Ecotoxicology and Environmental Safety 134 (2016) 11–16

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Genotoxicity evaluation of ionic liquid 1-octyl-3-methylimidazolium bromide in freshwater planarian *Dugesia japonica* using RAPD assay

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ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form
18 August 2016
Accepted 22 August 2016
Available online 29 August 2016

Keywords:
1-octyl-3-methylimidazolium bromide
Dugesia japonica
Genotoxicity
RAPD

ABSTRACT

The randomly amplified polymorphic DNA (RAPD) assay has been used to detect DNA alternation and mutation recently. However, the effectiveness of this method in detecting DNA damage in planarians, a model organism for assessing the toxicity of environmental pollutants is unknown. In the present study, RAPD assay was used to detect the DNA damage in planarians treated by the ionic liquid 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) for the first time. Among the 20 test RAPD primers, 13 primers with 60–70% GC content produced unique polymorphic band profiles. A total of 60 bands were observed in the untreated control planarians. In comparison with the control group, the [C_8mim]Br-treated groups displayed differences in RAPD patterns in the band intensity, disappearance of normal bands and appearance of new bands. The variation of RAPD profiles showed both concentration- and time-effect relationships. Meanwhile, the genomic template stability (GTS) of treated planarians decreased and exhibited negative correlation to the exposure concentration and time of [C_8mim]Br. Our results suggested that [C_8mim]Br had genotoxic effects on planarians, and this DNA damage analysis would lay the foundation for further elucidating the toxicity mechanisms of ionic liquids on planarians. Furthermore, RAPD analysis was proved to be a highly sensitive method for the detection of DNA damage induced by environmental pollutants like toxic chemicals on planarians.

1. Introduction

Planarians play a key role in freshwater lotic ecosystems and they are one of the most abundant predators in streams and upper reaches of rivers (Thorp and Covich, 2001). Freshwater planarians are usually considered as ideal early warning indicators for the deterioration of aquatic ecosystem because of their cosmopolitan distribution and high sensitivity to low concentrations of environmental toxicants (Prá et al., 2005). Furthermore, they are easily collected in large numbers and inexpensively kept in laboratory. These entire characteristics make planarians one suitable organism for toxicological research. More and more reports confirmed that planarians are the favorable test organisms for assessing the toxicological effects of pollutants (Horvat et al., 2005; Kalafatić et al., 2006; Kovačević et al., 2009; Alonso and Camargo, 2011, 2015). *Dugesia japonica* is a common species of freshwater planarian distributed widely in the Far East, including China (Tamura et al., 1998). In view of the regeneration capacity and chemical sensitivity, *D. japonica* is used as a model organism in the research fields of regenerative medicine, stem cell biology, neurological disease and toxicology (Yuan et al., 2016).

Ionic liquids (ILs) are pure ionic compounds with melting points near room temperature (or by convention below 100 °C) (Stock et al., 2004; Weingärtner, 2008). Due to their preponderant properties, such as almost no vapor pressure and nonvolatility, high thermal stability and nonflammability, and high solvent capacity and chemical stability, ILs have been applied in various areas of chemical industry (Welton, 1999; Bubalo et al., 2014a). With the application popularized and the research deepened, however, their "greenness" was questioned. Literature concerning the biotoxicity and environmental hazards of ILs has increased rapidly in recent years (Bubalo et al., 2014b; Du et al., 2014; Jing et al., 2014; Liu et al., 2015; Guo et al., 2016). Among the known ILs, imidazolium-based ILs have been most extensively studied and applied to academic research and industrial uses (Zhang et al., 2011). The 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) is a kind of typical imidazolium-based IL and widely used in industrial production. At present, the toxic effects of [C_8mim]Br on some organisms such as earthworm (Li et al., 2010), duckweed (Zhang et al., 2013), diatom (Deng et al., 2015) and so on or in vitro cell lines (Li et al., 2012a) have been reported. In our previous study the effects of [C_8mim]Br on *D. japonica* were evaluated from the

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http://dx.doi.org/10.1016/j.ecoenv.2016.08.016
0147-6513/

mortality, behavior and antioxidant response (Shi et al., 2013; Zhang et al., 2015, 2016), and the results indicate a remarked dose-effect relationship between the IL and the animals. Nonetheless, to the best of our knowledge no studies have been reported about the genotoxicity or DNA damage of [C_8mim]Br on planarians until now.

The comet assay and chromosome aberration have been used to evaluate the genotoxic effects of toxic chemicals on planarians (Guecheva et al., 2001; Lau et al., 2007). Recent advances in molecular biology have led to the development of several PCR-based techniques, which can be used for DNA analysis in the field of genotoxicology. The randomly amplified polymorphic DNA (RAPD) method is a PCR-based technique that amplifies random DNA fragments with the use of single short primers of arbitrary nucleotide sequence under low annealing conditions. It has been initially used to detect polymorphism in genetic mapping, taxonomy and phylogenetic studies and later in genotoxicity and carcinogenesis (Atienzar and Jha, 2006). To date this technique has been successfully applied to detect genotoxicity of pollutants in animals, plants, microorganisms and in vitro cell lines (Süleyman et al., 2009; Zhou et al., 2011; Rocco et al., 2014; Aksakal and Esim, 2015; Baurand et al., 2015; Zaleska-Radziwill and Doskocz, 2016).

In the present study, the impact of IL [C_8mim]Br on planarian *D. japonica* in terms of DNA damage was studied using RAPD analysis for the first time. The principal objectives were to assess the genotoxicity of [C_8mim]Br on *D. japonica*, and to explore the potential of RAPD technique in genotoxicity detection on planarians.

2. Materials and methods

2.1. Test animals and chemicals

D. japonica was collected from Yu-quan stream (Qi County, China) in Nov. 2014 and maintained in autoclaved tap water at laboratory. Animals were fed with raw fish spleen once a week. Culture medium was renewed weekly after feeding. Planarians fasting for one week were used in the experiments.

Ionic liquid 1-octyl-3-methylimidazolium bromide ([C_8mim]Br) was purchased from Hubei Hengshuo Chemical CO., LTD. (Wuhan, China) with a chemical purity of 99%. Other reagents in analytical grade were obtained from various commercial sources.

2.2. Exposure and sampling

Based on the median lethal concentration (LC_{50}) of [C_8mim]Br on *D. japonica* determined by Shi et al. (2013), three concentrations of 74, 147 and 220 mg L^{-1} were designed for treatment groups, and the autoclaved tap water was used as the control. Stock solution of [C_8mim]Br was prepared on the day of the experiment and diluted to desired concentrations using autoclaved tap water. Twenty-four planarians with normal morphology and 10–15 mm in gliding length were divided into four groups averagely and exposed to the treatment groups and the control. IL solution and autoclaved tap water were renewed every day. After 1, 3 and 5 d exposure, one planarian from each group was randomly sampled for genomic DNA extraction.

2.3. Genomic DNA extraction

Total DNA was extracted following the method of Zhang and Qiao (2008) with minor modifications. Single planarian was rinsed three times with double-distilled water and then homogenized in STE (30 mM Tris-HCl, 200 mM EDTA, 50 mM NaCl, pH 8.0). Tissue homogenates were incubated at 55 °C in lysis buffer (30 mM Tris-HCl, 200 mM EDTA, 50 mM NaCl, 1% SDS, 0.5 mg mL^{-1} proteinase K, pH 8.0) for 2–4 h, followed by a standard phenol-chloroform-isoamylalcohol (PCI) extraction. DNA was precipitated from the supernatant with cold ethanol, and then centrifuged, washed, dried and dissolved in 40 μL TE buffer (30 mM Tris-HCl, 200 mM EDTA, pH 8.0). RNA was digested with RNase A at 37 °C for 30 min. The quality of the obtained DNA was checked by staining 5 μL DNA sample and resolving it in an electrophoresis system and observing the DNA bands under UV light. After the concentration and the index of purity (OD_{260}/OD_{280}) were measured via a NanoDrop 2000 Spectrophotometer (Gene Company Limited), DNA samples were sub-packaged and stored at −20 °C until use.

2.4. RAPD procedures

An initial screening of 20 random primers was performed in order to test amplification profiles for polymorphism and reproducibility. The sequences of 13 primers utilized in this study were shown in Table 1. PCR reactions were performed in reaction mixtures of 15 μL containing 20 ng of genomic DNA, 0.2 μM primer (Sangon Biotech, China), 2 × Taq Master Mix containing blue dye (Zhengzhou Science Tech-trade, China). Amplifications were implemented in a DNA thermocycler (Biometra, Germany) programmed for 5 min at 94 °C (initial denaturing step), 40 consecutive cycles each consisting of 1 min at 94 °C (denaturing), 1 min at 37 °C (annealing), 2 min at 72 °C (extension), and followed by 5 min at 72 °C for the final extension. After amplification, the PCR products were analyzed by electrophoresis on 1.5% agarose gels in 1 × TBE buffer. Then the electrophorograms were photographed under a GIS-1000 Gel Auto-photograph System (Guangzhou Fangtong Biotech, China). All amplifications were repeated twice in order to confirm the reproducible RAPD patterns. Only repeatable and clear amplification bands were scored for the construction of the data matrix.

2.5. Estimate of genomic template stability

Genomic template stability (GTS) was calculated for each primer as the formula: GTS (%)=(1 − a/n) × 100, where “a” is the number of polymorphic bands detected in each treated sample, and “n” is the number of total bands in the control. Polymorphism observed in RAPD profile includes disappearance of a normal band and appearance of a new band in comparison with the control profile (Atienzar et al., 1999). To compare the sensitivity of parameter (GTS), changes in these values were calculated as a percentage of their control (set to 100%).

3. Results

3.1. The genomic DNA and the reproducibility of RAPD profiles

The purity index of the DNA isolated from the planarian samples was determined spectrophotometrically as 1.7–2.2. This indicates a high degree of DNA purity. The quality of the isolated DNA was checked and found to be single band (Fig. 1), which suggests good DNA integrity and no degradation. The consistency of the RAPD technique was checked (Fig. 2), and the results showed that the isolated DNA always gave the same banding pattern with the same primer.

3.2. Effect of [C_8mim]Br-modified DNA on RAPD profiles

Out of the total of 20 10-mer random primers, only 13 priming oligonucleotides (Table 1) yielded specific and stable results (Fig. 3, Table 2). The other primers showed no visible bands or only smear. The differences in RAPD patterns refer to band intensity,