Food Chemistry 216 (2017) 186–193

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Determination of irradiation histories of raw beef livers using liquid chromatography–tandem mass spectrometry of 5,6-dihydrothymidine

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ARTICLE INFO

Article history:
Received 7 April 2016
Received in revised form 13 July 2016
Accepted 13 August 2016
Available online 16 August 2016

Keywords:
Irradiated food
DNA
5,6-Dihydrothymidine
Beef livers
LC–MS/MS

ABSTRACT

A method for detecting irradiation histories of raw beef livers was developed by measuring 5,6-dihydrothymidine (DHdThd) using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Liver DNA was extracted using phenol–chloroform extraction followed by precipitation in 50% ethanol. DNA was then enzymatically digested and nucleosides were purified using an OASIS MCX column. DHdThd and thymidine (dThd) contents of resulting test solutions were analyzed using LC–MS/MS. DHdThd was detected specifically after γ-irradiation. Concentration ratios of DHdThd to dThd in the test solutions increased dose-dependently after irradiation at 1.0–11.3 kGy, which included the practical dose for sterilization of 2–7 kGy. Dose–response curves from beef livers of individual animals almost overlapped. Thus, this method is a candidate for the detection of irradiation histories of foods from which DNA can be extracted.

1. Introduction

Irradiation of food, an effective method for improving hygiene compliance and the shelf life by controlling microorganisms, is a popular preservation technique worldwide (Arvanitoyannis, Stratakos, & Mente, 2009; Arvanitoyannis, Stratakos, & Tsarouhas, 2009; Dempster, 1985; Diehl, 1992). Thus, irradiation of foods has been approved in >40 countries as a sterilizing or insecticidal method that avoids the quality-lowering effects of heating or fumigation on products such as spices, frozen seafood, and meats. Irradiation (2–7 kGy) of certain packaged meat products, without heating, can control meat-borne pathogens, including *Escherichia coli* O157:H7, *Salmonella enterica* serovar Enteritidis, *Campylobacter jejuni*, and *Yersinia enterocolitica* (Farkas, 1998). In 2011, serious food poisoning due to enterohemorrhagic *E. coli* (EHEC) was caused by consumption of raw beef dishes (*yukhoe*) at a barbecue restaurant in Japan (National Institute of Infectious Diseases, 2012; Watahiki et al., 2014; Yahata et al., 2015). Following this EHEC outbreak and successive food poisonings because of consumption of raw beef, including liver, the Ministry of Health, Labour, and Welfare (MHLW) of Japan revised in 2011 the standard of consumable raw meat to improve the process of trimming (National Institute of Infectious Diseases, 2012). Raw beef liver dishes (*liver sashimi*) are popular recipes, along with *yukhoe*, at barbecue restaurants in Japan. Thus, MHLW examined beef livers and concluded that contamination with EHEC could occur on the surface and internally via biliary ducts. Hence, revisions of the standard failed to control the risk of EHEC contamination in beef livers, and MHLW banned the sale of raw beef livers for *liver sashimi* in a revision of the Food Sanitation Act in 2012 (National Institute of Infectious Diseases, 2012). In the notification for the revision, MHLW stated the conditions of lifting the ban to sell, subject to the development of technologies that could confirm the elimination of EHEC contamination from beef livers. Irradiation is a candidate technology for decontamination of raw beef liver products in Japan.

Assessment of whether beef livers were irradiated requires an appropriate method for the determination of irradiation histories, and confirmation of the irradiation history is essential for determining regulatory compliance and facilitating international trading. Various detection methods for food irradiation have been developed and validated (Chauhan, Kumar, Nadanasabapathy, & Bawa, 2009). These methods have been based on the detection of physical or chemical changes following irradiation. Currently, 10 official standard methods to detect whether food was irradiated have been established in Europe. To determine the irradiation history of boneless meat, EN1785, which is one of the standard methods for detecting 2-alkylcyclobutanones (ACBs), would be suitable

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http://dx.doi.org/10.1016/j.foodchem.2016.08.036
0308-8146/

(CEN, 2003). ACBs are specific radiolytic products from food fats. Thus, the presence of a sufficient amount of fat is the most important requirement for the detection of ACBs in irradiated foods, and EN1785 is used to determine irradiation histories of chicken, liquid whole eggs, and pork, in which fat contents are >10%. In previous studies, we have developed a rapid and convenient method based on EN1785 for the detection of ACBs in irradiated fat-rich foods, including raw beef patties (Kitagawa et al., 2014). However, the fat content of the raw beef liver is approximately 4%, and it is approximately one-fourth of that in raw beef patties (17.7%) (United States Department of Agriculture, 2015). Thus, in subsequent pilot studies, we adapted the method to determine irradiation histories of raw beef livers.

Because all foods derived from living organisms contain DNA, physical and chemical changes to DNA following irradiation may be useful markers for the determination of irradiation histories, and these techniques do not require a sufficient content of fat. Currently, two such detection methods have been established, the DNA comet assay (EN13784) for the detection of irradiation-induced DNA strand breaks and immunological detection of modified DNA bases using an enzyme-linked immunosorbent assay (ELISA) (CEN, 2001; Delincée, 1996a). The DNA comet assay is applicable to various foods from which cells with intact DNA can be isolated. However, complicated image analyses of electrophoretic data are required to obtain dose–response curves, which is essential for the determination of irradiation histories (Cetinkaya, Ercin, Özvatan, & Erel, 2016; Miyahara, Saito, Ito, & Toyoda, 2000; Verbeek, Koppen, Schaeken, & Verschaeve, 2008). Moreover, enzymatic degradation of DNA during food storage undermines the utility of the DNA comet assay and can lead to ambiguous results (Delincée, 1996b). Thus, although DNA comet assays are among the standard methods, validation of positive results is required using other official methods. To overcome the problem of enzymatic DNA degradation, it has been proposed that strand breaks are electrophoretically analyzed in mitochondrial DNA, which is more stable than nuclear DNA (Marchioni, Tousch, Zumsteeg, Kuntz, & Hasselmann, 1992). However, complicated analyses of electrophoretic images could still be required to generate dose–response curves, similar to the DNA comet assay. As an alternative, an ELISA of modified DNA bases can be used to generate dose–response curves. Tyreman et al. and Kikuchi et al. successfully determined irradiation histories of prawns by detecting 5,6-dihydrothymidine (DHdThd) residues in DNA using ELISA (Tyreman, Bonwick, Smith, Coleman, Beaumont, & Williams, 2004) and those of meat by detecting 8-oxo-2′-deoxyguanosine (8-oxo-dGuo) in DNA using chemiluminescence ELISA (Kikuchi et al., 2007), respectively. DHdThd can be formed following irradiation of thymidine (dThd) residues (Furlong, Jorgensen, & Henner, 1986; Sharpatyi, Cadet, & Teoule, 1978). Moreover, unlike the DNA adducts 5,6-dihydroxy-5,6-dihydrothymidine, 5-formyl-2′-deoxyuridine, 5-hydroxymethyl-2′-deoxyuridine, and 8-oxo-dGuo, which can be formed via oxidizing reactions without irradiation (Cadet, Wagner, Shafirovich, & Geacintov, 2014; Hua et al., 2001), DHdThd is formed specifically by irradiation, without reactive oxygen species (Furlong et al., 1986). Thus, DHdThd may be a more suitable marker of the irradiation history than the above-listed nucleosides. However, ELISA methods require a stable supply of high-quality antibodies for routine use, and preparation of antibodies that specifically recognize DHdThd is time consuming (Hubbard, Ide, Erlanger, & Wallace, 1989). Alternatively, liquid chromatography–tandem mass spectrometry (LC–MS or LC–MS/MS) has become a powerful tool for detecting chemically modified nucleosides (Cadet et al., 2002). Indeed, Berger et al. detected DHdThd in irradiated dThd solutions using LC–MS (Berger, Cadet, Berube, Langlois, & van Lier, 1992), and Dawidzik et al. detected dihydrothymine lesions in the DNA of X-irradiated cells using LC–MS/MS (Dawidzik et al., 2004). These findings suggest that this technique could be used to dose-dependently detect DHdThd formation in foods. Furthermore, LC–MS/MS has been universally used at quarantine stations and analytical laboratories for analyses of pesticides and veterinary medicines in foods. Thus, in this study, we developed a method for the determination of irradiation histories of raw beef livers using LC–MS/MS analysis of DHdThd.

2. Materials and methods

2.1. Beef liver

Fresh raw beef livers from 4 individual animals (Livers I–IV) were obtained from a meat retailer in Osaka. The livers were cut into cubes of 1–1.5 cm (approximately 3 g per cube). The 3 cubes were wrapped in a polyvinylidene chloride film for food. The 3 wrapped livers (9 cubes) were placed in 50 mL polypropylene (PP) tubes (Thermo Fisher Scientific K.K.; Yokohama, Japan) and were stored at −20 °C until γ-ray irradiation.

2.2. Chemicals

DHdThd and 5-ethyl-2′-deoxyuridine (EtdUrd) were purchased from Berry & Associate Inc. (Dexter, MI) and Sigma-Aldrich Co. (St. Louis, MO), respectively. DHdThd has an asymmetric carbon at the 5 position that causes the formation of diastereomers. Hence, the standard for DHdThd comprised 15% 5*R*- and 85% 5*S*-isomers, and the total-DHdThd was expressed as the sum of these isomers. dThd, salmon sperm DNA, proteinase K, and nuclease P1 were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Alkaline phosphatase was obtained from Toyobo Co., Ltd. (Osaka, Japan). Phenol saturated with Tris-EDTA buffer (TE-saturated phenol) and TE-saturated phenol/chloroform/isoamyl alcohol (PCI solution; 25/24/1, v/v) were purchased from Nippon Gene Co. Ltd. (Tokyo, Japan). Other chemicals were of the highest commercially available grade. Methanol eluent for LC–MS/MS was of LC–MS grade and was purchased from Wako. Radiochromic films (FWT-60-1P) were obtained from Far West Technology, Inc. (Goleta, CA).

2.3. γ-Irradiation and storage of samples

γ-Irradiation of samples was conducted at the radioisotope centers of Osaka Prefecture University or Koga Isotope Ltd. (Koka, Japan) using ^{60}Co. Subsequently, 1 mL aliquots of frozen dThd aqueous solutions (1.0 mg/mL; 4.1 mM), 1 mL of frozen aqueous salmon sperm DNA solution (5.0 mg/mL) in 1.5-mL PP tubes, and beef liver samples in 50-mL PP tubes were irradiated at −20 °C under aerobic conditions. Planning irradiation doses were 2, 5, 8, and 11 kGy, and actual irradiation doses were determined using radiochromic film dosimetry patches on the surfaces of the tubes. After irradiation, samples were stored at −20 °C until analysis. Storage periods of Livers I–IV prior to DNA extraction are summarized in Table 1.

Table 1
Irradiation doses and storage periods of Livers.

	Livers[a]				
	I	II	III	IVa	IVb
Irradiation dose (kGy)	1.8; 5.5; 8.2; 11.0	2.2; 5.2; 8.1; 11.2	1.0; 3.5; 7.5; 11.3	1.5; 4.9; 8.2; 10.3	1.5; 4.9; 8.2; 10.3
Storage periods (day)[b]	22	11	25	6	220

[a] Livers-I and II were irradiated at Koga Isotope Ltd. Livers-III and IV were irradiated at Osaka Prefecture University.
[b] Days after γ-ray irradiation till DNA extraction.