Food Chemistry 207 (2016) 233–238

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the analysis of 6-benzylaminopurine and its ribose adduct in bean sprouts

Wei Zhang [a], Lishan He [a], Rui Zhang [a], Suqin Guo [a], Huanfang Yue [a], Xiangxue Ning [a], Guiyu Tan [a,*], Qing X. Li [b], Baomin Wang [a,*]

[a] College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China
[b] Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Honolulu, HI 96822, USA

ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form 27 March 2016
Accepted 28 March 2016
Available online 30 March 2016

Chemicals studied in this article:
6-Benzylaminopurine (PubChem CID: 62389)
6-Benzylaminopurine riboside (PubChem CID: 92208)
Benzyladenosine (PubChem CID: 250699)

Keywords:
6-Benzylaminopurine
Monoclonal antibody
ELISA
Food safety
Plant growth regulator

ABSTRACT

6-Benzylaminopurine (6-BA), a cytokinin plant growth regulator, has been banned for use in bean sprout production in China. An indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed with a specific monoclonal antibody (mAb 3E5). The assay showed a half-maximum inhibition concentration (IC_{50}) and detection range of 18.9 ng/mL and 3.6–106 ng/mL, respectively. Recoveries of 6-BA spiked in home cultured bean sprout samples averaged from 75% to 89% with a correlation coefficient (R^2) of 0.998 between the results determined by icELISA and those by liquid chromatography–electrospray ionization quadrupole Orbitrap mass spectrometry (LC–ESI-MS). LC–ESI-MS showed that 6-BA had been partially metabolized to 6-benzylaminopurine riboside (6-BAR) in the positive samples. The content of 6-BA determined by icELISA was about 5–70 times higher than that of LC–ESI-MS because mAb 3E5 had 315% cross-reactivity with 6-BAR. Such icELISA being ultra-sensitive to 6-BAR would allow quick monitoring of 6-BA by detecting 6-BAR as a potential biomarker.

1. Introduction

6-Benzylaminopurine (6-BA), also called benzyl adenine, was the first synthetic cytokinin plant growth regulator (Fig. 1). Owing to its high efficiency and low cost, 6-BA had been widely used in China. The physiological functions were similar to its natural counterparts. 6-BA can promote cell division, accelerate fruit growth, stimulate shoot formation and increase fruit-set and yield (Crosby, Aung, & Buss, 1981; Pan & Xu, 2011; Polanco, Pelaez, & Ruiz, 1988; Wismer, Proctor, & Elfving, 1995). 6-BA can also be converted into numerous metabolites in different plants tissues. Riboside, benzyladenosine, ribotide, benzyladenylic acid, urea and ureide were formed from 6-BA in the leaves of *Xanthium pensylvanicum* (cocklebur) (McCalla, Morre, & Osborne, 1962). Deleuze, McChesney, and Fox (1972) and Wilson, Gordon, Letham, and Parder (1974) had identified that 6-benzylamino-7-glucosylpurine, 6-benzylamino-7-glucofuranosylpurine and 6-benzylamino-9-glucosylpurine were the derivatives of 6-BA. 6-Benzylamino-3-β-D-glucopyranosylpurine, the compound with a glycosidic linkage at position 3 and β-(6-benzylaminopurin-9-yl)alanine, the amino acid conjugate at position 9 were isolated from plant tissues (Letham, Summons, Parker, & MacLeod, 1979; Letham et al., 1975). The 3,7,9-glucosides (N-glucosides), 9-riboside and 9-ribosylglucoside were characterized as metabolites of 6-BA (Blakesley, Lenton, & Horgan, 1991; Letham & Gollnow, 1985). The ribosyl-CTKs were mainly present in xylem and phloem of plants.

According to the United States Environmental Protection Agency (US EPA) report, 6-BA can cause eye irritation, damage to esophageal and gastric mucosa and stimulation to skin. The Korean Food and Drug Administration (KFDA) had set a maximum residue limit for 6-BA at 0.1 mg/kg in fruits (KFDA, 2009). In 2011, the Ministry of Health of People's Republic of China (GB2760-2011) had banned use of 6-BA in bean sprout production. However,

* Corresponding authors.
E-mail addresses: tanguiyu@cau.edu.cn (G. Tan), wbaomin@263.net (B. Wang).

http://dx.doi.org/10.1016/j.foodchem.2016.03.103
0308-8146/

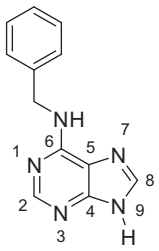


Fig. 1. Chemical structure of 6-BA.

because of its low cost and high efficiency, illegal uses of 6-BA still exist.

A number of methods were developed for the detection of 6-BA, including electrochemical technique (Sun & Zhang, 2006), overlapping of second order scattering and frequency double scattering spectra method and resonance Rayleigh scattering method (Qiao et al., 2014), liquid chromatography–mass spectrometry (LC–MS) and pyrolysis MS (Jin, Ren, & Chen, 2007; Kim & Liu, 2009). A polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was developed for the analysis of the 6-BA metabolite 6-benzylaminopurine riboside (6-BAR) (Constantinidou, Steele, Kozlowski, & Upper, 1978; Strnad, 1996). The sensitivity of high performance liquid chromatography (HPLC) with ultraviolet visible (UV–VIS) detection was not adequate for trace analysis of 6-BA. Electrochemical technique was very complicated when functionalized electrodes were fabricated. The instrumental analyses were expensive and required highly trained expertise. Therefore, a convenient and sensitive method is needed for the analysis of 6-BA residues in bean sprouts.

As a sensitive and high throughput analysis method, ELISA had become a major method to analyze specific chemicals in food, medical drugs and health care products as well as environmental matrices (Goryacheva, Saeger, Eremin, & Peteghem, 2007; Kuhn et al., 2012; Watanabe, Miyake, & Yogo, 2013). An indirect competitive enzyme-linked immunosorbent assay (icELISA) is an immobilized antigen immunoassay, which is based on competition between the immobilized antigen and an unknown amount of analyte for a small fixed amount of antibody. The antibody bound to the immobilized antigen is quantitated by the activity of an enzyme-labeled second antibody. The color intensity developed from the substrate is inversely proportional to the amount of analyte being investigated (Zhao, Li, Yi, et al., 2006). In this study, a sensitive and effective icELISA based on mAbs had been developed to quantitatively determine 6-BA residues in food.

2. Materials and methods

2.1. Reagents and apparatus

Reagents purchased from Sigma-Aldrich (St. Louis, MO) included 6-BA, 6-BAR, goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP), potassium periodate, o-phenylenediamine, citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$).

The 4 °C, −20 °C and −40 °C biomedical freezers were purchased from Sanyo (Osaka, Japan). Cell microscope was purchased from Olympus (CKX41, Tokyo, Japan). The centrifugal machine (3H16RI) was purchased from Hunan Haosi Apparatus Co. Ltd. (Hunan, China). The myeloma and hybridoma cell were incubated in the direct heat CO_2 incubator (311, Thermo, Franklin, MA, USA).

2.2. Experimental animals, myeloma cell line and cell culture mediums

Balb/c mice were obtained from the Laboratory Animal Center of the Institute of Genetics (Beijing, China). The animal husbandry conditions and animal treatment procedures were performed strictly according to the standards of the Animal Care Committee of China Agricultural University (CAU). SP2/0-Ag14 (HAT-sensitive mouse myeloma cell line) was provided by College of Veterinary Medicine, CAU. The myeloma and hybridoma cell complete culture medium were made from L-glutamine (0.2 M), penicillin (50,000 U/L), streptomycin (50 mg/L), cell culture medium (Dulbecco's modified Eagle's medium, DMEM) containing 10–20% (v/v) Fetal bovine serum (FBS) (Gibco BRL, PaisLey, Scotland). HAT selection medium was complete culture medium with hypoxanthine, aminopterin and thymidine (HAT) medium supplements (100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine).

2.3. Preparation of complete antigen

The immunogen and coating antigen had been synthesized via a periodate oxidation method (Eberle, Arnscheidt, Klix, & Weiler, 1986). Two milligram of 6-BAR were dissolved in 2 mL of methanol, followed by dropwise addition of 1 mL 0.01 M $NaIO_4$. After the reaction mixture was stirred for 20 min at room temperature, 180 μL 0.1 M ethylene glycol was added, and stirred for another 10 min at ambient temperature. The mixture was added dropwise to 2 mL protein solution (10 mg of BSA or 8 mg of OVA). The reaction mixture was adjusted to pH 9.3 with 5% K_2CO_3 and was stirred for another hour. Two aliquots of 1 mg of $NaBH_4$ were added in a 30 min interval and the solution was stirred at 4 °C. After the pH was adjusted to 6.5 with 0.1 N HCl, the solution was stirred for another hour. The mixtures were dialyzed against 2.5 L of PBS (1.5 mM KH_2PO_4, 8.3 mM $Na_2HPO_4{\cdot}12H_2O$ and 154 mM NaCl, pH 7.5) with a change per 5 h for 3 days at 4 °C. The dialyzed conjugates were stored at −20 °C. The hapten to BSA/OVA molar ratios were estimated with UV spectroscopy. The conjugate of hapten-BSA and hapten-OVA was used as coating antigen and immunogen, respectively.

2.4. Monoclonal antibody production, purification and characterization

The animal experimental procedure was approved by the Beijing Experimental Animal Management Office. The protocols of monoclonal antibody production, purification and characterization were previously described (Zhao, Li, Wang, et al., 2006). The immunogen (6-BAR-OVA) was emulsified with complete or incomplete Freund's adjuvant before injection into six female Balb/c mice (6 weeks old), and subsequently three boosts were performed. Four days after the third injection, mouse antisera collected from the retrobulbar plexus were tested for anti-6-BA antibody titer and for 6-BA recognition properties by indirect ELISA and icELISA, respectively. Four days after the fourth injection, the best-performing mouse was boosted intraperitoneally with 0.1 mL immunogen (6-BAR-OVA conjugate in PBS, 1 mg/mL) and was used for hybridoma production. The spleen cells harvested from the mouse were fused with SP2/0-Ag14 cell line using PEG-2000 at a ratio of 10:1 of spleen to myeloma cells. The hybridoma cells were developed in 96-well plates (Corning, NY, USA). The plates were incubated at 37 °C and 5% CO_2 in an incubator. About one week after fusion, the supernatant was screened by indirect ELISA. Positive hybridoma cells were cloned by limiting dilution. The resulting clones were further selected by icELISA.

The clone (mAb 3E5) performing better with antibody titer and sensitivity than other clones was expanded in mice treated with mineral oil for production of ascites. The antibody was purified by ammonium sulfate precipitation. The class and subclass of the isotypes of mAb 3E5 were determined using a mouse antibody isotyping kit (Pierce, Rockford, IL, USA). The specificity of mAb 3E5

was evaluated by cross-reactivity with 6-BA and its analogues in icELISA (He et al., 2009).

2.5. Indirect competitive ELISA

The protocol for icELISA was previously described (Zhao, Li, Wang, et al., 2006). The microtiter plate (96-well) (Corning, NY, USA) was first coated with 100 μL 6-BAR-BSA conjugate, which was diluted in coating buffer (14.2 mM Na_2CO_3 and 35.8 mM $NaHCO_3$, pH 9.6) at a ratio of 1:500. The plate was then left in an electric heating constant-temperature incubator (ZDP-A2270A, Zhicheng, Shanghai, China) at 37 °C for 3 h. The coating solution was poured away. After being washed by the automatic 96-wellplate washer (Wellwash 4 MK2, Thermo, Finland) with 250 μL PBST (Tween-20 diluted in PBS, 0.1% (v/v)) four times, 50 μL of standard sample or analytes was added to each well, followed by 50 μL of mAb. After incubation at 37 °C for 0.5 h, the contents were poured away and the plate was washed as before. And then, 100 μL per well of goat anti-mouse IgG-HRP was added, which was diluted in PBSTG (gelatin diluted in PBST, 0.1% (w/v)) at a ratio of 1:1000. After incubation at 37 °C for 0.5 h, the plate was emptied and washed as before, followed by addition of 100 μL of fresh substrate solution (24.3 mM $C_6H_8O_7{\cdot}H_2O$, 51.5 mM $Na_2HPO_4{\cdot}12H_2O$, 18.5 mM o-phenylenediamine, 0.1% Tween-20 (v/v) and 400 μL/L of 30% H_2O_2, pH 5.5) to each well for color development. Finally, 15 min later, 50 μL per well of the stop solution (2.0 M sulfuric acid) was added, and the absorbance was read with the microplate reader (Multiskan FC, Thermo, Finland) at 492 nm. Data were calculated with OriginPro8.5 (OriginLab) software.

2.6. Preparation, extraction and analysis of samples

One negative control group and two positive control groups were treated as follows. Each group of mung bean (*Vigna radiata* (L.) Wilczek) seeds (8.0 g) was carried out for different treatment in triplicate. Those seeds were immersed in a 10% solution of H_2O_2 for surface sterilization for 15 min, and rinsed six times with sterile deionized water (Miche & Balandreau, 2001). Subsequently, seeds were immersed in solutions containing three concentration levels of 6-BA (0 μg/mL, 5 μg/mL, 10 μg/mL), respectively, for 6 h at 23 °C, and were allowed to germinate in 120 mm × 120 mm × 50 mm germination boxes. The bottom of the boxes with many small holes lined with a triple layers of gauze. The germination boxes were placed in a dark growth chamber, where temperature was maintained at 23 ± 1 °C and humidity was 80 ± 5%. The bean sprouts were showered with tap water every 6 h and the pots were randomly moved every day to minimize positional effects. After 5 days, bean sprouts were sampled and stored in a −40 °C refrigerator.

6-BA was extracted as previously described (Jin et al., 2007). The extraction steps were as follows: 4.0 g of bean sprout sample were manually ground with a mortar and pestle. Subsequently, the tissue homogenates were transferred into a 50 mL centrifuge tube, followed by addition of 15.0 mL of extracting solution (methanol with 0.1% acetic acid). The centrifuge tubes were partially immersed into the KH-500E ultrasonic cleaning bath (Kunshan Hechuang, Jiangsu, China). The samples were extracted in 200 W for 10 min. The extract was centrifuged for 15 min at 6000*g*. The supernatant was collected into a 50 mL volumetric flask. The ultrasound extraction and centrifugation were repeated once again. The supernatants were combined and the volume was adjusted with acidified methanol.

Five milliliter of extracts taken from the 50 mL volumetric flask was dried under a gentle stream of nitrogen gas at 40 °C. The residues were dissolved in 0.4 mL of PBS through vigorous vortexing for 5 min. The solution was diluted 10-fold in PBS, and then was used for icELISA analysis. One milliliter of extracts taken from the 50 mL volumetric flask was filtered with 0.22-μm membrane prior to the LC–electrospray ionization (ESI)-MS analysis.

2.7. Sample fortification for recovery tests

6-BA was fortified at varying concentrations in 6-BA-free bean sprout samples (4.0 g) for recovery studies. The fortification levels were 0, 10, 20, 40, 100, 400 ng/g, respectively. The sample preparation, extraction and analysis procedures were the same as above.

2.8. LC–ESI-MS analysis of 6-BA

The Waters Acquity UPLC H-class biosystem (Waters, MA, USA) interfaced to a Thermo Scientific Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher, Franklin, MA, USA) was used to analyze 6-BA in extracts. The supernatants of extracts taken from the 50 mL volumetric flask were filtered with 0.22-μm membrane and then were detected by LC–MS. Separation was carried out with a 5 μL injection loop by a Poroshell 120 EC-C18 column (75 mm × 3.0 mm i.d., 2.7 μm) (Agilent, USA). The mobile phase was aqueous methanol containing 0.1% (v/v) formic acid at a flow rate of 300 μL/min. The methanol content in mobile phase was gradually increased from 40% to 80% in 5 min, and then decreased to 40% in 12 s. Until the next sample injection, methanol percentage at 40% was remained for 3.8 min. The retention time of 6-BA was at 3.6 min.

3. Results and discussion

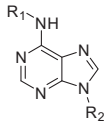
3.1. Screening of antisera and production of mAbs

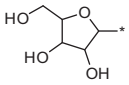
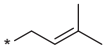
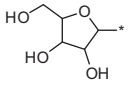
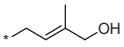
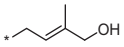
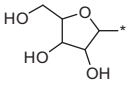
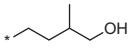
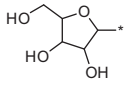
The antigen was successfully synthesized for production of highly selective antibodies. The strategy to link 6-BAR with a carrier protein was via a periodate oxidation method. The hapten loads were estimated to be 12:1 and 8:1 for 6-BAR-BSA and 6-BAR-OVA in molar ratios, respectively. After the fourth injection, the spleen cells coming from the mouse with the highest titer of approximately 5.7×10^4 and myeloma cells were fused to develop monoclonal antibody. All hybridomas cells on 96-well microtiter plates were screened with icELISA. Among them, three wells were positive and cloned three times by limiting dilution. Two clones (designated as 3E5 and 2D6) were obtained. The mAbs were tested in icELISA and the IC_{50} value of mAb 3E5 was remarkably lower than 2D6 (data not shown). Therefore, mAb 3E5 with higher sensitivity was chosen to develop an icELISA for the analysis of 6-BA.

3.2. Characteristics of mAb 3E5

The mAb 3E5 was IgG1 with κ light chains. The titer (the dilution ratio of serum that gave an absorbance of 1.0 in the non-competitive assay conditions) of the ascites was about 8×10^3. The specificity of mAb 3E5 was evaluated by cross-reactivity with 6-BA and its analogues in icELISA. Table 1 shows the cross-reactivity with 7 purine analogues. Although natural cytokinins and its synthetic analogues shared the same purine ring, the cross-reactivity of mAb 3E5 with 6-BAR, kinetin (KT), isopentenyladenine (iP) and isopentenyladenine riboside (iPR) was approximately 315%, 5.6%, 1.2% and 3.4%, respectively. No competitive inhibition was observed up to 10,000 ng/mL of *trans*-zeatin (Z), *trans*-zeatin riboside (ZR) and dihydrozeatin riboside (DHZR). The cross-reactivity result suggested that mAb 3E5 was highly specific to 6-BA and 6-BAR.

Table 1
Relative cross-reactivity of 6-BA and related analytes.



Analytes	Structure		IC_{50} (ng/mL)	Cross-reactivity[a] (%)
	R_1	R_2		
6-BA		*—H	18.9 ± 2.3[b]	100 ± 0.1
6-BAR			6.0 ± 0.4	315 ± 6.6
KT		*—H	337 ± 11	5.6 ± 0.4
iP		*—H	1536 ± 40	1.2 ± 0.1
iPR			552 ± 24	3.4 ± 0.1
tZ		*—H	NI[c]	0
tZR			NI	0
DHZR			NI	0

*Binding site.
[a] Relative cross-reactivity (%) = (IC_{50} of 6-BA/IC_{50} of other compound) × 100.
[b] Data represented means of triplicate samples ± standard deviations.
[c] No inhibition was observed up to 10,000 ng/mL of the analytes.

3.3. Optimization of icELISA

To obtain an optimal combination of concentrations of coating antigen and mAb, a two-dimensional checkerboard titration was performed in icELISA. Under the optimum working condition, the dilution ratios of coating antigen (0.5 mg/mL) and mAb 3E5 (1.0 mg/mL) were at 1:500 and 1:4000, respectively. The standard sigmoidal inhibition curves for 6-BA (R^2, 0.997) and 6-BAR (R^2, 0.998) were generated by icELISA using the optimum reagent dilutions (Fig. 2). The IC_{50} values of 6-BA and 6-BAR were 18.9 and 6.0 ng/mL, respectively, and the detection range of 6-BA (based on 20–80% of inhibition) was 3.6–106 ng/mL.

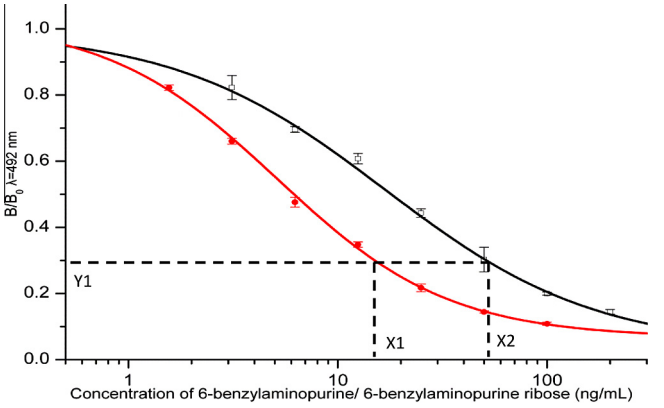


Fig. 2. Standard inhibition curves for 6-benzylaminopurine (□) and 6-benzylaminopurine riboside (●) in icELISA under optimized conditions. B_0 and B were optical density (OD) in the absence and presence of 6-BA and 6-BAR, respectively. Each value represented the mean of three replicates. The method to convert content of 6-BAR into equivalent content of 6-BA was as follows: finding Y1 (an OD value) from X1 (a content of 6-BAR) in the standard inhibition curve of 6-BAR, finding X2 from Y1 in the standard inhibition curve of 6-BA. X2 was the equivalent content of 6-BA, which was converted from 6-BAR.

3.4. Analyses of 6-BA in spiked bean sprout samples

Table 2 shows the average recoveries of 6-BA from bean sprout samples. The matrix interference was almost inevitable for any instrument detection methods (Matuszewski, Constanzer, & Chavez-Eng, 2003), as well as ELISA analysis (Cairoli, Arnoldi, & Pagani, 1996). Elaborated sample pretreatment and proper dilution had become common methods to minimize matrix interference. The sample solution was centrifuged at 6000*g* for 10 min twice to remove the solid particles and then diluted 12.5-fold (4.0 g–50 mL). This procedure would effectively decrease the matrix effect on icELISA analysis. The average recoveries of icELISA were ranged from 76% to 99%, and the average recoveries of LC–MS were ranged from 104% to 109%. The correlation coefficient (R^2) between icELISA and LC–MS results was 0.998. The result indicated that negligible cross-reactivity of the matrices with mAb 3E5, and showed that the assay was sufficiently sensitive for monitoring 6-BA residues in practical samples.

Table 2
Average recoveries of 6-benzylamonipurine spiked in bean sprout samples.

Spiked concentrations (ng/g)	Concentrations detected (ng/g)		Mean recoveries (%, n = 3)	
	icELISA[a]	LC–MS[a]	icELISA	LC–MS
0	0 ± 0	0 ± 0	0	0
10	8.2 ± 1.3	10.7 ± 0.3	82.0 ± 13.0	107.0 ± 3.0
20	17.3 ± 0.8	21.1 ± 1.9	86.5 ± 4.0	105.5 ± 9.5
40	34.5 ± 3.2	42.7 ± 2.5	86.3 ± 8.0	106.6 ± 6.2
100	75.7 ± 5.2	109.1 ± 2.2	75.7 ± 5.2	109.1 ± 2.2
400	355.7 ± 3.5	416.5 ± 4.6	88.9 ± 0.9	104.1 ± 1.2

[a] Data were the means of triplicate samples ± standard deviations.

3.5. Analyses of 6-BA residues in different bean sprout samples

This new icELISA was compared with LC–MS for the analysis of 6-BA in negative control bean sprout samples (chamber cultivated without 6-BA), positive control samples (chamber cultivated with 6-BA), doubted positive samples (practical samples provided by Hebei Province Department of Police) and practical samples (purchased from local markets). The comparative results showed that 6-BA residues were present in the positive samples and some of the doubted positive samples, but were absent in the samples from local markets. Most of the bean sprouts purchased from the local markets in Beijing were from intensive farming under strict supervision. The negative results of the 10 practical samples were reasonable. The LC–MS analysis showed that 6-BA was partially metabolized to 6-BAR in the bean sprouts (Table 3). The cross reactivity of mAb 3E5 with 6-BAR was approximately 315%. To compare the apparent concentrations of 6-BA determined by icELISA with those by LC–MS, the high cross reactivity of 6-BAR with the icELISA would need to be calibrated. The 6-BA equivalent in icELISA would be the 6-BA content plus 6-BA equivalent converted from the concentrations of 6-BAR determined by LC–MS (Table 3). The 6-BA equivalents by icELISA ranged approximately from 30% to 78% of the apparent values as measured by icELISA using 6-BA as the standard. Such disagreement was probably due to cross-reactivity of the icELISA with other 6-BA metabolites. Metabolites being possibly highly recognized by mAb 3E5 can result in the high icELISA values. In addition, the disagreement can be plausibly explained by no interference by icELISA as confirmed by comparison between icELISA and LC–MS in the spiked samples (R^2, 0.998) (Table 2).

The antibodies of cytokinins were usually not specific to N^3- and N^9-substituted cytokinin analogues and have cross-reactivity with their metabolites (Strnad, Veres, Hanus, & Siglerova, 1992; Todor, Ivailo, & Iordanka, 1996). For example, the polyclonal antibodies recognized 6-BAR, 6-BA, 6-BA-3-glucoside, 6-BA-9-glucoside, 6-BAR-5′-monophosphate, N^6-(meta-hydroxybenzyl)adenosine, N^6-(meta-hydroxybenzyl)adenine, N^6-(meta-hydroxybenzyl)adenine 9-glucoside with cross-reactivity of 100%, 72.2%, 23.3%, 79.5%, 86%, 6.8%, 2.8%, and 4.7%, respectively (Strnad, 1996). MAb 3E5 also likely lacks specificity for N^3- and N^9-substituted 6-BA analogues. Conjugated CTKs-sugar was the main form in storage and inactivation (Normanly, Slovin, & Cohen, 2010). It can be reasonably hypothesized that mAb 3E5, evoked by 6-BAR-OVA, can highly recognize conjugates of 6-BA, as demonstrated by 6-BAR. The icELISA results suggest that 6-BA metabolites, in addition to 6-BAR, were formed in the bean sprouts.

6-BA is quite sensitive to be metabolized. The content of 6-BA residues in the bean sprouts as determined by LC–MS was very low. In bean sprouts, 6-BA can be metabolized to 6-BAR and other metabolites. Since mAb 3E5 had high cross-reactivity with 6-BAR, the icELISA could sensitively detect 6-BAR and other metabolites. This bioassay, therefore, would offer a great advantage for detecting trace level of 6-BA in bean sprouts.

Food safety has been a serious issue in China. Pesticide residues, plant growth regulator residues and food additives must be lower than the maximum residue limit (MRL) of China. Although there was no report about the toxicity of 6-BA derivatives and metabolites, China Food and Drug Administration had reiterated that 6-BA had been prohibited to use in bean sprout in 2015's 11th announcement. The advantages of high throughput of icELISA make it possible for rapid screening of a large number of samples. The suspicious samples by icELISA monitoring would then be subjected to a confirmatory analysis of 6-BA and its metabolite 6-BAR together by LC–MS methods. The above results suggested that the icELISA based on mAb 3E5 against 6-BA be a quick and easy method to monitor 6-BA residues in bean sprouts. In addition, the monoclonal antibody is very sensitive to 6-BAR that is potentially a biomarker indicating 6-BA. This bioassay can be therefore used to detect 6-BAR for biomonitoring of 6-BA in bean sprouts.

Table 3
Comparison of icELISA and LC–MS analysis of 6-BA in bean sprout samples.

Sample No.	Conc. determined by icELISA[a] using 6-BA as the standard (ng/g)	Conc. by LC–MS (ng/g)		Conc. equiv.[c] of 6-BA in icELISA calculated from 6-BA and 6-BAR conc.[d] determined by LC–MS (ng/g)	Percent[e] of 6-BA conc. equiv. as those determined by icELISA (%)
		6-BA[b]	6-BAR[b]		
1	0	0	0	–	–
2	229.0 ± 12.5	3.2 ± 0.1	24.1 ± 1.8	93.7	40.9
3	44.6 ± 6.4	1.7 ± 0.2	5.3 ± 0.8	17.6	39.5
4	119.0 ± 13.7	7.5 ± 0.1	11.5 ± 0.5	48.0	40.3
5	52.2 ± 3.5	0.8 ± 0.0	6.3 ± 0.7	20.5	39.3
6	102.0 ± 10.8	6.2 ± 0.1	9.5 ± 0.8	38.7	37.9
7	149.9 ± 7.9	32.5 ± 0.2	10.4 ± 1.1	68.3	45.6
8	310.6 ± 24.1	52.5 ± 0.2	11.6 ± 1.3	93.4	30.1
9	201.9 ± 17.5	40.8 ± 0.2	31.5 ± 1.7	158.4	78.5
10–19	0	0	0	–	–

Sample 1: Bean sprout sample were cultivated in a chamber without 6-BA.
Sample 2–6: Bean sprout samples were provided by Hebei Province Department of Police.
Sample 7–9: Bean sprout samples applied with 6-BA were cultivated in lab.
Sample 10–19: Bean sprout samples were purchased from local markets.
[a] The samples were detected by icELISA under optimized conditions.
[b] Data was the means of triplicate samples ± SD.
[c] The theoretical content of icELISA was 6-BA content plus equivalent 6-BA content of 6-BAR.
[d] The residual content of 6-BAR had been converted into the equivalent content of 6-BA in Fig. 2.
[e] Proportion (%) = (theoretical content of icELISA/experimental content of icELISA) × 100.

4. Conclusions

To our knowledge, this is the first paper on mAb-based icELISA for 6-BA in bean sprouts. The icELISA had an IC_{50} value of 18.9 ng/mL of 6-BA and a working range of 3.6–106 ng/mL and the mAb 3E5 had low cross-reactivity with natural cytokinin analogues. The fortification tests of 6-BA in blank bean sprout samples by icELISA had average recoveries of 76–99%, and the average recoveries determined by LC–MS were from 104% to 109%. The results by icELISA agreed well with those by LC–MS. Overall, some 6-BA had converted into 6-BAR in bean sprouts; the icELISA were very sensitive to 6-BAR and possibly or other metabolites of 6-BA. The results indicated that this bioassay based on mAb 3E5 is sufficient for high throughput monitoring of residual 6-BA and 6-BAR in bean sprouts.

Acknowledgment

This work was supported in part by the US National Institute of Occupational Safety and Health grant 2U54OH007550.

References

Blakesley, D., Lenton, J. R., & Horgan, R. (1991). Uptake and metabolism of 6-benzylaminopurine in shoot cultures of *Gerbera jamesonii*. *Physiologia Plantarum, 81*, 343–348.

Cairoli, S., Arnoldi, A., & Pagani, S. (1996). Enzyme-linked immunosorbent assay for the quantitation of the fungicide tetraconazole in fruits and fruit juices. *Journal of Agricultural and Food Chemistry, 44*, 3849–3854.

Constantinidou, H. A., Steele, J. A., Kozlowski, T. T., & Upper, C. D. (1978). Binding specificity and possible analytical applications of the cytokinin-binding antibody, anti-N^6-benzyladenosine. *Plant Physiology, 62*, 968–974.

Crosby, K. E., Aung, L. H., & Buss, G. R. (1981). Influence of 6-benzylaminopurine on fruit-set and seed development in two soybean, *Glycine max* (L.) Merr. genotypes. *Plant Physiology, 68*, 985–988.

Deleuze, G. G., McChesney, J. D., & Fox, J. E. (1972). Identification of a stable cytokinin metabolite. *Biochemical and Biophysical Research Communications, 48*, 1426–1432.

Eberle, J., Arnscheidt, A., Klix, D., & Weiler, E. W. (1986). Monoclonal antibodies to plant growth regulators. III. Zeatinriboside and dihydrozeatinriboside. *Plant Physiology, 81*, 516–521.

Goryacheva, I. Y., Saeger, S. D., Eremin, S. A., & Peteghem, C. V. (2007). Immunochemical methods for rapid mycotoxin detection: Evolution from single to multiple analyte screening: A review. *Food Additives and Contaminants, 24*, 1169–1183.

He, S., Tan, G., Li, G., Tan, W., Nan, T., Wang, B., ... Li, Q. (2009). Development of a sensitive monoclonal antibody-based enzyme-linked immunosorbent assay for the antimalaria active ingredient artemisinin in the Chinese herb *Artemisia annua* L. *Analytical and Bioanalytical Chemistry, 393*, 1297–1303.

Jin, M., Ren, Y., & Chen, X. (2007). Determination of 6-benzyladenine in bean sprout by LC–ESI-IT-MS–MS. *Chromatographia, 66*, 407–410.

Kim, S. W., & Liu, J. R. (2009). A rapid, simple method for the determination of 6-benzyladenine by pyrolysis mass spectrometry. *Journal of Plant Biology, 52*, 625–629.

Korea Food and Drug Administration (2009). *MRLs for pesticides in foods*. Seoul, Korea.

Kuhn, K. G., Falkenhorst, G., Ceper, T., Dalby, T., Ethelberg, S., et al. (2012). Detection of antibodies to *Campylobacter* in humans using enzyme-linked immunosorbent assays: A review of the literature. *Diagnostic Microbiology and Infectious Disease, 74*, 113–118.

Letham, D. S., & Gollnow, B. I. (1985). Regulators of cell division in plant tissues. XXX. Cytokinin metabolism in relation to radish cotyledon expansion and senescence. *Journal of Plant Growth Regulation, 4*, 129–145.

Letham, D. S., Summons, R. E., Parker, C. W., & MacLeod, J. K. (1979). Regulators of cell division in plant tissues. XXVII. Identification of an amino-acid conjugate of 6-benzylaminopurine formed in *Phaseolus vulgaris* seedlings. *Planta, 146*, 71–74.

Letham, D. S., Wilson, M. M., Parker, C. W., Jenkins, I. D., MacLeod, J. K., & Sunnons, R. E. (1975). Regulators of cell division in plant tissues. XXIII. The identity of an unusual metabolite of 6-benzylaminopurine. *Biochimica et Biophysica Acta, 399*, 61–70.

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Analytical Chemistry, 75*, 3019–3030.

McCalla, D. R., Morre, D. J., & Osborne, D. J. (1962). The metabolism of a kinin, benzyladenine. *Biochimica et Biophysica Acta, 55*, 512–522.

Miche, L., & Balandreau, J. (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Applied and Environmental Microbiology, 67*, 3046–3052.

Ministry of Health of People's Republic of China (2011). *Standards for uses of food additives*. Beijing, China.

Normanly, J., Slovin, J. P., & Cohen, J. D. (2010). Auxin biosynthesis and metabolism. In P. J. Davies (Ed.), *Plant hormones: Biosynthesis, signal transduction, action* (3rd ed., pp. 36–62). The Netherlands: Kluwer Academic Publishers.

Pan, B., & Xu, Z. (2011). Benzyladenine treatment significantly increases the seed yield of the biofuel plant *Jatropha curcas*. *Journal of Plant Growth Regulation, 30*, 166–174.

Polanco, M. C., Pelaez, M. I., & Ruiz, M. L. (1988). Factors affecting callus and shoot formation from in vitro cultures of *Lens culinaris* Medik. *Plant Cell Tissue and Organ Culture, 15*, 175–182.

Qiao, M., Liu, S., Liu, Z., Yang, J., Zhu, J., & Hu, X. (2014). Overlapping of second order scattering and frequency double scattering spectra method and resonance Rayleigh scattering method for the determination of 6-benzyladenine in bean sprout. *Food Analytical Methods, 7*, 1737–1744.

Strnad, M. (1996). Enzyme immunoassays of N^6-benzyladenine and N^6-(meta-hydroxybenzyl)adenine cytokinins. *Journal of Plant Growth Regulation, 15*, 179–188.

Strnad, M., Veres, K., Hanus, J., & Siglerova, V. (1992). Immunological methods for quantification and identification of cytokinins. In M. Knaminek, D. W. S. Mok, & E. Zazimalova (Eds.), *Physiology and biochemistry of cytokinins in plants* (pp. 437–446). The Hague: SPB Academic Publishing.

Sun, D., & Zhang, H. (2006). Voltammetric determination of 6-benzylaminopurine (6-BAP) using an acetylene black-dihexadecyl hydrogen phosphate composite film coated glassy carbon electrode. *Analytica Chimica Acta, 557*, 64–69.

Todor, G., Ivailo, I., & Iordanka, I. (1996). Analysis of cytokinins by immunoassay and high performance liquid chromatography of in vitro cultivated dianthus caryophyllus. *Bulgarian Journal of Plant Physiology, 22*, 95–104.

United States Environmental Protection Agency (2009). *The US EPA pesticide toxicity database*. <http://www3.epa.gov/pesticides/chem_search/ppls/072639-00003-20091019.pdf>.

Watanabe, E., Miyake, S., & Yogo, Y. (2013). Review of enzyme-linked immunosorbent assays (ELISAs) for analyses of neonicotinoid insecticides in agro-environments. *Journal of Agricultural and Food Chemistry, 61*, 12459–12472.

Wilson, M. M., Gordon, M. E., Letham, D. S., & Parder, C. W. (1974). Regulators of cell division in plant tissues. XIX. The metabolism of 6-benzylaminopurine in radish cotyledons and seedlings. *Journal of Experimental Botany, 25*, 725–732.

Wismer, P. T., Proctor, J. T. A., & Elfving, D. C. (1995). Benzyladenine affects cell division and cell size during apple fruit thinning. *Journal of American Society for Horticultural Science, 120*, 802–807.

Zhao, J., Li, G., Yi, G., Wang, B., Deng, A., Nan, T., ... Li, Q. (2006). Comparison between conventional indirect competitive enzyme-linked immunosorbent assay (icELISA) and simplified icELISA for small molecules. *Analytica Chimica Acta, 571*, 79–85.

Zhao, J., Li, G., Wang, B., Liu, W., Nan, T., Zhai, Z., ... Li, Q. (2006). Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the analysis of glycyrrhizic acid. *Analytical and Bioanalytical Chemistry, 386*, 1735–1740.