Expression and characterization of a codon-optimized butyrylcholinesterase for analysis of organophosphate insecticide residues

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Abstract
Organophosphate insecticide residues on vegetable, fruit, tea and even grains are primary cause of food poisoning. Organophosphate compounds can cause irreversible inhibition of the activity of acetylcholinesterase and butyrylcholinesterase (BChE, EC 3.1.1.8), which are both candidates for rapid detection of organophosphate pesticides. To develop an easy-to-handle method for detecting organophosphate pesticides using BChE, BChE from human was optimized according to the codon usage bias of Pichia pastoris and successfully expressed in P. pastoris GS115. The codon-optimized cDNA shared 37.3% of the codon identity with the native one. However, the amino acid sequence was identical to that of the native human butyrylcholinesterase gene (hBChE) as published. The ratio of guanine and cytosine in four kinds of bases ((G+C) ratio) was simultaneously increased from 40 to 47%. The recombinant hBChE expression reached a total protein concentration of 292 mg mL⁻¹ with an activity of 14.7 U mL⁻¹, which was purified 3.2×10³-fold via nickel affinity chromatography with a yield of 68% and a specific activity of 8.1 U mg⁻¹. Recombinant hBChE was optimally active at pH 7.4 and 50°C and exhibited high activity at a wide pH range (>60% activity at pH 4.0 to 8.0). Moreover, it had a good adaptability to high temperature (>60% activity at both 50 and 60°C up to 60 min) and good stability at 70°C. The enzyme can be activated by Li⁺, Co⁺, Zn²⁺ and ethylene diamine tetraacetic acid (EDTA), but inhibited by Mg²⁺, Mn²⁺, Fe²⁺, Ag⁺ and Ca²⁺. Na⁺ had little effect on its activity. The values of hBChE of the Michaelis constant (Kₘ) and maximum reaction velocity (Vₘₕ) were 89.4 mmol L⁻¹ and 1 721 mmol min⁻¹ mg⁻¹, respectively. The bimolecular rate constants (K) of the hBChE to four pesticides were similar with that of electric eel AChE (EeAChE) and higher than that of horse BChE (HoBChE). All values of the half maximal inhibitory concentration of a substance (IC₅₀) for hBChE were lower than those for HoBChE, but most IC₅₀ for hBChE were lower than those for EeAChE except dichlorvos. The applicability of the hBChE was further verified by successful detection of organophosphate insecticide residues in six kinds of vegetable samples. Thus, hBChE heterologously over-expressed by P. pastoris would provide a sufficient material for development of a rapid detection method of organophosphate on spot and produce the organophosphate detection kit.

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1. Introduction

One of the most concerns in our daily life is food safety. Foods and food materials are vulnerable to be contaminated by foodborne pathogenic microorganism, illegal food additive, heavy metals and pesticides. Organophosphate compounds are broad spectrum insecticides used for crop protection to control agricultural pests. The primary toxicity of these compounds results in irreversible inhibition of nervous system enzyme, for example, cholinesterase (CHE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). CHE serves as a key enzyme mainly distributed in the central and peripheral nervous systems, plasma, liver and kidney, where it regulates the level of choline as well as transmits nerve impulse by catalyzing the hydrolysis of choline (Massoulie et al. 1993). In terms of different substrates, cholinesterase is classified in acetylcholinesterase (ACHE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). ACHE functions in cholinergic nerve catalyzing the hydrolysis of acetylcholine, whereas BChE is considered as a toxic scavenger and substitute for ACHE (Li et al. 2000; Mesulam et al. 2002).

BChE, which is also called pseudocholinesterase, belongs to the serine esterase family. It is mainly distributed in serum and liver, and even little in muscle and brain (Taylor and Radic 1994). Compared with the catalytic specificity of ACHE, BChE possesses a broad hydrolytic spectra of choline ester substrates (Taylor and Radic 1994) because of the weak steric hindrance in the active center (Harel et al. 1992; Ordentlich et al. 1993; Radic et al. 1993). The function of BChE is to protect ACHE from inactivation and to hydrolyze many esters, amides and peptides (Small et al. 1996). Moreover, it involves in some metabolic processes of some drugs and even cell growth process (Lockridge 1990; Alber et al. 1994; Mattes et al. 1997). In particular, as the target of organophosphorus pesticide (OP) and carbamate pesticide (CB), BChE could be inhibited by pesticides to detect OP and CB residues (Boublik et al. 2002; Pritchard et al. 2004; Vakurov et al. 2004). However, BChE purified from tissues or blood is both time and cost intensive. Therefore, BChE is highly necessary for heterologous expression in a new host fleetly and economically.

*Pichia pastoris* is now widely used for heterologous production of recombinant protein owing to its high level expression, efficient secretion, superior post-translational modification and potential to dense cultivation (Macauley et al. 2005; Mchunu et al. 2009). Due to the difference of codon usage between the host and their original organisms, researchers have utilized codon optimization to increase expression of a variety of protein (Wang et al. 2008; Zhu et al. 2011). Moreover, the recombinant plasmid pPIC9K/nhBChE of the native human BchE gene (*hBChE*) constructed by our research group expressed poorly in *P. pastoris* after repeated experiments.

Therefore, in this assay, we optimized the codon usage of *hBChE* cDNA fragment based on *P. pastoris* preferred codons to increase the production level. As a result, *hBChE* was successfully expressed in *P. pastoris* with a high-level expression. The biochemical characteristics of the recombinant enzyme were also determined.

The study is to produce a codon-optimized butyrylcholinesterase with low-cost preparation for achieving promising biomaterials to detect organophosphatase insecticide residues. The expressed enzyme could be an ideal tool for detection of insecticides in agricultural products and their derivatives.

2. Results

2.1. Synthesis of codon-optimized *hBChE* and bioinformatics analysis for gene optimization

We successfully synthesized a codon-optimized *hBChE* with 6×His-Tag at N-terminal according to the prefer codons of *P. pastoris*. Here, it is clear that the native gene containing tandem rare codons leads to poor expression. So, we changed the codon usage frequency in *P. pastoris* by upgrading the codon adaptation index (CAI), which is a measurement of the relative adaptiveness of the codon usage of a gene compared with the codon usage of highly expressed genes, from 0.79 to 0.87 (Fig. 1). Moreover, the ratio of guanine and cytosine in four kinds of bases ((G+C) ratio) increased from 0.40 to 0.47 and unfavorable peaks were optimized to prolong the half-life of the mRNA (Fig. 2). The secondary structures, which affect ribosomal binding and stability of mRNA, have been removed as well. In addition, optimization process has successfully screened and removed the splice sites, polyadenylation signal, instability elements, and all the cis-acting sites that may have a negative influence on the expression rate. Finally, the codon-optimized cDNA (*hBChE*) shared 37.3% of codon identity with the native one, however, amino acid sequence of the synthetic gene was aligned with amino acid sequence of the native one (Fig. 3).
2.2. Construction of transformants and verification of phenotypes

The synthetic gene (hBChE) was cloned into the expression vector pPIC9K, yielding the recombinant plasmid pPIC9K/hBChE for integrating into the genome of P. pastoris GS115 competent cells through electrotransformation. Several His+ transformants were obtained on minimal dextrose medium (MD) plates. After transferring 67 strains onto minimal medium (MM) plates and MD plates separately, it revealed that all of the positive colonies were His+Muts phenotype. Meanwhile, six colonies from yeast extract peptone dextrose medium (YPD) plate containing 4.0 mg mL⁻¹ geneticin 418 showed high hBChE activity.

2.3. Induced expression and purification of the recombinant hBChE

Six highly expressed transformants were induced with methanol at the final concentration of 1% for 120 h at 30°C under the control of the methanol-inducible alcohol oxidase promoter. The expression levels were similar in different transformants floating within a certain range from 231 to 292 mg mL⁻¹ total protein. The enzyme activity of concentrated culture supernatants reached up to 14.7 U mL⁻¹. Since the secretion of active hBChE within 6×His-Tag into the medium, purification was greatly simplified by one-step Ni-NTA affinity chromatography column. This procedure was revealed to be highly efficient in gaining large amounts of pure hBChE, and 2.3 mg pure hBChE were obtained from 100 mL culture supernatant with a specific activity of 8.1 U mg⁻¹. The protein concentration in purified enzyme solution was up to 98% determined using the Bio-Rad dye agent with BSA as the standard. Besides, the purified hBChE migrated one single band of 68 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was more than the predicted molecular weight 65 kDa, probably due to N-glycosylation (Fig. 4).

2.4. Characterization of the purified hBChE

The optimal pH of the purified recombinant hBChE for enzyme activity was pH 7.4 in the McIlvaine buffer (Fig. 5-A) at 37°C. It was stable over a pH range of 4.0–9.0, retaining...
Fig. 3 Alignment of codon between the codon-optimized cDNA (hBChE) and the native one.

Fig. 4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified hBChE with 6×His-tagged from pre-extracted suspension by one-step Ni-NTA affinity chromatography column (QIAGEN) under denaturation condition. Lane M, molecular weight markers; CS, the culture supernatant of recombinant P. pastoris GS115; pp, the purified hBChE with 6×His-tagged after elution.

tetraacetic acid (EDTA) at a final concentration of 1 mmol L⁻¹ on hBChE were also investigated. The enzyme activity was remarkably inhibited by Cu²⁺ losing 79.78% of the activity and K⁺ losing 59.81% activity, respectively; Li⁺, Co⁺, Zn²⁺ and EDTA partially enhanced more than 20% activity of the enzyme. Four kinds of metal ions (Mg²⁺, Mn²⁺, Fe²⁺, Ag⁺) slightly inhibited the enzyme activity by 22.47, 9.85, 10.11, 16.85%, respectively; Ca²⁺ and Na⁺ had little or no effect on the enzyme activity (Table 1).

The Michaelis-Menten constants of hBChE were determined by S-butylthiocholine iodide. The values of Michaelis constant (Kₘ) and maximum reaction velocity (Vₘₚ) were 89.4 μmol L⁻¹ and 1721 μmol min⁻¹ mg⁻¹, respectively.

2.5. Sensitivity analysis of hBChE

We tested the values of bimolecular rate constants (Kᵢ) of recombinant butyrylcholinesterase to four pesticides. Our results indicated (Table 1) that the hBChE had similar sensitivity with electric eel AChE (EeAChE) and higher than sensitivity of horse BChE (HoBChE) to dichlorvos, omethoate, paraoxon and paraoxon methyl. The result suggested that recombinant butyrylcholinesterase to four pesticides. Our results indicated (Table 1) that the hBChE had similar sensitivity with electric eel AChE (EeAChE) and higher than sensitivity of horse BChE (HoBChE) to dichlorvos, omethoate, paraoxon and paraoxon methyl. The result suggested that recombinant butyrylcholinesterase to four pesticides. Our results indicated (Table 1) that the hBChE had similar sensitivity with electric eel AChE (EeAChE) and higher than sensitivity of horse BChE (HoBChE) to dichlorvos, omethoate, paraoxon and paraoxon methyl. The result suggested that...
2.6. hBChE-based insecticide residues assay for vegetable samples

Six vegetable samples randomly collected from a local market in Beijing (China) were analyzed by hBChE-based insecticide residues assay. The samples were in parallel analyzed by GC. According to the Chinese national standard, the samples are considered to be positive if 50% of the enzyme activity is inhibited after 2.5 mL of extracts incubating with 0.1 mL of hBChE solution for 15 min at room temperature. The results of hBChE-based assay of the spiked samples (Table 2) indicated that the hBChE can be useful for detection of organophosphate insecticide residues.

3. Discussion

Compared with the prokaryotic expression system and other eukaryotic expression systems, P. pastoris expression system has its own synonymous codon usage bias in the process of protein translation. For example, there are six kinds of synonymous codon coding arginine (AGA, CGT, CGC, CGA, CGG and AGG). However, 48% of arginine was encoded by AGA while the rest (CGT, CGC, CGA, CGG and AGG) are used in equally low frequency (the usage frequency is about 10%) in the yeast genome. If we had used other five codons instead of AGA in yeast, the opportunity that arginine was normally added to the polypeptide chain would be reduced to approximately one fifth of in using AGA. Thus, the speed of synthesis of entire polypeptide chain is restricted. Graham et al. (2002) successfully expressed glucocerebrosidase in P. pastoris and gave evidence that both the codon optimization and the content increase of G+C in genes contributed to high level expression of glucocerebrosidase, which the adjustment of GC content contributed more.

In this study, we provided more evidence to support this
conclusion by having performed high-level expression of recombinant human butyrylcholinesterase in P. pasteuris by codon optimization. First of all, The (G+C) content and the CAI of hBChE were adjusted from 0.40 to 0.47 and from 0.79 to 0.87, respectively, which are more appropriate for P. pasteuris system. Then, compared with the recombinant plasmid pPIC9K/nhBChE of the native hBChE, the recombinant vector pPIC9K/hBChE by codon optimization has higher enzyme expression up to 292 mg mL⁻¹, which is enough to prove that codon optimization leads to the improvement of protein expression. Moreover, the enzyme activity in P. pasteuris was determined to be 14.7 U mL⁻¹, which was comparable to that of the native gene.

P. pasteuris expression system takes unique advantages of higher expression, stronger secretion, better protein stability and post-translational processing, easier to achieve high density fermentation compared with the prokaryotic expression system. Therefore, P. pasteuris expression system is more suited for the expression of proteins which have more modifications, for example human butyrylcholinesterase. Post-translational processing function aimed at heterologous protein in P. pasteuris expression system makes the conformation of the heterologous protein closer to the natural one, and this will ensure that the heterologous expressed protein has activity similar to the native one.

The eukaryotic expression system commonly used in gene engineering currently includes the yeast expression system, mammalian expression system and insect cell expression system. This research obtained the optimal pH 7.4 using the yeast expression system, which is a little higher than the optimal pH 7.2 of the hBChE expressed in Chinese hamster ovary (CHO) reported by Ilyushin et al. (2013), comparable to the expression in silk worm reported by Wei et al. (1998). Interestingly, the optimal temperature was 50°C, which is much higher than 25 and 37°C, respectively. Maybe, the difference between natural protein conformation and that formed by post-translational modification does exist in the process of protein expression in P. pasteuris, which resulted in the changes of some enzyme properties.

In 2011, Zhu et al. (2011) discovered Zn²⁺, Mn²⁺, Cu²⁺ had significant inhibition on butyrylcholinesterase derived from duck liver. Compared with the above result, our research show that: Li⁺, Co⁺, Zn²⁺ and EDTA can activate the activity of the enzyme, Mg²⁺, Mn²⁺, Fe²⁺, Ag⁺ can inhibit the activity of the enzyme, while Ca²⁺ and Na⁺ had no obvious effect. The results will provide reference for us on the study of enzyme inhibitor as well as activator.

The Kᵣ of human butyrylcholinesterase was 89.4 μmol L⁻¹ obtained by our study with S-butyrylthiocholine iodide as substrate, which is higher than the 25 μmol L⁻¹ determined by Ilyushin et al. (2013) but lower than the 185 μmol L⁻¹ determined by Wei et al. (1998). The above values are higher than the 23 μmol L⁻¹ from serum butyrylcholinesterase. Meanwhile, the hBChE sensitivity to four typical pesticides was higher than that of the horse BChE and EeAChE because of its higher Kᵣ values.

Comparing to the GC assay, the hBChE-based assay was more sensitive in detecting organophosphate insecticide residues in vegetable samples. This fact indicated that the hBChE-based test could definitely meet the demand of current legislated standards in China. Thus, the over-expressed hBChE will potentially provide us sufficient material for developing and producing hBChE-based assay kit for the detection of organophosphate pesticides on spot.

4. Conclusion

This study allowed us to construct an efficient system for the expression of active human butyrylcholinesterase in P. pasteuris. The codon optimization made it possible to increase production of the recombinant protein significantly. The calculated kinetic constants indicate that the active site of the enzyme is intact. The next step of this work will be focused on further improvement of the stability of the recombinant enzyme by chemical modifications, such as PeGylation (Chilukuri et al. 2008) or sialylation (Jain et al. 2003). The hBChE-based assay method could be used for rapid screening for large numbers of agricultural products containing organophosphate insecticides. Furthermore, it would help to enforce national regulation to limit insecticide contamination in agricultural products on spot.

Table 2 Organophosphate insecticide residues analysis based on the inhibition rate of hBChE

<table>
<thead>
<tr>
<th>Code</th>
<th>Vegetable</th>
<th>hBChE-based assay (inhibition rate of hBChE, %)</th>
<th>GC analysis (insecticide detected, mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cabbage</td>
<td>97.8</td>
<td>Pirimicarb, 1.07</td>
</tr>
<tr>
<td>2</td>
<td>Coriander (Coriandrum sativum)</td>
<td>18.8</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Tomato (Lycopersicon esculentum)</td>
<td>22.3</td>
<td>Omethoate, 0.54</td>
</tr>
<tr>
<td>4</td>
<td>Cauliflower (Brassica oleracea var. botrytis)</td>
<td>15.6</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>Chinese spinach (Amaranthus tricolor)</td>
<td>14.3</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>Com chrysanthemum (Chrysanthemum segetum)</td>
<td>91.2</td>
<td>Methamidophos, 1.92</td>
</tr>
</tbody>
</table>

ND, not detected.
5. Materials and methods

5.1. Materials

The biochemical reagents such as the restriction endonucleases EcoRI, NotI, SacI and DNA marker were purchased from TaKaRa (Dalian, China). T4 DNA ligase was purchased from Promega (Beijing, China). The oligonucleotides were synthesized by Sangon Biotech (Beijing, China). PageRuler™ Prestained Protein Ladder was purchased from Fermentas (Beijing, China). The S-butyrylthiocholine iodide (BTChI) and 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (Beijing, China). All other reagents and analytical grade chemicals were obtained from local suppliers. Capillary gas chromatography (GC) was performed on a 30 m×0.32 mm, 0.25 mm HP-1 column (Agilent, Waldbronn, Germany), equipped with a nitrogen-phosphorus detector (NPD). All other reagents were analytical grade products from various sources.

5.2. Bacterial strains, plasmids, and media

*Escherichia coli* strain DH5α (TaKaRa, China) and plasmid pUC57 vector (Promega, USA) were used for construction of cloning plasmid. Expression plasmid pPIC9K and expression host *P. pastoris* (GS115/His′Mut′) purchased from Invitrogen (Beijing, China) were used for hBChE expression. The recombinant plasmid pPIC9K/nhBChE of the native hBChE gene (nhBChE) preserved by our laboratory. *E. coli* cells were cultured in LB medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride). Yeast cells were cultured in YPD (1% yeast extract, 1% peptone, 2% glucose), BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base without amino acids, 0.1 mol L⁻¹ potassium phosphate (pH 7.0), 4×10⁻²% biotin, 1% glycerol), or BMMY (BMGY medium with 0.5% methanol instead of 1% glycerol) media. Where necessary, ampicillin (Invitrogen, Beijing, China) was added to the media at a final concentration of 100 mg mL⁻¹. For solid media, 2% agar was added to the media.

5.3. Optimization of hBChE cDNA and sequence analysis

The hBChE cDNA fragment with glycosylphosphatidylinositol (GPI) signal peptide sequence was redesigned according to the usage frequency of triplet codon in *P. pastoris*: avoiding repeated sequences, improving GC content and CAI without substitution in amino acid sequence. For cloning, we introduced the restriction sites EcoRI and NotI at the ends of the synthetic sequence, respectively. The optimized hBChE cDNA was synthesized by GENEWIZ (Beijing, China) and confirmed by sequencing. DNA and protein sequences were aligned using the BLASTx and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1997), respectively.

The sequence assembly was performed using the Vector NTI Advance 10.0 software (Invitrogen, Beijing, China) to evaluate the identity and to predict the molecular mass of the mature protein. The signal peptide was analyzed by the Signal P 4.0 server. The potential N-glycosylation sites were predicted online (http://www.cbs.dtu.dk/services/NetNGlyc/).

5.4. Construction of expression plasmid and transformation

The optimized cDNA fragment was cut with EcoRI and NotI, then was inserted into the EcoRI-NotI-digested expression vector pPIC9K for generating recombinant plasmid pPIC9K/hBChE. After verifying the sequence, the plasmid was linearized with SacI to be introduced into *P. pastoris* strain GS115 by electroporation for homologous transformation. His′ yeast transformants were grown on MD plate and then spotted on YPD plates containing G418 at a final concentration of 0, 1.0, 2.0, 4.0 mg mL⁻¹ to be incubated at 28°C for 5 days to screen high expression transformants.

5.5. Verification of transformant phenotypes

His′ yeast transformants were picked out from MD plates and transferred onto MM and MD plates. The MM and MD plates were incubated at 28°C for 3 days for differentiating the His′Mut′ recombinants (where Mut refers the slow-methanol-utilization phenotype) that grew normally on the MD plates but showed little or no growth on MM plates and His′Mut′ recombinants whose growing speed are the same on MD and MM plates.

5.6. Expression and purification of hBChE

The single colonies of recombinant *P. pastoris* clone were isolated from 4.0 mg mL⁻¹ G418 YPD plates. The yeast transformants were pre-cultivated in YPD medium at 28°C with being shaken at 200 r min⁻¹ to early logarithmic growth phase. 200 mL cultured cells were inoculated into 20 mL of BMGY medium and shaken at 30°C (200 r min⁻¹) until OD₆₀₀ reached 2.0–6.0. The cells were harvested by centrifugation (4 000 r min⁻¹, 10 min) at room temperature, resuspended in 40 mL BMMY medium and grown at 30°C (20 r min⁻¹) for 5 days. Throughout the induction phase, 100% methanol was added to a final concentration of 1.0% (v/v) for induced expression of the target protein every 24 h. The concentration of protein in supernatant was measured by the method of Bradford (Bradford 1976). The culture supernatant
obtained by centrifugation (12 000 r min⁻¹, 10 min at 4°C) was purified with Ni-NTA affinity chromatography column (QIAGEN, Beijing, China). The procedure was carried out at 4°C. The purity of the protein sample was checked by SDS-PAGE.

5.7. Enzyme activity assay

hBChE catalytic activity was determined by measuring the amount of yellow anion NTB₂⁻ generated by DTNB and active hydrosulfuryl released from hBChE according to the Ellman method (Ellman et al. 1961). The reaction system comprised 400 μL of appropriately diluted enzyme and 2.3 mL of 0.43% (v/v) BTChI-DTNB (1:2) in 100 mmol L⁻¹ sodium phosphate at pH 7.4. After incubation at 37°C for 15 min, the reaction was terminated by ice-bath. After centrifugation at 0°C, the absorbance at 412 nm was monitored. For the control sample, 400 μL diluted enzyme boiled for 15 min was added to the reaction system. All experiments were conducted in triplicate. One unit (U) of hBChE activity was defined as the amount of enzyme that released 1 μmol of yellow anion NTB₂⁻ per minute under standard assay conditions.

5.8. Characterization of hBChE

The optimal pH of purified hBChE was determined at 37°C for 10 min under various pH ranging from 2.0 to 10.0. The buffers used included 100 mmol L⁻¹ of McIlvaine buffer (pH 3.0–8.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.0–10.0). To estimate the pH stability, the enzyme was pre-incubated in different buffers as described previously without substrate at 37°C for 1 h, and then the residual activities were monitored at 37°C and pH 7.4 for 15 min.

The optimal temperatures were determined at optimal pH over the temperature range of 10–80°C for 15 min. To investigate the thermal stability, the purified enzyme was pre-incubated at 50, 60 and 70°C without substrate for 5–60 min at pH 7.4. Then the residual enzyme activities at specific time points were detected under the standard conditions.

The effects of various metal ions and EDTA on the recombinant hBChE activities were determined by adding 1 mmol L⁻¹ of various metal ions and chemical reagents (Na⁺, K⁺, Ca²⁺, Li⁺, Ag⁺, Co²⁺, Cu²⁺, Mg²⁺, Fe²⁺, Mn²⁺, Zn²⁺, EDTA) to the reaction solution. The reaction system without any additive was performed as the blank control. The residual activities were measured as described above.

The kinetic parameters of hBChE were carried out in McIlvaine buffer (pH 7.4) by measuring the initial velocities of the enzyme at 50°C for 3 min, containing 0.1–10 mg mL⁻¹ BTChI as substrate. The Michaelis-Menten constant (Kₘ) and maximal reaction velocity (V_max) were calculated using the non-linear regression computer program GraFit (Biosoft, Cambridge, UK).

5.9. Sensitivity analysis of hBChE against pesticides

The bimolecular rate constants (Kᵢ) for pesticides was detected in the PBS (50 mmol L⁻¹, pH 7.4), using four concentrations of each pesticide in the range of 0.01–100 mg mL⁻¹. Pesticides were previously dissolved in acetone and then diluted with water. The maximum acetone concentration in reaction buffer was 1%. hBChE was incubated in PBS (50 mmol L⁻¹, pH 7.4) at 25°C with the inhibitor for various time before addition of 1 mmol L⁻¹ BTChI. A plot of the natural logarithm of residual activity ([E]/[E₀]) vs. time was linear for a given inhibitor concentration. Kᵢ values were obtained by dividing the slope of the curve by the concentration.

The value of IC₅₀ of each pesticide was determined from the inhibition curve as 50% hBChE activity inhibited. The enzyme sample was incubated with the inhibitor for 60 min at 25°C. The residual hBChE activity was assayed according to the method mentioned above and plotted as a function of the concentration of inhibitor. The experiment was repeated three times with duplicates for each concentration.

5.10. hBChE-based assay of practical samples

The hBChE-based assay was developed and applied to detect insecticide residues in vegetable samples. Sample extract (2.5 mL) was incubated with 0.1 mL of hBChE for 15 min at room temperature. The inhibition rate was deduced from this formula, [(E₀–E)/E₀]×100%. E₀ was the initial hBChE activity obtained with 2.5 mL of 50 mmol L⁻¹ PBS (pH 7.4) instead of sample extract. Spiked samples were prepared by adding 10 mL of the pesticide working solution to 5 g of vegetable. The pesticide levels ranged from 0.01 to 2 mg kg⁻¹, depending on its inhibitive potency. The solvent was evaporated at room temperature for 4 h. The sample was immersed in 10 mL of 50 mmol L⁻¹ PBS (pH 7.4) and extracted for 15 min in an ultrasonic bath. After a brief settling period, the clear extract was removed for hBChE-based assay.

During GC analysis, the injection port and detector working temperatures were 260 and 270°C, respectively, with nitrogen as the carrier gas at a flow rate of 1.0 mL min⁻¹. The oven working temperature was 240°C, and the injection volumes were 3 μL. The constant flow rates of hydrogen and air were 100 and 50 mL min⁻¹, respectively. The concentration of each compound in tested samples was determined by comparing the peak areas obtained in the samples with the standards. Chromatographic standards were prepared by spiking blank samples with known amounts of pesticides. The extraction procedure selected was based on a method previously applied for the deter-
mination of organophosphorus pesticides in vegetables by GC analysis (Sasaki et al. 1987).

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