Large-scale Purification and Acute Toxicity of Hygromycin B Phosphotransferase[1]

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Objective To provide the acute toxicity data of hygromycin B phosphotransferase (HPT) using recombinant protein purified from *E. coli*. **Methods** Recombinant HPT protein was expressed and purified from *E. coli*. To exclude the potential adverse effect of bacteria protein in recombinant HPT protein, bacterial control plasmid was constructed, and bacteria control protein was extracted and prepared as recombinant HPT protein. One hundred mice, randomly assigned to 5 groups, were administrated 10 g/kg, 5 g/kg, or 1 g/kg body weight of HPT or 5 g/kg body weight of bacterial control protein or phosphate-buffered saline (PBS) respectively by oral gavage. **Results** All animals survived with no significant change in body weight gain throughout the study. Macroscopic necropsy examination on day 15 revealed no gross pathological lesions in any of the animals. The maximum tolerated dose (MTD) of HPT was 10 g/kg body weight in mice and could be regarded as nontoxic. **Conclusion** HPT protein does not have any safety problems to human health.

Key words: Hygromycin B phosphotransferase; Selectable marker; Acute toxicity; Safety assessment

INTRODUCTION

Hygromcycin B phosphotransferase (HPT) gene (*hpt*) is a selectable marker widely used in prokaryotic and eukaryotic transformation systems. The *hpt* gene seems to be of special value for selecting transformed cereal cells (such as those of rice) in comparison with the first and most frequently used kanamycin antibiotic gene (*npt II*) since various cereals are resistant to kanamycin or G418 and sensitive to low doses of hygromcyin B[1]. Therefore, HPT has become the second most selectable antibiotic selectable marker on transgenic crops after NPT II[2]. All selectable marker genes should be subjected to careful and thorough safety assessment as target genes. The questions related to the biosafety of all marker genes are the same. Do they code for toxic products or allergen? Will they create unwanted changes in the composition of the crop? Will they compromise use of therapeutic drugs? Will there be horizontal gene transfer to relevant organisms and pathogens? Can gene transfer to other plants create new weeds or compromise the value of non-target crops? All these questions are related to the biosafety of all marker genes. Therefore, all marker genes have to be assessed individually. Studies are available on the *npt II* gene[3-4] and the WHO workshop concluded that use of the *npt II* marker gene in genetically modified plants has no risk to human health[5] and NPTII has been approved by the US Food and Drug Administration (FDA) as a food additive for tomato, cotton, and oilseed rape. Since no report on the safety of the *hpt* gene is available at present, safety evaluation of the *hpt* gene is very critical for all crops with hygromycin B selective system. The expression levels of selectable marker genes are relatively low. In order to obtain enough protein for safety assessment, foreign proteins are generally expressed and purified from prokaryotic expression systems for further safety assessment. In previous works, we developed a non-fusion HPT protein expression and purification method. The protein purified using this procedure is consistent with the HPT of transgenic rice on molecular weight, immuno-reactivities, N-terminal acid sequences and biological activities[6]. In this study, HPT protein was expressed and purified by fermentation and acute oral toxicity evaluation of the HPT was conducted.

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MATERIALS AND METHODS

Fermentation

One milliliter frozen *E. coli* cell with *pET41 HPT* vector was inoculated to 2L LB medium containing 30 μg/mL kanamycin. After shaken at 37 ℃ overnight at 200 rpm, the culture was transferred into an 80 L bioreactor (BioFlo 5000, New Brunswick Scientific, Edison, NJ) with 50 L fermentation medium containing 10 g/L tryptone and yeast extract, 5 g/L glucose, 3 g/L NaCl, 4 g/L KH_2PO_4, 8 g/L Na_2HPO_4, 1 g/L NH_4Cl and $MgSO_4$, 0.1 g/L $CaCl_2$, and 0.04 g/L $FeSO_4$. Fermentation was performed at 37 ℃ and the pH was maintained at 7.0 by addition of 25% (vol/vol) NH_3 and 1 mmol/L HCl. The airflow rate was kept at 1 volume of air/volume of medium/min. One milliliter antiform (Sigma) was added at beginning and thereafter when needed. The dissolved oxygen was maintained at 25% saturation by automatic adjustment of the stirrer speed (300-600 rpm). When OD_{600} reached 3, the feeding medium (200 mL/L glycerol, 50 g/L tryptone and yeast extraction, 2 g/L $MgSO_4$) was added at 20-60 mL/h. When OD_{600} reached 12, IPTG was added to a final concentration of 0.1 mmol/L. Fermentation continued to cultivate for 4 h and cells were harvested by centrifugation at 5 000 rpm for 20 min. Cell pellet was weighed and stored at –80 ℃.

Large Scale Purification of HPT from E. coli

The purification process was scaled-up as described previously[6]. In brief, 200 g pellets was thawed on ice and resuspended in 5 000 mL STE (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L EDTA). The suspension was lysed by five passes through a high-pressure homogenizer (EmulsiFlex-C55, AVESTIN, Canada) at a flow rate of 500 mL/min, 80 MPa. The cell lysate was centrifuged at 10 000 g for 10 min. The pellets were washed 5 times with 2% Triton X-100, 1% Triton X-100, 2 mol/L NaCl, 1 mol/L NaCl and distilled water, respectively, to release the trapped protein. Pellets were finally resuspended in 1 000 mL STE containing 0.3% sarkosyl and incubated at room temperature with continuous stirring for 30 min. After centrifugation at 18 000 g for 30 min, the supernatant was diluted 10 times with STE and dialyzed against 10L STE at 4 ℃ for 24 h, buffer was changed every 8 h. The dialysate was centrifuged at 18 000 g for 30 min. The supernatant was applied to a column (XK 50/60, Amersham Pharmacia Biotech, Sweden) packed with 500 mL anion-exchange resin (DEAE Sephroase Fast Flow, Amersham Pharmacia Biotech, Sweden) using AKTA prime system (Amersham Pharmacia Biotech, Sweden) at 4 ℃. The column was pre-equilibrated with 5 000 mL Start buffer (20 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0), 5 000 mL elute buffer (1 mol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0) and 5 000 mL start buffer, respectively. Elution was achieved with 0.02-1 mol/L NaCl gradient. The peak fractions were monitored by SDS-PAGE and concentrated by Labscale TFF system (Millipore, USA) at 4 ℃ and further lyophilized by ALPHA1-4 LSC (Crist, Gemany).

Hygromycin Phosphotransferase Assay

HPT functional activity was measured using the continuous coupled spectrophotometric assay as previously described[6].

Preparation of Bacterial Control Protein

For preparation of bacterial control protein, non-fusion control plasmid-*pET 41 control* was constructed (Fig. 1). The *pET41 EK* control plasmid was cleaved with Nde I and *Eco*R V to remove the tag, subsequently reacted with T4 DNA polymerase to form two blunt ends. after gel purified, the tag free vector was ligated overnight at 16 ℃ and then transformed into *DH5 α* competent cells. After identified by PCR using the vector primers-T7 promoter and T7 terminator, the positive plasmid was transformed into the expression strain *BL21(DE3)*. This *E. coli* cell with the *pET 41 control* plasmid was used to produce bacterial control protein. The bacterial protein preparation steps were the same as the HPT fermentation and purification procedure except for ion-exchange chromatography.

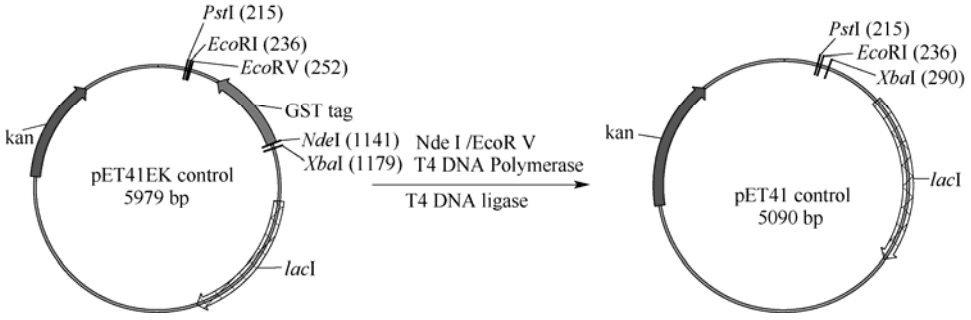

FIG. 1. Construction scheme of tag free control plasmid *pET 41 control*.

Acute Oral Toxicity Study

One hundred Kunming strain mice (50 males and 50 females), weighing 18-22 g, were obtained from the Animal Center of National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The testing facility provided appropriate environmental conditions (24 ℃ ±2 ℃ room temperature, 12 h light/dark cycle and 50%±10% relative humidity). After acclimatization for three days, the mice were randomized into five groups: high, medium, low dose groups of HPT, bacterial protein control group and control group, 10 male and 10 female mice in each group. Five mice were kept in one acrylic cage with free access to standard rodent chow diet and water and fasted overnight prior to the experiment. All the proteins were dissolved in sterilized PBS buffer. The mice administered the designated amount of HPT or bacterial control protein or PBS only by gavage. The mice in the high, medium, and low HPT groups were given HPT protein at the dose of 10 g/kg, 5 g/kg, 1 g/kg body weight, respectively. The mice in the bacterial protein control group were given control protein at the dose of 5 g/kg body weight and the mice in the control group were given PBS buffer only. Because the HPT protein was only soluble at the concentration of 133.3 mg/mL and the volume/body weight ratio in the high dose HPT group would be 1.5 mL/20 g, it was divided into three equal doses. The dose of bacterial protein was fixed at 5 g/kg body weight due to its lower solubility than HPT. All the mice were gavaged three times every 4 hours on the administration day. The gavage volume was the same for each mouse. After gavage, mortality and clinical signs were monitored twice daily. Food intake was recorded twice every week. Body weight was measured on days 0 (the day of gavage administration), 3, 7, and 14. After fasting overnight, the mice were sacrificed on day 15. All animals were necropsied. Internal cavities were opened and organs were examined *in situ*. Absolute and ratio of organ *vs* body weight for the following organs was recorded for all animals: brain, heart, lungs, kidney, liver, spleen thymus and sex glands. If any mouse died during the experiment, it was examined to find the cause of death.

Statistical Analysis

Results were expressed as mean±standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) and *post hoc* least-significant difference (LSD) test. $P<0.05$ was considered statistically significant.

RESULTS

Fermentation and Large-scale Purification of HPT

The fermentation and purification results are presented in Fig. 2. After 4 h induction with IPTG, the final cell density (A_{600}) was 30, the final fermentation volume was 56 L, and the total cell wet weight was 2170 g. The expression level of the recombinant protein reached about 30% of the total cellular proteins. After several washing steps, the purity of solubilized HPT protein determined by optical densitometry on SDS-PAGE was about 70%. After dilution, dialysis, and ion-exchange chromatography, the purity of the HPT protein was about 90% and the bioactivity was about 4-6 U/mg protein. About 10 g HPT protein was obtained from the fermentation.

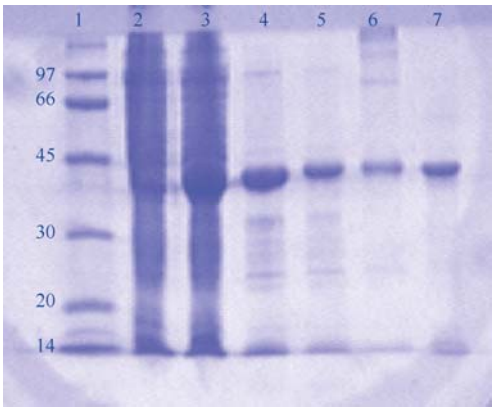

FIG. 2. SDS-PAGE analysis of fermentation and purification of HPT protein.
Lane 1: protein molecular weight marker; lane 2: uninduced whole cell lysate; lane 3: induced whole lysate at 4 h; lane 4: inclusion bodies after washings; lane 5: retentate after diluting; lane 6: sample after dialysis; lane 7: sample after AEC.

Clone and Preparation of Bacteria Control Protein

PCR identification of the *pET 41 control* is presented in Fig 3. The PCR fragment size from the *pET 41 EK l* and *pET 41* controls was about 1 200 bp and 350 bp, respectively. The theoretical fragment size from the site of Nde I and EoR V was 857 bp. The results of PCR suggest that the fusion tag of the vector had be thoroughly removed. After induction, no obvious expression band was found. The final fermentation volume was 54 L, and the total cell wet weight was 1 328 g. Through cell disruption, washing, dissolving, dilution, dialysis, concentration, and lyophilization, the final amount of bacterial control protein was about 8 g.

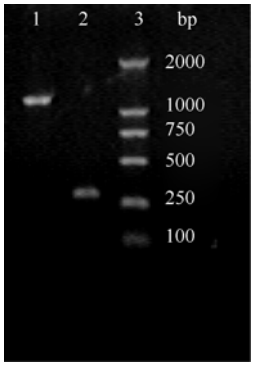

FIG. 3. PCR analysis of pET41 control plasmid. Lane 1: PCR fragment from *pET 41EK* control; lane 2: PCR fragment from *pET 41* control; lane 3: DL2000 DNA molecular weight marker.

Acute Oral Toxicity Study

The mice in all groups survived during the 14-day observation period. No evident changes were noted in behavior, activity, posture, gait or external appearance of any group or in either sex. There were no statistically significant differences in body weight and weight gain of male and female mice in any group (Tables 1 and 2). Food consumption was generally similar in mice of all groups (Table 3).

Data about relative organ weights (organ weight data as a percentage of body weight) were comparable among all groups (Table 4). The relative kidney weight was significantly decreased in female mice of the medium HPT dose group and the bacterial protein group, and significantly increased in the high HPT dose group compared to the bacterial protein and PBS groups. The relative brain weight was significantly increased in male of the medium HPT dose group and PBS group compared to the high and low HPT dose groups.

Therefore, the maximum tolerable dose (MTD) of HPT was more than 10 g/kg in mice, and oral HPT in mice was not toxic according to the criteria for acute toxic classification of Ministry of Health of P. R. China[7].

TABLE 1

Body Weight Change in Mice (g) ($\bar{x} \pm s$)

Administration Day	10 g/kg HPT (*n*=10)	5 g/kg HPT (*n*=10)	1 g/kg HPT (*n*=10)	Bacterial Protein (*n*=10)	PBS (*n*=10)
Female					
0	20.7±1.5	20.7±1.5	20.7±1.4	20.6±1.2	20.9±1.5
3	25.6±1.1	25.3±1.2	25.6±1.4	25.3±1.5	26.0±2.0
7	29.3±1.3	28.1±1.4	28.0±1.4	28.7±1.3	29.6±2.3
14	33.0±2.6	30.9±1.9	31.4±1.3	31.45±1.47	32.1±1.9
Male					
0	20.2±1.3	20.2±1.2	20.3±1.0	20.3±1.0	20.3±1.4
3	25.8±2.4	26.3±2.1	26.6±0.9	26.3±1.3	26.1±1.5
7	30.9±2.9	31.7±2.3	32.3±1.1	31.6±1.1	31.4±2.0
14	36.9±2.9	38.1±2.8	38.8±2.4	37.6±1.0	37.9±2.9

Note. There were no statistically significant differences among the five groups.

TABLE 2

Body Weight Gain of Mice (g) ($\bar{x} \pm s$)

Body Weight Gain at	10 g/kg HPT (*n*=10)	5 g/kg HPT (*n*=10)	1 g/kg HPT (*n*=10)	Bacterial Protein (*n*=10)	PBS (*n*=10)
Female					
3 Days	5.0±1.3	4.5±0.8	4.8±1.0	4.7±0.8	5.0±1.0
7 Days	8.6±2.2	7.4±1.6	7.3±1.3	8.1±1.1	8.7±1.5
14 Days	12.3±3.1	10.2±2.4	10.7±1.7	10.7±1.7	10.9±0.6
Male					
3 Days	5.6±1.6	6.1±2.0	6.3±0.9	6.0±1.3	5.7±0.7
7 Days	10.8±2.4	11.4±2.0	11.9±1.2	11.3±1.2	11.8±1.1
14 Days	16.7±2.6	17.9±2.7	18.4±2.6	17.2±1.2	17.5±2.1

Note. There were no statistically significant differences among the five groups.

TABLE 3

Food Intake of Mice ($\bar{x} \pm s$)

Test Group	Sex	Food Consumption (g)
10 g/kg HPT	Female	5.0±0.2
5 g/kg HPT	Female	5.2±0.8
1 g/kg HPT	Female	5.0±0.5
Bacteria Protein	Female	4.8±0.3
PBS	Female	4.7±0.8
10 g/kg HPT	Male	5.9±0.8
5g/kg HPT	Male	6.0±0.9
1 g/kg HPT	Male	6.4±1.1
Bacteria protein	Male	6.3±1.3
PBS	Male	6.1±0.8

Note. There were no statistically significant differences among the five groups.

TABLE 4

Relative Weight of Mice ($\bar{x} \pm s$)

Parameter	10 g/kg HPT	5 g/kg HPT	1 g/kg HPT	Bacterial Protein	PBS
Female	(*n*=10)	(*n*=10)	(*n*=10)	(*n*=10)	(*n*=10)
Heart (%)	0.476±0.053	0.441±0.050	0.478±0.046	0.473±0.053	0.492±0.049
Lung (%)	0.565±0.065	0.562±0.077	0.580±0.067	0.617±0.055	0.657±0.094
Spleen (%)	0.422±0.101	0.387±0.076	0.440±0.123	0.422±0.081	0.429±0.061
Kidney (%)	1.178±0.166	1.047±0.103$^{\#}$	1.144±0.105	1.181±0.066	1.171±0.166
Liver (%)	5.218±0.543$^{*,\#}$	4.680±0.327	4.896±0.283	4.687±0.327	4.725±0.404
Brain (%)	1.324±0.134*	1.322±0.105*	1.319±0.100*	1.335±0.134*	1.176±0.185
Thymus (%)	0.386±0.090	0.544±0.324	0.468±0.063	0.487±0.055	0.406±0.094
Ovaries (%)	0.039±0.016	0.029±0.007	0.034±0.012	0.040±0.016	0.037±0.018
Male	(*n*=10)	(*n*=10)	(*n*=10)	(*n*=10)	(*n*=10)
Heart (%)	0.540±0.089	0.523±0.059	0.493±0.057	0.497±0.059	0.485±0.034
Lung (%)	0.704±0.080	0.674±0.083	0.662±0.096	0.640±0.0750	0.683±0.099
Spleen (%)	0.449±0.167	0.433±0.140	0.409±0.065	0.420±0.120	0.447±0.107
Kidney (%)	1.495±0.204*	1.526±0.085*	1.399±0.194	1.476±0.150	1.345±0.150
Liver (%)	5.443±0.766	5.732±0.894	5.676±0.363	5.488±0.491	5.374±0.291
Brain (%)	0.873±0.145	0.822±0.103	0.796±0.072	0.842±0.044	0.849±0.105
Thymus (%)	0.273±0.098	0.257±0.068	0.230±0.034	0.228±0.101	0.214±0.060
Testes (%)	0.513±0.056	0.547±0.083	0.528±0.074	0.499±0.062	0.507±0.070

Note.*Indicates statistically significant differences with respect to PBS group (P<0.05), $^{\#}$ indicates statistically significant differences with respect to bacteria protein group (P<0.05).

DISCUSSION

Subchronic toxicity test, immunotoxicity assessment, teratogenic study and nutritional assessment of the transgenic rice harboring the Cowpea trypsin inhibitor (*cpti*) and *hpt* gene developed by scientists of the Chinese Academy of Sciences[8] were conducted, showing that there are no adverse effects[9-12]. However, the disadvantage of these assessments is that the exposure level is much lower than the safety margin (animal exposure/human exposure) required in classical hazard assessment studies. Since the typical safety margin is 100-fold or above, it is not suitable to assess the foreign proteins by whole food feeding. To reach the safety margin for toxicity assessments, the method for obtaining enough foreign protein should be explored. In this study, about 10 g active HPT protein above 90% purity was obtained that would be sufficient for toxicity study in mice. Because the protein purity is

less than 100%, there must be some bacterial protein. The high dose HPT protein was 10 g/kg body weight, so about 1 g/kg body weight bacterial protein was incorporated. If any adverse effect was observed, it was hard to decide whether it was from HPT or from bacterial protein. Therefore, the tag free bacterial control plasmid was constructed and the control protein was produced for excluding the adverse effect from the bacterial protein.

The results of this acute oral toxicity study indicate that the maximum tolerable dose (MTD) of HPT was more than 10 g/kg in both male and female mice. This dose did not induce mortality, body weight gain and food consumption changes. Though some slight significant differences were observed in the relative organ weights of mice between the HPT groups and bacteria control protein group or PBS group, all the data were within the normal range, which are consistent with the reported findings[13] and not dose-related, suggesting that the differences are not related with treatment.

The rice consumption is rather high in many countries of Asia. According to the data obtained from "Survey on the Status of Nutrition and Health of the Chinese People" in 2002, the rice consumption is 238.3 g per standard person per day in China[14]. The expression level of HPT was extremely low in the transgenic rice line. The HPT level was not detectable in the GM rice grain by ELISA because it is below the lower limit of the detectable range of 0.03 μg/mL[15-16]. The amount of HPT protein in the GM rice seeds is only about 0.000008%-0.000015% (0.08-0.15 μg/g) detected by Western blot[6] and even lower than that of another antibiotic selector marker NPT II[17]. The non-effective dose of 10 g/kg HPT protein in mice is equivalent to that of the potential human exposure (238.3×0.15=35.7 μg) to consumption of transgenic rice (approximately 280 000-fold). This calculation is based on the very conservative assumption that no HPT protein is lost during processing of rice and 100% of the rice consumed is derived from transgenic rice.

In conclusion, HPT protein, as a selectable marker of the transgenic rice, does not pose any safety problem to human health.

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