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RESEARCH ARTICLE

Synthesis of haptens and production of antibodies to bisphenol A

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Abstract Three immunizing haptens of bisphenol A (BPA), including two new haptens, were used to produce highly sensitive and specific polyclonal antibodies. The spacer arms of haptens for coupling to the protein carrier were located at different positions in BPA, and different length spacer arms were tested. Highly sensitive polyclonal antibodies were obtained and characterized using indirect competitive enzyme-linked immunosorbent assay (icELISA). Under optimized conditions, the half maximal inhibitory concentration (IC_{50}) value of the best polyclonal antibody was 2.1 $\mu g \cdot L^{-1}$, based on coating heterogeneous antigens, and this optimal polyclonal antibody was highly sensitive toward BPA and displayed negligible cross-reactivity with bisphenol B and bisphenol E. A sensitive icELISA method utilizing the polyclonal antibody was developed for the determination of BPA in milk. In spiked samples (5, 10 and 20 $\mu g \cdot L^{-1}$), the recovery ranged from 80% to 102% with a coefficient of variation (CV) value below 15.8%. The limit of detection of icELISA was 1.95 $\mu g \cdot L^{-1}$. These results indicate that the icELISA method is suitable for the detection of BPA in milk.

Keywords bisphenol A, cross-reactivity, hapten, indirect competitive ELISA, polyclonal antibody

1 Introduction

Endocrine disrupting chemicals that bind receptors for estrogen, progesterone, androgen and other steroid hormones have been identified as potential estrogenic substances[1,2], of which bisphenol A (BPA), generally known as bisphenol, is one example[1]. BPA shows a weak estrogenic effect similar to 17β-estradiol[3]. Furthermore, BPA is reported to induce secretion of prolactin[4] and is postulated to cause reproductive disorders including a decline in sperm count[5]. In addition, BPA is a key constituent of epoxy resins that are used as protective coating on metal cans to maintain the quality of canned food and beverages, and traces can be present in the enclosed food or drink[1,6]. BPA has also been found in air and dust samples in residential and commercial environments in Massachusetts[7] and in leachates from a waste water treatment plant and river water in Japan[6,8,9]. To reduce the hazard from ingesting food contaminated by BPA, a simple, rapid and sensitive analytical method for the detection of BPA is necessary.

Several methods for detecting BPA have been reported, including gas chromatography coupled with mass spectrometry[10] and liquid chromatography coupled with mass spectrometry[11]. These methods are highly sensitive and specific, but time-consuming and costly. Some highly sensitive immunoassays have been reported that are more convenient and faster than standard chromatographic methods[12–18], but antibodies with a high affinity and sensitivity are essential for immunoassay development, and hapten design is a critical step in the production of suitable antibodies[19]. Researchers have derivatized the hydroxyl group of BPA with 2–5 carbon straight chains[12,15,20] and used these derivatives, as well as 4,4-bis(4-hydroxyphenyl)pentanoic valeric acid (BHPVA)[18], as haptens for coupling to carrier proteins (Fig. 1). However, adding haptens at other positions using different

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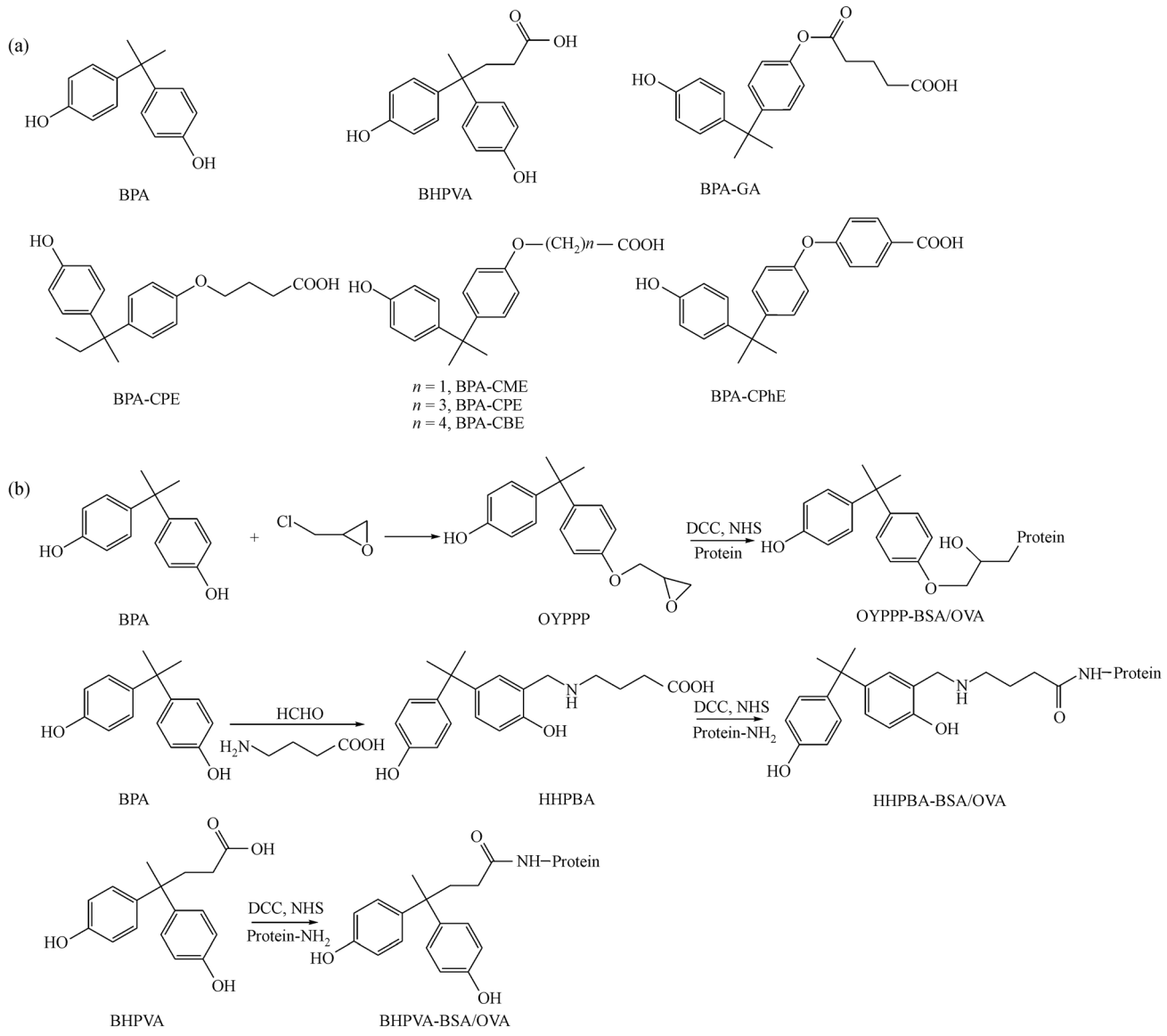

Fig. 1 Haptens from the literature (a) and the synthesis of haptens and antigens (b)

length spacer arms for producing highly sensitive antibodies have not been fully explored. Thus, the objectives of this study were to synthesize two new haptens for preparing highly sensitive polyclonal antibodies, and to develop an indirect competitive enzyme-linked immunosorbent assay (icELISA) method for detecting BPA in milk.

2 Materials and methods

2.1 Materials and equipment

Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant, Freund's incomplete adjuvant, urea hydrogen peroxide, 3,3′,5,5′-tetramethylbenzidine (TMB), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxy-succinamide (NHS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-rabbit IgG-HRP was purchased from Jackson ImmunoResearch (West Grove, PA, USA). BPA, epichlorohydrin, and 4-aminobutyric acid were purchased from Beijing Ouhechem Technology Co., Ltd. (Beijing, China). BHPVA was purchased from Beijing Hvsco Technology Co., Ltd. (Beijing, China). Other reagents and solvents were of analytical grade or higher. A Multiskan MK3 microplate absorbance reader was obtained from Thermo Inc. (Shanghai, China), and microtiter plates were purchased from Costar Inc. (Cambridge, MA, USA).

New Zealand white rabbits were provided by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Rabbits were euthanized by cervical dislocation and

manipulated in compliance with Chinese laws and guidelines (GKFCZ2001545) and according to the China Agriculture University (Beijing, China) regulations concerning protection of animals used for scientific purposes (2010-SYXK-0037).

2.2 Buffers and solutions

Coating buffer (CB) of 0.05 mol·L^{-1} carbonate solution (pH 9.6) and phosphate buffered saline (PBS, 0.01 mol·L^{-1}, pH 7.4) were used for antibody dilution and goat anti-mouse IgG-HRP dilution (PBS-BSA 1%). Blocking buffer was 2% casein in PBS (w/v). The washing buffer was PBS containing 0.05% Tween 20 (PBST). Substrate solution was prepared as described previously[21]. Solution A (pH 5) contained 1.0 g urea hydrogen peroxide, 18.0 g $Na_2HPO_4 \cdot 2H_2O$ and 10.3 g citric acid·H_2O per liter of water. Solution B (pH 2.4) contained 0.5 g TMB, 40 mL dimethyl sulfoxide, 10.3 g citric acid·H_2O and 960 mL water. Both solutions were stored separately in the dark at 4°C. Before the assay, equal volumes of A and B were mixed, and 2 mol·L^{-1} H_2SO_4 was used as a stop solution.

2.3 Hapten synthesis

2.3.1 Synthesis of 4-(2-(4-(oxiran-2-ylmethoxy)phenyl) propan-2-yl)phenol

BPA (228 mg, 1 mmol·L^{-1}) was dissolved in 5 mL of DMF, and 100 μL of epichlorohydrin (1.1 mmol·L^{-1} was added dropwise. The reaction mixture was stirred for 3 h at 25°C. Subsequently, 10 mL of deionized water was added, and the mixture was extracted with 10 mL of ethyl acetate three times. The organic phase was dried using a nitrogen stream at 60°C, and residues were further purified via thin-layer chromatographic (TLC) separation with dichloromethane-ethyl acetate 5:1 (v/v) as developing solution to yield 4-(2-(4-(oxiran-2-ylmethoxy)phenyl)propan-2-yl) phenol (OYPPP). The hapten OYPPP was characterized by NMR.

2.3.2 Synthesis of 4-(2-hydroxy-5-(2-(4-hydroxyphenyl) propan-2-yl)benzyl- amino)butanoic acid

BPA (228 mg, 1 mmol·L^{-1}) and 4-aminobutyric acid (103 mg, 1 mmol·L^{-1}) were dissolved in 5 mL of absolute ethanol, and 10 μL of formaldehyde and 10 μL of acetic acid were added dropwise. The mixture was allowed to react at 25°C for 3h, and subsequently evaporated to dryness under reduced pressure. Residues were further purified by TLC separation with dichloromethane-ethyl acetate 5:1 (v/v) plus 1.5% acetic acid as developing solution as described above to obtain4-(2-hydroxy-5-(2-(4-hydroxyphenyl)propan-2-yl)benzyl- amino)butanoic acid (HHPBA). The hapten HHPBA was also characterized by NMR.

2.4 Preparation of immunogens and coating conjugates

Haptens of OYPPP, HHPBA and BHPVA were covalently attached to BSA and OVA for preparing the immunizing and coating conjugates, respectively. To attach OYPPP to BSA or OVA, 66 mg of BSA (or 90 mg of OVA) and sodium bicarbonate (168 mg) were dissolved in 10 mL of deionized water, and 1 mL of DMF containing 30 mg of OYPPP was added dropwise and allowed to react for 2 days at room temperature.

HHPBA and BHPVA haptens were conjugated to BSA or OVA via an active ester method[22]. Briefly, HHPBA (34.3 mg, 0.1 mmol·L^{-1}), or BHPVA (28.6 mg, 0.1 mmol·L^{-1}), was dissolved together with NHS (22 mg, 0.15 mmol·L^{-1}) and DCC (31.8 mg, 0.15 mmol·L^{-1}) in 1 mL of DMF and stirred at room temperature for 8 h. Precipitates were removed by centrifugation and the supernatant was added drop-wise to 66 mg of BSA (OVA, 90 mg) in 10 mL of PBS and stirred for 12 h at 4°C.

All conjugates were purified by dialysis against PBS for 3 days and stored at – 20°C. MALDI-TOF/MS was used to measure the hapten-protein molar ratios.

2.5 Polyclonal antibody production

Nine female New Zealand white rabbits (each weighing 2–3 kg) were used for immunization. The rabbits were injected intradermally at multiple sites on a shaved area of the back (about 30 sites) with 1 mg of immunogen in 0.2 mL of PBS emulsified with 0.2 mL of Freund's complete adjuvant. For booster injections, the same amount of immunogen was emulsified in incomplete Freund's adjuvant and injected subcutaneously at 10 sites at 3-week intervals for 18 weeks[23]. Blood specimens were collected 7 days after immunization. Antisera were tested by indirect ELISA and icELISA as described below.

2.6 ELISA optimization

The polyclonal antibody was characterized in terms of titer and sensitivity by indirect ELISA (iELISA) and indirect competitive ELISA (icELISA), and after confirmation of sensitivity, the antibody was used in subsequent experiments. The optimized method was consistent with that published previously[22]. The checkerboard method was used to optimize the concentration of coating antigen, and the coating buffer, temperature, pH and ionic strength were evaluated to establish a sensitive icELISA procedure. Lower IC_{50} values and a suitable absorbance value (from 1.5 to 2.0) were selected as the criteria. The icELISA method was essentially carried out as described previously[22,24]. Briefly, 96-well microplates were coated with 100 μL per well of coating antigen in different buffer and incubated at 37°C for 2 h or 4°C overnight. Plates were washed three times with PBST and blocked with 200 μL of blocking buffer and incubated at 37°C for 1 h. After

removal of blocking buffer, 50 μL per well of standard solution and 50 μL per well of antibody were added and incubated for 30 min at 37°C. Following a washing step, goat anti-mouse IgG-HRP (diluted 1:5000 in PBS, 100 μL per well) was added and incubated for another 30 min. After another washing step, 100 μL per well of substrate solution was added and incubated for 15 min. The reaction was stopped using 50 μL per well of stop solution. The optical density was measured at 450 nm and 630 nm with a Multiskan MK3 microplate absorbance reader.

The IC_{50} value of BPA was calculated using a titration curve. The binding inhibition was determined for BPB, BPE, BHPVA, 17β-estradiol, diethylstilbestrol and nonylphenol. Cross-reactivity (CR) values were calculated as follows[24]:

$$CR = (IC_{50} \text{ of BPA}/IC_{50} \text{ of competitor}) \times 100$$

2.7 Sample preparation

BPA-free skimmed milk was supplied by the National Reference Laboratory for Veterinary Drug Residues (Beijing, China). Milk samples were centrifuged at 3500 *g* for 10 min at 4°C, and the upper fat layer was completely removed. Fat-free milk samples were then diluted twice with 0.1% Tween 20 prior to immunoassay. Twenty blank samples were subjected to icELISA as control, and the limit of detection (LOD) was calculated using the following equation:

$$LOD = \overline{X} + 3SD$$

3 Results and discussion

3.1 Synthesis of haptens and conjugates

The design of haptens is important in the development of a specific antibody. A suitable hapten for immunization should exhibit a near perfect mimic of the target molecule structure, and share similar electronic and hydrophobic properties. Tethering of the hapten to an antigenically inert handle at a location distal to the determinant group(s) should then be performed to avoid masking or altering any functional groups[25,26]. Two basic approaches have been used for preparing BPA conjugates; one involving BHPVA[1,13,18], and the other involving the attachment of various spacer arms between the hydroxyl group of BPA and the terminal carboxyl group of the carrier protein used for coupling, as demonstrated for BPA-GA, BPA-CPE, BPA-CME, BPA-CBE and BPA-CPE (Fig. 1a)[12,13,15,16,20]. In this study, to preserve both potential immunodominant epitope phenolic groups, BHPVA was used as the hapten for conjugation, and hapten OYPPP, which has one remaining phenolic group and the methyl group of BPA, was initially synthesized and coupled to the carrier protein. Furthermore, in order to avoid any other functions of BPA, 4-aminobutyric acid was introduced into the benzene ring in hapten HHPBA. The reaction scheme is shown in Fig. 1b. The ^{1}H NMR for OYPPP and HHPBA were ^{1}H NMR (D-DMSO) δH:1.67 (s, 6H, –CH3), 2.38 (m, 1H, epoxyethane–CH2–), 2.63 (m, 1H, epoxyethane–CH2–), 3.04 (t, H, –CH–O), 3.95 (m, 1H, –O–CH2–), 4.21 (m, 1H, –O–CH2–), 4.80 (s, H, –OH), 6.65–6.71 (m, 4H, H3 + H5 + H3′ + H5′), 6.95–7.10 (m, 4H, H2 + H6 + H2′ + H6′) and ^{1}H NMR (D-DMSO) δH: 1.68 (s, 6H, –CH3), 1.73 (t, 2H, –CH2–), 2.27 (t, 2H, –CH2–COOH), 2.62 (t, 2H, –NH–CH2–), 3.80 (t, 2H, Ar–CH2–NH–), 4.58 (s, H, –OH), 4.74 (s, H, –OH), 6.50–6.65 (m, 3H, H2 + H5 + H6), 6.90–7.00 (m, 2H, H2′ + H6′), 6.68–6.85 (m, 2H, H3′ + H5′), respectively, indicating that both haptens were synthesized successfully. And hapten-protein molar ratios were determined by MALDI-TOF/MS and were 4.36, 2.52 and 5.81 for HHPBA-BSA, OYPPP-BSA and BHPVA-BSA, respectively, indicating successful conjugations.

3.2 Screening of polyclonal antibodies

All nine antisera were detected following the sixth immunization using non-competitive ELISA to determine the optimum dilution of coating antigens and antibodies, and icELISA was then used to determine the sensitivity using homologous coating antigens. As shown in Table 1, IC_{50} values and suitable absorbance values (from 1.5 to 2.0) were used as selection criteria. The three immunogens could induce a significant immune response, but the immunogen used OYPPP as hapten was the most active, compared with haptens of HHPBA and BHPVA. Although the hapten of HHPBA was generated purposely to avoid any other functions of BPA, the sensitivity of these

Table 1 Optical density (OD_{450}) and IC_{50} of polyclonal antibodies[a]

Code	OYPPP-BSA/OVA		HHPBA-BSA/OVA		BHPVA-BSA/OVA	
	OD_{450}	IC_{50}/(ng·mL^{-1})	OD_{450}	IC_{50}/(ng·mL^{-1})	OD_{450}	IC_{50}/(ng·mL^{-1})
1	1.251	37.4	–	–	1.643	36.3
2	1.867	38.2	1.487	45.5	1.589	40.2
3	1.948	23.1	1.248	126.2	–	–

Note: [a], the antisera were evaluated by icELISA for primary screening. The antiserum dilution was 20000 and coating conjugate dilution was 5000. "–" indicates that the rabbit died.

antibodies was lower than that of other haptens, partly due to the lower hapten-protein molar ratio. The sensitivity of antibodies derived from the BHPVA hapten was lower than those derived from OYPPP, presumably because the two phenolic groups act as potential immunodominant epitopes, and the structure of BPA was altered slightly following coupling with the carrier protein. Serum from the rabbit induced by OYPPP-BSA was eventually selected for subsequent experiments.

3.3 Comparison of homogeneous and heterogeneous icELISA

Heterogeneous formats can greatly enhance the affinity of antibodies toward their targets compared with coating antigen or tracer hapten[19,24,27,28] by substantially improving the sensitivity. In this study, HHPBA-OVA and BHPVA-OVA were used as heterogeneous coating antigens, and OYPPP-OVA was used as a homogeneous coating antigen, in order to compare their sensitivities. The IC_{50} value of icELISA with HHPBA-OVA as a heterogeneous coating antigen was 3.83 $\mu g \cdot L^{-1}$, compared with 22.6 $\mu g \cdot L^{-1}$ for OYPPP-OVA (Fig. 2a). However, an IC_{50} value of 81 $\mu g \cdot L^{-1}$ was obtained when BHPVA-OVA was used as the coating antigen, further indicating that the phenolic and methyl groups of BPA act as the major epitopes. HHPBA-OVA was therefore selected for subsequent experiments.

3.4 ELISA optimization

The effects of various factors on icELISA (Table 2) were investigated using B_0 and IC_{50} as selection criteria. CB (0.05 $mol \cdot L^{-1}$, pH 9.6), 37°C and pH 7.4 were chosen for the coating buffer, the incubation temperature and the optimal pH of the assay buffer, respectively. The ionic strength had a strong effect on the immunoassay. Specifically, increasing the NaCl concentration from 0.1 to 0.4 $mol \cdot L^{-1}$ resulted in lower B_0 values. However, a higher NaCl concentration (0.4 $mol \cdot L^{-1}$) may affect antigen-antibody binding, hence, 0.1 $mol \cdot L^{-1}$ NaCl in PBS buffer was considered the optimal ionic strength.

Table 2 Effects of various factors on the sensitivity of the icELISA

Factor		B_0 (OD_{450})	IC_{50}/($ng \cdot mL^{-1}$)
Coating buffer	0.01 $mol \cdot L^{-1}$ PBS, pH 7.4	1.796	6.4
	0.05 $mol \cdot L^{-1}$ CB, pH 9.6	2.058	3.1
Temperature/°C	4	1.989	5.6
	25	2.012	5.4
	37	2.103	5.2
pH	6.0	1.584	3.2
	7.4	2.083	2.1
	8.0	2.307	2.7
NaCl concentration /($mol \cdot L^{-1}$)	0.1	1.980	2.2
	0.2	1.682	2.4
	0.4	1.489	2.9

3.5 Sensitivity and specificity of the immunoassay

Based on the optimized conditions, standard solutions were diluted in PBS at concentrations of 0, 0.33, 1, 3, 9, 27, 81 $\mu g \cdot L^{-1}$, and a standard curve for icELISA was determined (Fig. 2b), resulting in an IC_{50} value of 2.1 $\mu g \cdot L^{-1}$. IC_{50} values of other structurally related compounds and CR values when the cross reactivity with BPA was set as 100% are shown Table 3. The antibody displayed lower CR values than BPB and BPE, but differences in CR values for other related compounds were negligible ($< 0.2\%$), confirming the high specificity of the polyclonal antibody for BPA.

In addition, we summarized in Table 4 the haptens and

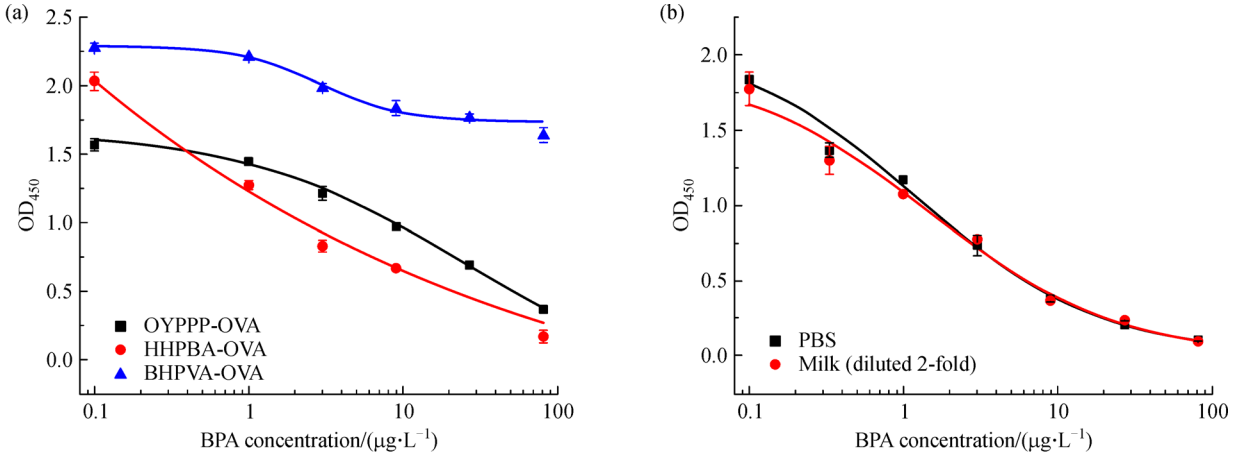

Fig. 2 Standard curves. (a) The comparison of homogeneous and heterogeneous icELISA; (b) the standard curve of BPA in PBS and milk. $n = 3$.

Table 3 The IC_{50} values and cross-reactivity (CR) of the polyclonal antibody

Compound	IC_{50}/(ng·mL^{-1})	CR/%
BPA	2.1	100
Bisphenol B	27.5	7.6
BHPVA	12.7	16.5
Bisphenol E	80.7	2.6
17β-estradiol	> 1000	< 0.2
Diethylstilbestrol	> 1000	< 0.1
Nonylphenol	> 1000	< 0.1

Table 4 Comparison of IC_{50} values reported in the literature and in this study

Antibody name	Hapten	IC_{50}/(μg·L^{-1})
Polyclonal antibody (this study)	BPA-AMA	2.1
Polyclonal antibody[29]	BPA-CME	1.25
Polyclonal antibody[16]	BPA-CME	5.4
Monoclonal antibody BBA-2187[20]	BPA-CPE	0.59
Monoclonal antibody[30]	BHPVA	2.2
Monoclonal antibody[31]	BHPVA	140
Polyclonal antibody[18]	BHPVA	200
Polyclonal antibody[1]	BHPVA	1100
Polyclonal antibody[13]	BPA-GA	1.2
Monoclonal antibody BPAB-11[15]	BPA-GA	230
Chicken immunoglobulins[12]	BPA-GA	570

IC_{50} values reported recently in the literature. It can be concluded that the icELISA we developed was more sensitive (expressed by IC_{50} values) than some in the literature[1,12,15,16,18,31], but similar to others in published literature[13,29,30]. We suggest that the new immunogens OYPPP-BSA and heterogeneous coating antigens HHPBA-OVA both contributed to enhance the sensitivity of the icELISA for the detection of BPA.

3.6 Assay performance in milk

To demonstrate the practicality of the icELISA method, milk samples were spiked with various concentrations of BPA, and the resulting LOD was 1.95 μg·L^{-1}. Recovery and coefficient of variation (CV) values are shown in Table 5. The mean recovery values ranged from 80% to 102%, with CV values below 15.8%. These results confirmed that the icELISA method developed is both suitable and convenient for detecting BPA in milk.

Table 5 Recoveries and coefficient of variation (CV) values for BPA in milk by the icELISA (n = 3)

Added/(ng·mL^{-1})	Observed/(ng·mL^{-1})	Recovery/%	CV/%
5	5.1 ± 0.75	102.0 ± 15.0	15.8
10	8.0 ± 0.12	80.0 ± 11.2	13.0
20	16.7 ± 0.19	83.5 ± 9.5	9.8

4 Conclusions

Three BPA immunizing haptens were used to produce polyclonal antibodies and all three immunogens were able to induce a significant immune response against BPA. Hapten OYPPP proved to produce the best antibody, but the sensitivity was improved significantly when HHBPA-OVA was used as the heterogeneous coating antigen. Using the optimized conditions, icELISA is a sensitive method for rapidly screening milk for BPA.

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Compliance with ethics guidelines Xiya Zhang, Xiaoyun Dong, Sijun Zhao, Kai Wen, Suxia Zhang, Zhanhui Wang, and Jianzhong Shen declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

References

1. Kim A, Li C R, Jin C F, Lee K W, Lee S H, Shon K J, Park N G, Kim D K, Kang S W, Shim Y B, Park J S. A sensitive and reliable quantification method for bisphenol A based on modified competitive ELISA method. *Chemosphere*, 2007, **68**(7): 1204–1209
2. Choi K C, Jeung E B. The biomarker and endocrine disruptors in mammals. *Journal of Reproduction and Development*, 2003, **49**(5): 337–345
3. Iso T, Watanabe T, Iwamoto T, Shimamoto A, Furuichi Y. DNA damage caused by bisphenol A and estradiol through estrogenic activity. *Biological & Pharmaceutical Bulletin*, 2006, **29**(2): 206–210
4. Diel P, Schulz T, Smolnikar K, Strunck E, Vollmer G, Michna H. Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity. *Journal of Steroid Biochemistry*, 2000, **73**(1–2): 1–10
5. Steinmetz R, Mitchner N A, Grant A, Allen D L, Bigsby R M, Ben-Jonathan N. The xenoestrogen bisphenol A induces growth, differentiation, and *c-fos* gene expression in the female reproductive tract. *Endocrinology*, 1998, **139**(6): 2741–2747
6. Markey C M, Rubin B S, Soto A M, Sonnenschein C. Endocrine disruptors: from Wingspread to environmental developmental biology. *Journal of Steroid Biochemistry*, 2002, **83**(1–5): 235–244
7. Rudel R A, Brody J G, Spengler J C, Vallarino J, Geno P W, Sun G, Yau A. Identification of selected hormonally active agents and animal mammary carcinogens in commercial and residential air and dust samples. *Journal of the Air & Waste Management Association*, 2001, **51**(4): 499–513

8. Behnisch P A, Fujii K, Shiozaki K, Kawakami I, Sakai S. Estrogenic and dioxin-like potency in each step of a controlled landfill leachate treatment plant in Japan. *Chemosphere*, 2001, **43**(4–7): 977–984
9. Fürhacker M, Scharf S, Weber H. Bisphenol A: emissions from point sources. *Chemosphere*, 2000, **41**(5): 751–756
10. Meesters R J W, Schroder H F. Simultaneous determination of 4-nonylphenol and bisphenol A in sewage sludge. *Analytical Chemistry*, 2002, **74**(14): 3566–3574
11. Inoue K, Kato K, Yoshimura Y, Makino T, Nakazawa H. Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection. *Journal of Chromatography. B, Biomedical Sciences and Applications*, 2000, **749**(1): 17–23
12. De Meulenaer B, Baert K, Lanckriet H, Van Hoed V, Huyghebaert A. Development of an enzyme-linked immunosorbent assay for bisphenol A using chicken immunoglobulins. *Journal of Agricultural and Food Chemistry*, 2002, **50**(19): 5273–5282
13. Lu Y, Peterson J R, Gooding J J, Lee N A. Development of sensitive direct and indirect enzyme-linked immunosorbent assays (ELISAs) for monitoring bisphenol A in canned foods and beverages. *Analytical and Bioanalytical Chemistry*, 2012, **403**(6): 1607–1618
14. Maiolini E, Ferri E, Pitasi A L, Montoya A, Di Giovanni M, Errani E, Girotti S. Bisphenol A determination in baby bottles by chemiluminescence enzyme-linked immunosorbent assay, lateral flow immunoassay and liquid chromatography tandem mass spectrometry. *Analyst*, 2014, **139**(1): 318–324
15. Moreno M J, D'Arienzo P, Manclus J J, Montoya A. Development of monoclonal antibody-based immunoassays for the analysis of bisphenol A in canned vegetables. *Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes*, 2011, **46**(6): 509–517
16. Ohkuma H, Abe K, Ito M, Kokado A, Kambegawa A, Maeda M. Development of a highly sensitive enzyme-linked immunosorbent assay for bisphenol A in serum. *Analyst*, 2002, **127**(1): 93–97
17. Wu X L, Wang L B, Ma W, Zhu Y Y, Xu L G, Kuang H, Xu C L. A simple, sensitive, rapid and specific detection method for bisphenol A based on fluorescence polarization immunoassay. *Immunological Investigations*, 2012, **41**(1): 38–50
18. Zhao M P, Li Y Z, Guo Z Q, Zhang X X, Chang W B. A new competitive enzyme-linked immunosorbent assay (ELISA) for determination of estrogenic bisphenols. *Talanta*, 2002, **57**(6): 1205–1210
19. Wang Z H, Liu M X, Shi W M, Li C L, Zhang S X, Shen J Z. New haptens and antibodies for ractopamine. *Food Chemistry*, 2015, **183**: 111–114
20. Nishi K, Takai M, Morimune K, Ohkawa H. Molecular and immunochemical characteristics of monoclonal and recombinant antibodies specific to bisphenol A. *Bioscience, Biotechnology, and Biochemistry*, 2003, **67**(6): 1358–1367
21. O'Keeffe M, Crabbe P, Salden M, Wichers J, Van Peteghem C, Kohen F, Pieraccini G, Moneti G. Preliminary evaluation of a lateral flow immunoassay device for screening urine samples for the presence of sulphamethazine. *Journal of Immunological Methods*, 2003, **278**(1–2): 117–126
22. Jiang W X, Luo P J, Wang X, Chen X, Zhao Y F, Shi W, Wu X P, Wu Y N, Shen J Z. Development of an enzyme-linked immunosorbent assay for the detection of nitrofurantoin metabolite, 1-amino-hydantoin, in animal tissues. *Food Control*, 2012, **23**(1): 20–25
23. Wang J J, Liu B H, Hsu Y T, Yu F Y. Sensitive competitive direct enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip for detecting aflatoxin M_1 in milk. *Food Control*, 2011, **22**(6): 964–969
24. Zhang X, Wen K, Wang Z, Jiang H, Beier R C, Shen J. An ultrasensitive monoclonal antibody-based fluorescent microsphere immunochromatographic test strip assay for detecting aflatoxin M_1 in milk. *Food Control*, 2016, **60**(2): 588–595
25. Goodrow M H, Hammock B D, Goodrow M H, Hammock B D. Hapten design for compound-selective antibodies: ELISAs for environmentally deleterious small molecules. *Analytica Chimica Acta*, 1998, **376**(1): 83–91
26. Goodrow M H, Sanborn J R, Stoutamire D W, Gee S J, Hammock B D. Strategies for immunoassay hapten design. *ACS Symposium Series*, 1995, **586**: 119–139
27. Lei H T, Shen Y D, Song L J, Yang J Y, Chevallier O P, Haughey S A, Wang H, Sun Y M, Elliott C T. Hapten synthesis and antibody production for the development of a melamine immunoassay. *Analytica Chimica Acta*, 2010, **665**(1): 84–90
28. Kim Y J, Cho Y A, Lee H S, Lee Y T, Gee S J, Hammock B D. Synthesis of haptens for immunoassay of organophosphorus pesticides and effect of heterology in hapten spacer arm length on immunoassay sensitivity. *Analytica Chimica Acta*, 2003, **475**(1–2): 85–96
29. Kaddar N, Bendridi N, Harthé C, de Ravel M R, Bienvenu A L, Cuilleron C Y, Mappus E, Pugeat M, Déchaud H. Development of a radioimmunoassay for the measurement of Bisphenol A in biological samples. *Analytica Chimica Acta*, 2009, **645**(1–2): 1–4
30. Yang F, Xu L, Zhu L, Zhang Y, Meng W, Liu R. Competitive immunoassay for analysis of bisphenol A in children's sera using a specific antibody. *Environmental Science and Pollution Research International*, 2016, **23**(11): 10714–10721
31. Huang P, Zhao S, Eremin S A, Zheng S, Lai D, Chen Y, Guo B. Fluorescence polarization immunoassay method for bisphenol A residue in environmental water samples based on monoclonal antibody and 4′-(aminomethyl) fluorescein. *Analytical Methods*, 2015, **7**(10): 4246–4251