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

QIN ZHUO, JIAN-HUA PIAO, YUAN TIAN, JIE XU, AND XIAO-GUANG YANG[2]

*Institute for Nutrition and Food Safety, Chinese Center for Disease Control and Prevention, Beijing 100050, China*

**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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[2]Correspondence should be addressed to Xiao-Guang YANG, Tel: 86-10-83132798. Fax: 86-1083132808. E-mail: xgyangcdc@vip.sina.com

Biographical note of the first author: Qin ZHUO, female, born in 1969, research associate, majoring in food safety of genetically modified organisms.

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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.

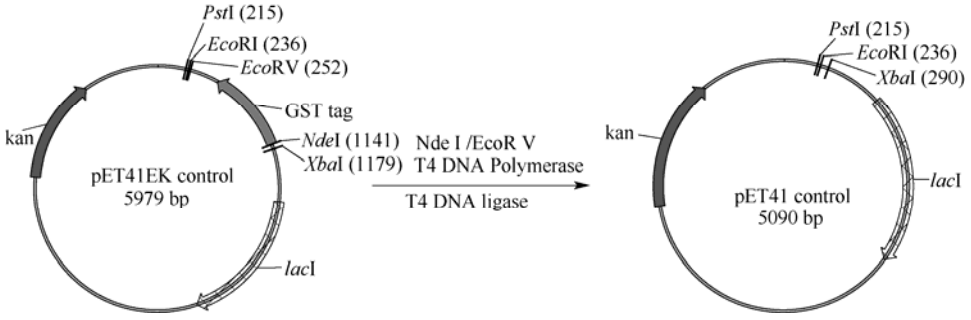


FIG. 1. Construction scheme of tag free control plasmid *pET 41 control*.