A new measurement approach of ionizing radiation in irradiated trout (*Oncorhynchus mykiss*) by Randomly Polymorphic DNA-Polymerase Chain Reaction

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Revised: 15 December 2015 / Accepted: 30 December 2015 / Published online: 21 April 2016
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Abstract Trout (*Oncorhynchus mykiss*) were irradiated at doses of 0.250, 0.500, 1, 3, 5, 7 and 9 kGy in gamma cell. DNAs were extracted from the irradiated samples before and after storage. 1ERP primers were designed, and RAPD-PCR (Randomly Polymorphic DNA-Polymerase Chain Reaction) was applied to make randomly amplifications on the DNA of the irradiated samples. Agarose gel profiles of irradiated fish were obtained to determine change of band profiles. In addition, DNA fragmentation occurring in each dose was determined by comet assay for the verification of methodology developed in this study. The molecular methodology was developed to estimate ionizing radiation (IR) level in irradiated fish. This methodology allows the analysis of the trout irradiated up to the dose limit of around 0.5 kGy and stored for a period of three months.

Keywords *Oncorhynchus mykiss* · Irradiation dose · PCR · Comet assay · DNA damage

Introduction

There is a growing public concern about whether irradiated foods are safe and healthy or not. The negative perception of nuclear technology presence makes a controversial issue in the availability of food irradiation technology. Currently, the

development of alternative technologies have been aimed for the traceability of irradiated foods and measurement of radiation dose applied to foods for the production of safe food in the future [Stefanova et al. 2010; Şakalar and Mol 2013; Şakalar and Mol 2015].

The radiation treatment of foods like sea food is gaining importance throughout the world. Irradiated food should be adequately labelled according to international and national guidelines. Therefore, there is a need for methods that provide reliable dose estimation. In recent years, the comet assay has been a rapid technique for analyzing DNA damage in individual cells (Erel et al. 2009) but further this technique is required to extend the full potential with the use of new verification techniques (Şakalar 2013).

Gamma irradiation is known to cause molecular damage to DNA (Von Sonntag 1987; Villavicencio et al. 2004). For example, it induces closely spaced lesions, including double-stranded DNA (dsDNA) breaks and about twice as many single strand DNA (ssDNA) breaks (Sutherland et al. 2000; Lee and Levin 2008). For that reason, it is possible to identify DNA breaks using primers that target notably different-sized sequences for DNA amplification by PCR.

The purpose of this study was to develop new DNA-based methodologies to estimate the level of irradiation dose and to distinguish irradiated and non-irradiated rainbow trout by RAPD-PCR.

Material and methods

Samples

Fishes (*Oncorhynchus mykiss*) were bought from a fishmonger in Istanbul, Turkey. They were immediately transported to the Genetic and Bioengineering Research Laboratory of Fatih

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University. Twenty five to thirty gr muscular portions were taken from the tail region of trout to prevent bacterial contamination into sterile polyethylene bags for each dose level were packed in polyethylene bags, and labelled with its respective irradiation doses and transported along with ice bags to Saraykoy Nuclear Research and Training Center (Ankara, Turkey).

γ -irradiation

Fish samples were placed in uniform areas of the irradiation field to minimize any differences in the geometry of the radiation dose. The samples were then treated with radiation doses of 0.25 ± 0.01 , 0.5 ± 0.1 , 1 ± 0.2 , 3 ± 0.03 , 5 ± 0.15 , 7 ± 0.04 , and 9 ± 0.05 kGy in gamma cell (gamma cell 60Co, dose rate 1.31 kGy/h at a room temperature of $\sim 10^\circ\text{C}$). Harwell Amber 3042 dosimeters were used for the measurement of radiation dose. Immediately after irradiation, irradiation-treated and reference samples were distributed into 2 ml microcentrifuge tubes for DNA extraction. Two parts of samples were also kept in the freezer at -20°C for 1 and 3 month periods.

DNA extraction and quantification

DNA was extracted from exactly 25.0 mg of fish meat samples by DNeasy® Tissue Kit (Qiagen, Hilden, Germany) before and after storage. A nanodrop spectrophotometer (Thermo Scientific-2000) was used to determine the concentration of DNA in a solution. The samples were exposed to ultraviolet light at 260 nm and 280 nm. 260:280 was used to calculate the qualification of nucleic acids by the following equation: DNA concentration = $\text{OD}_{260} \times \text{extinction coefficient}$ ($50 \mu\text{g}/\text{ml}$) $\times$ dilution factor. All of the DNA samples were adjusted to just 40 ng by elution buffer (AE) and were stored at $+4^\circ\text{C}$ until next treatment.

Migration distribution of genomic DNA

The genomic DNA extracts (5 μl) from the fish meats were mixed with 2 μl of gel loading solution, and transferred into 1 % agarose gel containing 0.1 mg/ml ethidium bromide in 0.5 X TBE buffer, and electrophoresed for 25 min at 110 V. Genomic DNA was imaged by a UV transilluminator for the estimation of genomic DNA bp-fragment size after IR (Fig. not shown).

Detection limit

In order to determine the absolute detection limit of RAPD-PCR, reference fish DNA was serially diluted by ten-fold increment (yielding solutions of 40, 4, 0.4, 0.04, 0.004, 0.0004 and 0.00004 ng/ μl). To identify the dose detection limit of IR, fish meat samples that were exposed to 0.25 and 0.5 kGy were used.

Primer designing

Ten base 1ERP primers containing 80 %, 70 % and 60 % of GC content were designed using the RAPD primer generator (<http://www2.unijena.de/biologie/mikrobio/tipps/rapd.html>) in order to achieve a suitable profile on agarose gel for rainbow trout. 1ERP80 (CTCGCAGGCG), 1ERP70 (CGCTTGACGC) and 1ERP60 (GGCGATTACG) were synthesized by Metabion (Steinkirchen, Germany).

RAPD-PCR assay

PCR amplification was performed in a 25 μL reaction mixture. The mixture consisted of 2.5 μL Standard Taq Reaction Buffer, 2.5 μL MgCl_2 (25 mM), 1.5 μL primer (15 pmol), 2 μL dNTP (100 μM), 0.2 μL Taq DNA polymerase and 1 μL of template DNA. The amplifications were carried out by using a thermal cycler (VWR, UK) programmed at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min, a final extension step at 72°C for 5 min and stored at 4°C .

Selection of suitable primers

Primers were checked, and optimization was done for rainbow trout through RAPD-PCR amplification. The alterations in various PCR parameters influenced the reproducibility and patterns of RAPD amplification. Concentrations of reagents including DNA template, Taq polymerase, MgCl_2 , dNTPs, and primers were selected based on clear and scorable DNA bands produced. RAPD-PCR with each of the primers was applied on DNA of rainbow trout, and observed under the UV to select the suitable profile of bands.

Detection limit

To check the detection limit of RAPD-PCR, DNA in diluted form (40, 4, 0.4, 0.04, 0.004, 0.0004, and 0.00004 ng/ μl) from the non-irradiated samples of rainbow trout were amplified using primers 1ERP80. The samples irradiated by 0.25 and 0.50 kGy were used for detection limit of irradiation dose.

Visualization of PCR products

Following amplification, 2 μl of 6X DNA Loading Dye (Thermo Scientific, US) were added to 8 μl of the PCR products resulting in a total volume of 25 μl . Ten μl were loaded into the wells of 2 % agarose gels stained with 0.2 mg/ml of ethidium bromide. PCR reaction products were separated using a horizontal electrophoresis chamber in 0.5X of TBE buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA at pH 7.8). Electrophoresis was conducted at 100 V for

50 min. DNA bands in the gels were visualized with UV transillumination using a Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA).

DNA comet assay

Comet test as the authentication method to detect irradiation dose was applied to rainbow trout meat in the accredited DNA laboratory of Turkey Atomic Energy Authority SANAEM Food Unit according to Erel et al. (2009).

Results and discussion

Determination of suitable primers

1ERP80 was found to be the best primer with distinct bands and high polymorphism for rainbow trout and was selected due to the clearance of bands for rainbow trout (Fig. not shown) in the present assay. Other primers did not produce sufficient numbers and clear polymorphic bands.

RAPD-PCR detection limits

The detection limit of RAPD-PCR assay was determined using different concentrations (40 to 0.000004 ng/ μ l) of genomic DNA from the non-irradiated rainbow trout. In this way, the minimum concentration was determined as the detection limit of RAPD PCR. In Fig. 1a, significant band losses were observed on the RAPD profiles performed with 0.00004 and 0.000004 g/ μ l DNA. RAPD PCR detection limit was 0.001 % (0.0004 ng/ μ l) for the rainbow trout.

IR dose detection limits

To identify the detection limit of IR dose, DNAs, which were extracted from the fish meat samples exposed to 0.25, 0.5 and 1 kGy, were used. The result of the sensitivity test showed that 0.5 kGy could be detected with using 1ERP80 primers for rainbow trout (Fig. 1b and c).

Detection of irradiated fishes by RAPD PCR

The amplification reactions were performed by RAPD PCR for the DNA of rainbow trout irradiated with 0, 0.25, 0.5, 1, 3, 5, 7 and 9 kGy using primer 1ERP80. The PCR product was then run on gel electrophoresis. The successive decreases in band numbers were observed as the irradiation dose applied to the samples was increased. As seen in Fig. 2a and b, the band profiles of non-irradiated (0) and irradiated (from 1 to 9 KGy) samples were compared and quite obvious differences were determined. The fade and loss of bands is noteworthy in band profiles of irradiated products, and here irradiated and non-irradiated products can easily be determined. In addition, losses (breakage) in longer targets occurred with increase in irradiation dose. For example, bands obtained with the irradiation dose of 9 kGy seemed not to be amplified. These indicate that irradiation reduces the available template DNA molecules in irradiated fish and irradiation results in DNA damage, including base modification, abasic sites and strand breaks (Lim et al. 2008; Von Sonntag 1987). As DNA lesions are capable of stopping a thermostable polymerase on DNA template (Sikorsky et al. 2004; Lim et al. 2006), an increase in lesions will result in decreased PCR amplification of the target sequence. The results suggest that RAPD PCR method can be

Fig. 1 The detection limit of RAPD-PCR for rainbow trout (a). IR dose detection limits of irradiated rainbow trout before storage (a), after storage (b) at -20°C for three months. M: Marker

used to differentiate the irradiated fishes from the non-irradiated ones, and even predict the radiation dose.

The effect of storage on RAPD profiles of irradiated fishes

The experiment was also carried out with the samples of rainbow trout, which were stored at -20°C for three months to see the effect of irradiation dose on stored fish samples. RAPD-PCR profiles of stored samples also showed progressive reduction of the intensity of bands. A gradual loss of bands was observed at the top of the profiles (Fig. 2c). These findings suggest that RAPD-PCR with a suitable primer can be used as a new tool for the differentiation of DNA, which is isolated from the irradiated fishes. RAPD-PCR is a promising method for identifying the irradiation effect on fish DNA after storage for a long period of time.

RAPD-PCR technique requires pure DNA since contaminated DNA can inhibit PCR. The spectroscopic results of DNA have proved this needs (table not shown). In general, the DNA concentrations and purities of irradiated products were 40–140 ng and 1.85, respectively. We used three primers with G + C (%) content from 60 to 80 %, and one of the three primers (1ERP80) with 80 % G + C content has proved useful in amplifying rainbow trout at the primary stage of RAPD-PCR optimization.

Fig. 2 RAPD-PCR profiles of irradiated rainbow trout. **a** and **b** are the images obtained from the repeat of the experiments. Image **c** shows effect of storage on RAPD profiles of irradiated rainbow trout after storage at -20°C for three months. M: Marker

Fig. 3 Comets observed by DNA comet assay **a** 0 kGy; **b** 0.25 kGy; **c** 0.50 kGy; **d** 1 kGy; **e** 3 kGy; **f** 5 kGy; **g** 7 kGy; **h** 9 kGy

Migration distribution of genomic DNA

The extent of DNA degradation due to strand breaks was then evaluated by directly subjecting extracted genomic DNA to agarose gel electrophoresis. This allowed the mobility distribution of the fragmented DNA to be determined visually (Brody and Kern 2004; Lee and Levin 2008). DNA derived from fish meat exposed to 0, 1, 3, 5, 7 and 9 kGy exhibited a notable decrease in molecular weight with increasing of IR dose (Fig. not shown). In addition, the total intensity of fluorescence decreased with increasing of IR dose. This indicated a quantitative decrease in double stranded DNA which is required for maximum fluorescence of ethidium bromide DNA from non-irradiated control fish meat.

IR-treated fish meats determined by RAPD PCR techniques were confirmed by comet assay method. The length of comet tails increased whereas the amount in the comet head decreased with increasing radiation dose. The microscopic observation of irradiated fish meat samples showed that the distance of DNA migration “comet length” increased with the radiation dose for all samples. Likewise, the amount of the irradiation dose might be indicated by the comet length. Based on the size of the comets for different doses of radiation, a rough dose estimate

may be possible. Generally in the non-irradiated samples, cells or nuclei were present as round- or oval-shaped intact images along with heads with short and faint tails. In the irradiated samples, only distinct comets were observed. All the cells were damaged and fragmented and no single intact cell could be found in any irradiated sample (Fig. 3).

In conclusion, this study with RAPD-PCR can be used as a basis for the identification of the dose of irradiation by looking at the loss of specific bands at specific amounts of irradiation. Further studies are required for the detection of DNA degradation in fish meats caused by irradiation using real-time PCR assay. IR was found to cause an extensive reduction in the molecular size of DNA. The determination of DNA damage can be possible by real-time PCR analysis that provides a potent tool for the evaluation of fluorescence intensity related to application doses. This method can be applied widely to not only food products but also all biological materials containing DNA.

Acknowledgments The authors would like to thank the Office of Scientific Research Projects at Istanbul University for its financial support to this project with the Grant No. 18169, and the Biology Department of Fatih University, which provided necessary facilities.

References

- Brody JR, Kern SE (2004) History and principles of conductive media for standard DNA electrophoresis. *Anal Biochem* 333:1–13
- Erel Y, Yazici N, Ozvatan S, Ercin D, Cetinkaya N (2009) Detection of irradiated quail meat by using DNA comet assay and evaluation of comets by image analysis. *Radiat Phys Chem* 78:776–781
- Lee JL, Levin RE (2008) New approach for discrimination of *Vibrio vulnificus* by real-time PCR before and after γ -irradiation. *J Microbiol Methods* 73:1–6
- Lim S, Jung J, Kim M., Ryu S, Kim D (2008) Effect of ionizing radiation on the quantitative detection of salmonella using real-time PCR. *Radiat Phys Chem* 77:1112–1117
- Lim S, Yoon H, Rys S, Jung J, Lee M, Kim D (2006) A comparative evaluation of radiation-induced DNA damage using real-time PCR: influence of base composition. *Radiat Res* 165:430–437
- Stefanova R, Vasilev VN, Spassov SL (2010) Irradiation of food, current legislation framework and detection of irradiated food. *Food Anal. Method* 3:225–252
- Sikorsky JA, Primerano DA, Fenger TW, Denvir J (2004) Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method. *Biochem Biophys Res Commun* 323:823–840
- Sutherland BM, Bennett PV, Sidorkina O, Laval J (2000) Clustered DNA damages induced in isolated DNA and in human cells by low doses of ionizing radiation. *Proc Natl Acad Sci* 97:103–108
- Şakalar E, (2013) Estimation of radiation dose by DNA comet assay and electrophoresis of irradiated fish DNA, VI international bioengineering congress 2013 human welfare, abstract book, page: 70: 12–15 november kuşadası/türkiye
- Şakalar E, Mol S (2013) Estimation of irradiation dose in the flesh of irradiated fish by RAPD PCR, VI international bioengineering congress 2013 human welfare, abstract book, page: 69, 12–15 november kuşadası/türkiye
- Şakalar E, Mol S (2015) Determination of irradiation dose and distinguishing between irradiated and non irradiated fish meat by real-time PCR. *Food Chem* 182:150–155
- Villavicencio ALCH, Araujo MM, Marín-Huachaca NS, Mancini-Fiho J, Delincée H (2004) Identification of irradiated refrigerated poultry with the DNA comet assay. *Radiat Phys Chem* 71:189–191
- Von Sonntag C (1987) Free-radical-induced DNA damage and its repair A Chemical Perspective. Springer Verlag, Berlin, Heidelberg