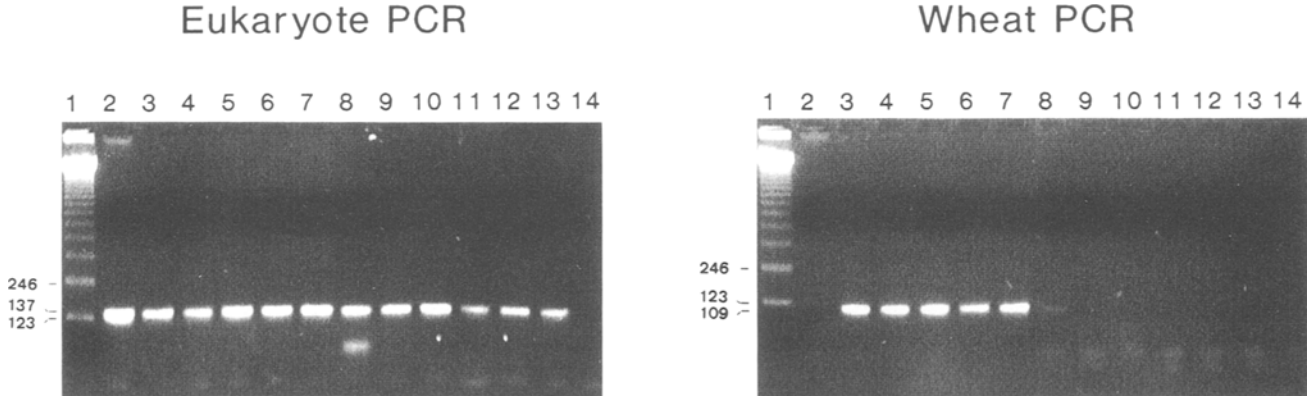

Fig. 1. Specifity of wheat and eucaryote polymerase chain reaction (PCR). Ethidium-bromide-stained agarose gel with amplification products obtained from different cereal samples: 10 ng cereal DNA and 1 µg yeast DNA were amplified by PCR. *Lane 1*, 123-bp ladder size standard; *lane 2*, yeast DNA (Promega); *lanes 3–7*, five wheat varietis; *lane 8*, rye; *lane 9*, oat; *lane 10*, barley; *lane 11*, rice; *lane 12*, maize; *lane 13*, millet; *lane 14*, negative control (no DNA). *Numbers* to the *left* of each panel are base pairs (bp). *Eukaryote PCR:* the size of the expected amplification product is 137 bp. *Wheat PCR:* the size of the expected amplification product is 109 bp

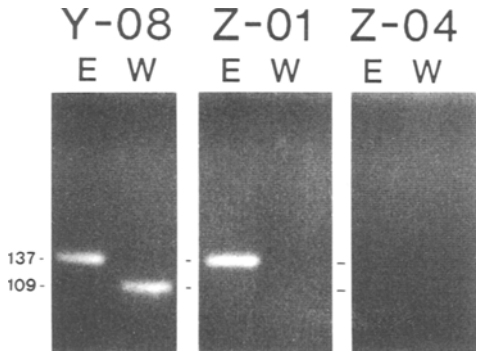

Fig. 2. Typical PCR amplification product profiles of food samples. Ethidium-bromide-stained agarose gel with amplification products obtained from different food samples: Y-08, instant sauce hollandaise; Z-01, condiment with herbs B; Z-04, curry. Lane *E*, eukaryote PCR; lane *W*, wheat PCR. *Numbers* to the *left* are base pairs (bp)

could not serve as a substrate for DNA isolation. The thickening and absorption effect made any extraction impossible. In contrast, processed, heated and fermented food products such as instant soups and sauces as well as breakfast cereals yielded DNA of adequate quality. When tested with eukaryote PCR tapioca, curry and malt extract did not yield a positive amplification signal, whereas all other food or ingredient samples were positive (Table 1). All samples positive in the eukaryote PCR were then analysed by the wheat PCR. Regarding quantification, we only differentiated strong amplification signals (+++) equal to 100 ng wheat genomic DNA or more, moderate (++) about 10 ng DNA, weak (+) 0.1 ng to 1 ng DNA and no (–) amplification less than 0.01 ng. PCR analysis of typical samples is shown in Fig. 2.

Third, the results of the gliadin analysis obtained with the Gluten Lab Kit provided us with those reliable quantitative results the distributor suggests. However, we found gliadin contaminations between 0 to 15 mg/kg on a dry matter basis could not be reliably detected. Contaminations exceeding 15 mg/kg were clearly and reproducibly detected. All results obtained with both wheat component analyses are listed in Table 1.

Discussion

The strategy for the judgement of the results obtained by PCR and immunochemical assay was a follows: a positive response with both genomic wheat DNA and gliadin analysis orginating from wheat flour contamination. This contamination was quantified based on gliadin analysis assuming a gliadin content of wheat around 4% [13]. A positive wheat DNA and a negative gliadin signal are considered indicative of contamination with gliadin-free wheat starch. Last, a positive signal with the gliadin assay alone is probably due to the use of purified gliadin as a food additive [14]. Therefore, as shown in Table 1, the three different rice flour samples (X-17 to X-19), the maize product polenta (Z-03) and six instant products (Y-05 to Y-09, Z-02) were all clearly contaminated or mixed with wheat flour in the range from less than 0.03 to over 0.25%, equal to less than 1 mg up to 100 mg per 100 g of food product. Three instant products (Y-01, Y-03, Y-04) were contaminated with gliadin up to 8 mg/100 g, which might not be considered safe for coeliac patients.

PCR technology was used to rapidly and sensitively detect either eucaryote or wheat genomic DNA. DNA samples only are screened for wheat genomic DNA when this sample has proved genomic-eukaryote-DNA positive. Hence, false negative results could be excluded due to insufficient DNA quality. A quick, easy and hazardless DNA isolation was presented providing an appropriate nucleic acid substrate for PCR reactions. However, food ingredients with a thickening and absorption function could not be reasonably extracted and therefore not reliably examined by either DNA or gliadin analysis. All other food samples were accessible for both analysis systems. When tapioca, curry and malt extract were DNA-extracted, other components were co-isolated that inhibited the PCR reaction. The sensitivity of DNA analysis by PCR is slightly better than gliadin determination by the Gluten Lab Kit applied to wheat contamination. Another method that determines and detects wheat in food based on measuring characteristic protein components (Leitpeptide) was published by Wieser et al. [15].

The PCR method can support and confirm food sample analysis and characterization by immunoenzymatic analysis when a single method is not sufficient or accepted. Although the DNA analysis based on PCR technology has no reliable quantification method as yet, its different approach to contamination problems is worth consideration as an alternative to or a confirmation of immunochemical methods.

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