Analytical Biochemistry 462 (2014) 51–59

Contents lists available at ScienceDirect

Analytical Biochemistry

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

A heterogeneous biotin–streptavidin-amplified enzyme-linked immunosorbent assay for detecting tris(2,3-dibromopropyl) isocyanurate in natural samples

Dan Bu [a], Huisheng Zhuang [a,*], Xinchu Zhou [b], Guangxin Yang [a]

[a] School of Environment Science and Technology, Shanghai Jiao Tong University, Minghang District, Shanghai 200240, People's Republic of China
[b] School of Agriculture and Biology, Shanghai Jiao Tong University, Minghang District, Shanghai 201101, People's Republic of China

ARTICLE INFO

Article history:
Received 6 March 2014
Received in revised form 14 May 2014
Accepted 3 June 2014
Available online 12 June 2014

Keywords:
Tris(2,3-dibromopropyl) isocyanurate
Biotin–streptavidin-amplified system
Enzyme-linked immunosorbent assay
Environmental monitoring

ABSTRACT

Tris(2,3-dibromopropyl) isocyanurate (TBC) is a novel brominated flame retardant (BFR) that is widely used to substitute the prohibited BFRs throughout the world. With the development of research, the potential environmental and ecological harms of TBC have been revealed. For sensitive and selective detecting TBC, an indirect competitive biotin–streptavidin-amplified enzyme-linked immunosorbent assay (BA–ELISA) has been established in this study. The small molecular TBC–hapten was synthesized first; it mimicked the chemical structure of TBC and possessed a secondary amine group. The as-obtained hapten was then conjugated with carrier proteins to prepare artificial antigen. After immunization, the anti-TBC polyclonal antibody was obtained from separating rabbit serum. The procedures of this BA–ELISA were optimized. Under the optimal conditions, the limit of detection (IC_{10}) was 0.0067 ng/ml and the median inhibitory concentration (IC_{50}) was 0.66 ng/ml. Cross-reactivity values of the BA–ELISA with the tested TBC analogues were ⩽5%. This immunoassay was successfully applied to determine the TBC residue in river water samples that were collected near a BFR manufacturing plant. Satisfactory recoveries (92.1–109.2%) were obtained. The results indicated that this proposed BA–ELISA is suitable for the rapid and sensitive determining of TBC in environmental monitoring.

With the increasing application of polymeric materials in electronic, construction, and household products, brominated flame retardants (BFRs)[1] have been used largely for the reason of fire safety. Global market demand for BFRs continues to increase, growing from 145,000 tonnes in 1990 [1] to 310,000 tonnes in 2000 [2]. BFRs can be mainly divided into two subgroups according to the way of incorporating into polymeric materials: reactive and additive flame retardants. The most commonly used BFRs include polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBPA), hexabromocyclododecanes (HBCDs), and tris(2,3-dibromopropyl) isocyanurate (TBC).

Recently, concerns about the potential environmental and ecological problems caused by BFRs have been raised. Studies have proved that many BFR materials could potentially harm ecosystems and human health [3–5]. Many types of BFRs have been found to exist in various environmental or biological samples such as breast milk [6,7], serum [8,9], living body [10,11], food [12,13], water [14,15], and sediment [16–18]. Currently, two types of polybrominated diphenyl ethers, penta- and octa-BDEs, are banned in Europe [19,20] and listed in the Stockholm Convention on persistent organic pollutants (POPs) [21], In 2013, HBCD was included in the Stockholm Convention as well. Meanwhile, TBBPA is listed in the convention on the protection of the marine environment of the North-East Atlantic as a hazardous substance. With growing stringent regulations on BFRs, other substitutes have been produced to fill the market vacancy of those obsolete BFRs. However,

* Corresponding author. Fax: +86 21 54740825/62419587.
E-mail addresses: huishengzhuang@126.com, hszhuang@sjtu.edu.cn (H. Zhuang).

[1] *Abbreviations used:* BFR, brominated flame retardant; PBDE, polybrominated diphenyl ether; PBB, polybrominated biphenyl; TBBPA, tetrabromobisphenol A; HBCD, hexabromocyclododecane; TBC, tris(2,3-dibromopropyl) isocyanurate; ELISA, enzyme-linked immunosorbent assay; BA–ELISA, biotin–streptavidin-amplified ELISA; SA–HRP, streptavidin–horseradish peroxidase; BSA, bovine serum albumin; OVA, egg albumin; BNHS, biotinylated *N*-hydroxysuccinimide ester; DMF, *N*,*N*-dimethylformamide; EDC·HCl, 1-ethyl-(3-dimethyllaminopropyl) carbodiimide hydrochloride; DMSO, dimethyl sulfoxide; TMB, 3,3′,5,5′-tetramethylbenzidine; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; CBS, carbonate buffer solution; PBST, PBS with 0.05% Tween 20; pAb–TBC, anti-TBC polyclonal antibody; Bi–pAb–TBC, biotinylated TBC antibody; LOD, limit of detection; SPE, solid phase extraction; HPLC, high-performance liquid chromatography; UV, ultraviolet; PEG, polyethylene glycol; PVA, polyvinyl alcohol; CV, coefficient of variation; CR, cross-reactivity; PBB15, 4,4′-dibrominated biphenyl.

http://dx.doi.org/10.1016/j.ab.2014.06.003
0003-2697/

these substitutes have similar semi-volatile properties and may also harm the environment. TBC is one of these alternative BFRs that had been identified in the environment first in 2009 [22].

TBC (Fig. 1) is a novel BFR that has a hexabrominated heterocyclic *s*-triazine structure. As an alternative BFR, TBC has the features of high thermal stability, low viscosity, and low tendency of photodegradation. In China, production and use of TBC were begun in 1980 [23]. The production amount of TBC in China is unknown, but judging from the usage and output information, its production quantity is relatively large [22]. So, it can be assumed that the potential for TBC to be released into the environment should not be ignored [22,24]. Recent studies have proved that TBC is a potential environmental hazard. High K_{ow} and bioaccumulation factor calculated results indicated that TBC has the characteristics of semi-volatility and bio-accumulation [25]. A study on zebrafish embryos showed that TBC could remarkably inhibit the expression of vitellogenin genes in liver and further affect gonadal development [26]. Therefore, it proved that TBC is a potential endocrine disruptor.

For TBC determination, the liquid chromatography tandem mass spectrometry method was commonly used [24,27]. It is well known that instrument methods are effective, accurate, and reliable. Nonetheless, some drawbacks exist. These methods are generally expensive, time-consuming, and labor-intensive and also require complex pretreatment procedures that restrict their widespread application for rapid detection in environmental studies. So, it makes sense to develop a more highly sensitive, selective, high-throughput, and simpler analytical method. Immunoassay, which is based on the principle of molecular biology, has the above advantages. Compared with instrumental methods, enzyme-linked immunosorbent assay (ELISA) is more suitable for detecting trace organic pollutants in the environment. Meanwhile, some ELISA methods have been established for detecting PBDEs [28–32] and other BFRs [33,34].

To further improve sensitivity, conventional ELISAs have been modified with a combination of chemiluminescence, fluorescence, or biotin–streptavidin system. Among these strategies, the biotin–streptavidin system is an effective technique and has been widely used for sensitivity improvement [35–39]. In biotin–streptavidin-amplified ELISA (BA–ELISA), several biotins are conjugated to one immunoglobulin without altering the biological activity of immunoglobulin. Then, streptavidin–horseradish peroxidase (SA–HRP) combines with biotins in the following step. The affinity of biotin and avidin is very strong, with an affinity constant of 10^{15} L/mol [40]. The signal intensity can be increased due to more enzyme molecules catalyzing the substrate.

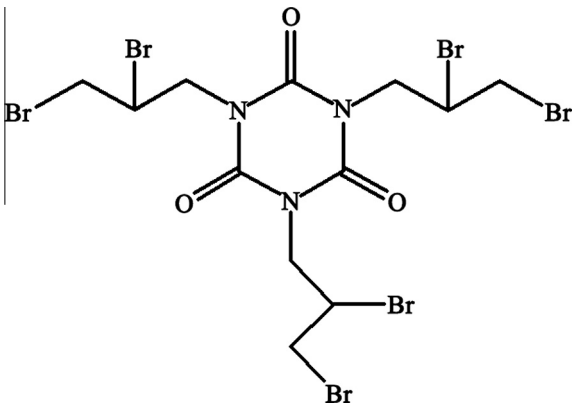

Fig.1. Molecular structure of tris(2,3-dibromopropyl) isocyanurate (TBC). CAS: 52434-90-9.

In this study, a highly sensitive and selective indirect competitive ELISA, using a biotin–streptavidin amplification system, was developed for detecting TBC. For establishing this proposed BA–ELISA, TBC–hapten and immunogens were prepared primarily. Based on the optimal immunization, polyclonal anti-TBC antibodies were produced. Procedures for BA–ELISA were optimized, and some influencing factors were also discussed. Subsequently, this BA–ELISA was implemented to detect TBC residues in environmental samples. The accuracy and sensitivity of the testing results were good. We believe that this proposed BA–ELISA will be useful for environmental studies.

Materials and methods

Reagents and apparatus

The TBC standard and organic materials for hapten synthesis, including 2,4,6-triallyloxy-1,3,5-triazine and liquid bromine, were purchased from J&K Chemical (Beijing, China). Hapten was purified through column chromatography using silica gel (40 μm average particle size) acquired from Shanghai Sanpont (China). Bovine serum albumin (BSA), egg albumin (OVA), biotinylated *N*-hydroxysuccinimide ester (BNHS), *N*,*N*-dimethylformamide (DMF), 1-ethyl-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl), glutaraldehyde, dimethyl sulfoxide (DMSO), hydrogen peroxide, Coomassie brilliant blue G250, Tween 20, complete and incomplete Freund's adjuvant, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sinopharm (China). SA–HRP was acquired from Sangon Biotech (Shanghai, China). All reagents were of analytic grade unless specified otherwise.

The ^{1}H nuclear magnetic resonance (NMR) spectrometer was an Avance III 400-MHz instrument (Bruker, Switzerland) with $CDCl_3$ solution. Fourier transform infrared spectrometry was performed on a Nicolet 6700 instrument (Thermo, USA). A Multiskan MK3 ELISA reader (Thermo) was used to determine absorbance in dual-wavelength mode (450/650 nm) with polystyrene 96-well microplates purchased from Sangon Biotech. TBC–protein conjugates were characterized on a UV-2012 PC spectrophotometer (UNICO, USA). Ultrapure water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

Buffers and solutions

Phosphate-buffered saline (PBS: 137 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4), carbonate buffer solution (CBS: 15 mmol/L Na_2CO_3 and 34.9 mmol/L $NaHCO_3$, pH 9.6), PBST (PBS with 0.05% Tween 20), phosphate–citrate buffer (0.1 mol/L citric acid and 0.2 mol/L Na_2HPO_4, pH 4.3), TMB substrate solution (0.4 ml of 2.5 g/L TMB ethanol solution, 10 ml of phosphate–citrate buffer, and 10 μl of 30% H_2O_2) were used.

Synthesis of TBC–hapten

Compound 1 was synthesized as Likhterov's method [41] and is shown in Fig. 2A. Here, 12.46 g (0.05 mol) of 2,4,6-triallyloxy-1,3,5-triazine, 0.9 g (0.05 mol) of water, and 0.38 g (2.25 mol) of $CuCl_2 \cdot 2H_2O$ were mixed in toluene. The mixture was sustained by stirring in a round-bottom flask and heating at 95 °C for 5 h. The reaction was monitored by thin-layer chromatography (TLC) in developing solvent (*n*-hexane/ethyl acetate = 5:1). After cooling to room temperature, the reaction solvent was evaporated to obtain the crude product. Then, the crude product was recrystallized from petroleum ether and dichloromethane. Finally, the pure compound 1 was obtained after being washed by diluted hydrochloric acid. M.P.: 146–148 °C; name: 1,3-diallyl isocyanurate; yield: 63.6%.