BIOMEDICAL AND ENVIRONMENTAL SCIENCES 16, 149-156 (2003)

Purification and Immunity Analysis of Recombinant 6His- HPT Protein Expressed in *E.coli* [1]

LI-CHEN YANG[#], ZHEN ZHU[*], AND XIAO-GUANG YANG[#]

[*]*Plant Genetic Manipulation Laboratory, Institute of Genetics, Chinese Academy of Sciences, Beijing 100101,China;* [#]*Key laboratory of Trace Element Nutrition of the Ministry of Health, China, Institute of Nutrition and Food Safety, Chinese Center for Diseases Control and Prevention, Beijing 100050, China*

Objective To obtain HPT protein (Hygromycin B Phosphotransferase), a kind of plant selective maker gene product expressed from *E.coli* and to prepare the polyclonal antibody (pAbs) against it. **Methods** HPT cDNA fragment was obtained by PCR and was inserted into the prokaryotic expressing vector pBV222. Then the constructed recombinant plasmid pBV222-HPT was transfered into *E.coli* DH5α for HPT expression. The recombinant expressing system was confirmed by restriction endonuclease digestion, DNA sequencing and protein expression. *E.coli* cells were lysed by sonication and detergent dissolution. After cell membrane was extracted, the inclusion bodies were denatured by 8 mol/L Urea and purified with metal chelate affinity chromatography on Ni-NTA agarose under denaturing condition. The purified 6His-HPT was characterized by SDS-PAGE, and used to immunize rabbit. The titer and specificity of antisera were detected by ELISA and Western blot respecitively. **Results** Analysis of DNA sequence and restricted enzymes showed that the sequence of PBV222-HPT plasmid was correct. The amount of recombinant HPT expressed in *E.coli* accounted for 30% of total cellular proteins. From 1 liter of fermentative bacteria about 22 milligrams of pure recombinant *HPT* was isolated with purity above 95%. The recombinant *HPT* protein could produce high titer antiserum in rabbits and show good immunity activity. Western blot showed specific binding reaction between the antiserum to the purified 6His-HPT protein and their expressed products (plants protein and bacterial protein). **Conclusion** HPT protein can be expressed and purified from *E.coli* by a relatively simple method, which has high immunity activity.

Key words: HPT; Expression and purification; *E. coli*; Immunity activity

INTRODUCTION

Selective marker genes are important for the production of genetically modified organisms (GMOs), and they are essential for the selection of modified cells at relatively low frequencies[1]. Marker genes are used to "tag" genes of interest and comprise several classes. Antibiotic resistance gene is one of the most commonly used markers in the modification process.

The enzyme Hygromycin B Phosphotransferas (HPT) is a widely used selective marker

[1]This work was supported by "973" Programme Funds (2001CB109001 and 2001CB109007) and "863" Programme Funds (2001AA212041, 2001AA212291, and 2002AA212041).

[*]Correspondence should be addressed to Zhen ZHU, Tel: 86-10-64873490, Fax: 86-10-64852890, E-mail: zzhu@genetics.ac.cn

Biographical note of the first author: Li-Chen YANG, female, born in 1974, Ph.D. candidate, dedicated to researches on safety assessment of genetically modified foods at the protein level.

0895-3988/2003
CN 11-2816

in various animal and plant transformation systems. Cells which had acquired foreign DNA were screened for their ability to grow in the presence of Hygromycin B (Hyg), an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes[2]. Hyg inhibits protein synthesis by blocking ribosomal translocation; it prevents polypeptide elongation by interfering with aminoacyl tRNA recognition and ribosomal A-site occupation[3]. Resistance can be conferred by a hygromycin B phosphotransferase (HPT) activity which was originally isolated from Streptomyces hygroscopicus[4]. HPT catalyzes phosphorylation of the 4-hydroxyl group on the hyosamine moiety, thereby inactivating Hyg[4].

Moreover, a host of experiments found that HPT was a marker gene for rice transformation with a higher efficiency than the traditional Kanamycin antibiotic gene (*NPTII*). Some kinds of insect-resistant rice with the selective maker HPT was developed by the Institute of Genetics, which showed excellent agronomic prospects and huge economic benefits and might provide one of the major avenues to meet the food need of the Chinese population. Certainly, the genetic modified products should be subjected to a careful and complete safety assessment before commercialization. Since the genetic modification ultimately results in the introduction of new proteins, including the marker gene protein, into the food plant, the safety of the newly introduced proteins must be assessed. Thus far, no study regarding the bio-safety of the HPT protein has ever been reported, which will limit its further application in GMOs and reaching the consumer marketplace. To facilitate the safety assessment of HPT protein, we need to develop a simple and efficient purification method to produce gram quantities of this protein in microbes, which could maintain its bioactivity and immunity.

MATERIAL AND METHODS

Chemicals

Molecular biological enzymes were purchased from Takala Ltd. (Daliang China,). Peroxidase–Conjugated Goat Anti-Rabbit IgG/(H+L) was purchased from Zhongshan Biotechnology Ltd. TMB Stabilized Substrate for HRP was bought from Promega Ltd. Ni-NTA Agarose was purchased from QIAGEN Ltd.

Animals

Adult male rabbits were gotten from Laboratory Animal Center, Institute of genetic, Chinese Academy of Sciences.

Cloning of HPT cDNA

To clone the translated region of the hygromycin B phosphotransferase (HPT), the method of PCR was used. Based on the published HPT gene sequence[5,6], two oligonucleotide primers (sense, 5′ GC GAA TTC CGG ATG GTG CCT ATC CAA AAA 3′; antisense, 5′CTA TTC CTT TGC CCT CGG ACG A 3′) were synthesized (Songon Biotech, Canada). The sense oligonucleotide was extended at the 5′ends (underlined above) containing recognition sequences for endonuclease XhoI. PCR amplification was performed for 35 cycles with 30 sec at 94℃, 30 sec at 63℃ and 2 min at 72℃. The sequence of the amplified PCR fragment was confirmed by the dideoxy chain-termination DNA sequencing method of double stranded DNA with the T7 Sequencing™ Kit (Pharmacia Boitech, Sweden).

Expression Vector Construction

The PBV222 vector was digested with *Pst*I at first and exonucleased with T4 DNA polymerases, and then digested with *Xho*I. The HPT gene fragment was subcloned into the corresponding site of pBV222 (National Laboratory of Gene Engineering, Virus research institute) to obtain pBV222-HPT by standard manipulation (Fig.1). Then the recombinant plasmid was transformed into the *E.coli* strain DH5α. The transformats for clonies that containing the cloned gene were screened by restriction enzyme analysis and protein expression.

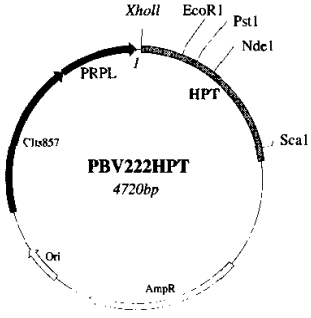

FIG.1. Structure of plasmid PBV222-HPT.

Protein Expression and Purification

An overnight culture of the *E.coli* DH5α containing pBV222-HPT at 30℃ was inoculated (1/10 volume) into fresh LB (Amp^+) medium and grown at 30℃ for 2 to 3 h. Then the temperature was increased to 42℃ continually for 4 to 6 h to induce the specific protein expression. After induction, the cells were pelleted by low-speed centrifugation and then resuspended in Sonication Buffer (0.01 mol/L Tris-HCl, 0.1 mol/L NaH_2PO_4, 1% Triton X-100, pH 8.0) at 5 mL per gram wet weight. Lysis was performed by sonication and detergent dissolution and the final inclusion body sediment was dissolved in Lysis Buffer (8 mol/L urea, 0.01 mol/L Tris-HCl, 0.1 mol/L NaH_2PO_4, pH 8.0)[7].

The Ni-NTA affinity column was assembled according to the manufacturer's instructions; the top adapter of the column was removed and the bottom outlet capped. A 50% Ni-NTA Superflow slurry was thoroughly resuspended and the slurry was poured into the column (5-10 mg protein per mL resin). The resin was allowed to settle. Top adapter was inserted and adjusted to top of bed. The column was equilibated with 5 column volumns of Lysis Buffer. Inclusion bodies lysate was applied to column and washed with Lysis Buffer until the A_{280} was below 0.01. Usually 5-10 column volumes were sufficient. Column was washed with wash buffer (8 mol/L Urea, 0.01 mol/L Tris-HCl, 0.1 mmol/L NaH_2PO_4, pH 6.3) until the A_{280} was below 0.01, which would remove proteins binding nonspecifically to the resin. The recombinant protein was eluted with Buffer A (8 mol/L Urea, 0.01 mol/L Tris-HCl, 0.1 mmol/L NaH_2PO_4, pH 5.9). Proteins usually elute in the second and third column volume. If elution is incomplete with Buffer A, it is followed by 4 times with 1 mL buffer B (8 mol/L Urea, 0.01 mol/L Tris-HCl, 0.1 mmol/L NaH_2PO_4, pH 4.5)[7]. The wash and the elute fractions were collected for SDS-PAGE analysis. The protein concentration was determined by method of Bradford.

Immunogenicity Assay on Recombinant Protein

Two rabbits were used to generate polyclonal antibody to the fusion protein. The 6His-HPT fusion protein was concentrated into 1 mg/mL. Each rabbit was injected with 300-500 ug denatured protein eluted from the Ni-NTA column. For the initial injection, the protein was mixed with equal volume complete adjuvant, and with uncompleted adjuvant for the following injections. Before injection to the selected rabbit blood was collected for pre-immunization. After inoculation for three times, some blood was collected from ear vein to detect antiserum titer with ELISA. If the titer was low, a fourth or more injections could be done. The entire blood was collected from the rabbit. The clear serum was divided into 1mL aliquots and was frozen at -20℃. One tube was kept at 4℃ for analysis.

Detection of the Antiserum

ELISA: The purified 6His-HPT protein were bound to the polystyrene microtiter plate at 100 ng each well. The final antiserum was diluted at 1:1 000 to 1:20 000 in PBS. The titer of the antiserum was to be detected[8].

Western Blot: After SDS-PAGE, moderate quantities of the total protein from transgenic rice and its contract, the purified recombinant protein and their expression bacterial proteins were transferred to the PVDF menbrance for the Western Blot. The anti-HPT antibody was rabbit polyclonal antibody. And the second antibody was sheep anti-rabbit IgG/HRP. The detailed procedures are shown in the Molecular Cloning.

RESULTS

Cloning of HPT cDNA

Fig. 2 showed that a gene fragment of about 1 026 bp gene fragment was obtained by PCR using primers derived from the published sequence of HPT gene. The gene fragment was cloned and sequenced in both directions, which proved the correctness of HPT sequence.

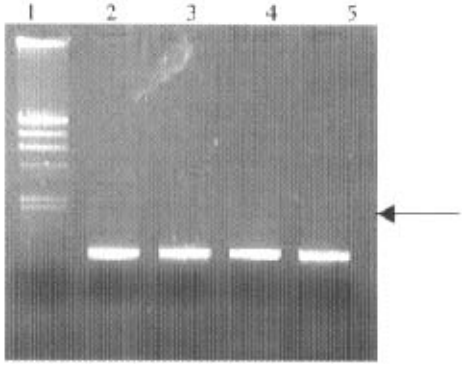

FIG. 2. Amplification of HPT gene fragment by PCR.

Lane 1: λDNA/Hind III marker. (from big size to small size:23 130 bp, 9 416 bp, 6 557 bp, 4 361 bp, 2 322 bp, 2 027 bp); Lanes 2-5: HPT DNA fragments amplified by PCR (Arrow indicates the HPT gene fragment).

Restriction Analysis of Recombinant Vector

The recombinant vector was identified by restriction analysis with *Bgl*II/*Nde*I, *Bam*HI/*Xho*I, *Sca*I and compared with that of λ/*Hin*dIII DNA maker in gel electrophoresis. (Fig. 3). Theoretically, *Bgl*II/*Nde*I, *Bam*HI/*Xho*I, *Sca*I digested the recombinant vector into 3956 bp/747 bp, 3694 bp/ 1026 bp, and 3813 bp/890 bp respectively. Fig. 3 showed that the recombinant plasmids were separated into the corresponding bands. The results of Fig. 3 indicated that the structure of recombinant plasmid was correct.

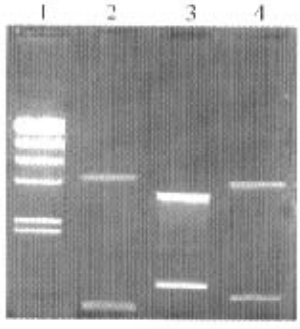

FIG.3. Map of digestion of recombinant plasmid pBV222-HPT by restriction enzymes
1. λ DNA /*Hin*dIII maker (from big size to small size:23 130 bp, 9 416 bp, 6 557 bp, 4 361 bp, 2 322 bp, 2 027 bp); 2. *Bgl*II/ *Nde*I (747, 3 956); 3. *Bam*HI/ *Xho*I (1 026/ 3 694); 4. *Sca*I (890,3 813).

Determination of Recombinant Expression System

The translated region of the HPT gene was cloned into the expression vector pBV222. Shifting the culture temperature from 30℃ to 42℃ led to a high level expression of 6His-HPT fusion protein, the amount of which accounted for 30% of total cellular proteins by UV scanning (Fig. 4), analyzed by SDS-PAGE. The molecular weight (MW) of the specific product was estimated to be about 36 kDa being equal to the MW of 6His-HPT, and mainly existed as inclusion body in the precipitate.

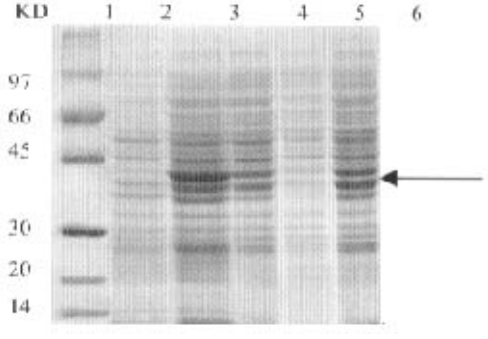

FIG. 4. *Expression of recombinant 6His-HPT gene product in E. coli DH5α*
Lane 1 standard middle weight proteins maker; Lane 2 bacteria including PBV222-HPT non-induced by temperature change; Lane 3 bacteria including PBV222-HPT induced by temperature change; Lane 4 total bacteria proteins with PBV222-HPT after sonication; Lane 5 supernatant of the lysate ; Lane 6 proteins of inclusion body in the precipitate.

Recombinant HPT Expression and Purification

The HPT gene product expressed in *E.coli* existed primarily as inclusion body and was purified using Ni-NTA agarose under denaturing conditions. Subsequent differential fractionation filtered on Ni-NTA column were shown in Fig. 5. The isolated protein was greater than 95% pure examined by UV scanning and from one liter of fermentative bacteria 22 milligrams of pure recombinant HPT could be obtained.

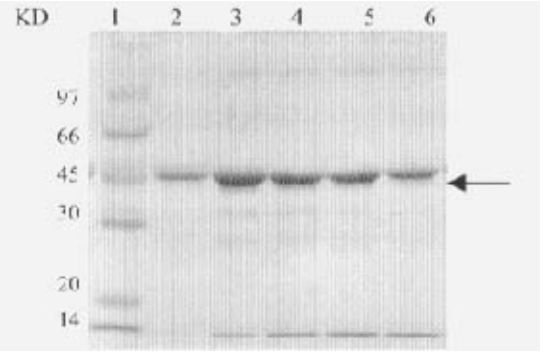

FIG.5. Isolation of recombinant HPT from *E.coli* DH5αby SDS-PAGE.
Lane 1: standard middle weight proteins maker; Lane 2-6: the 6His-HPT protein fractions during purification.

Detection of Antiserum With ELISA and Western Blot

After three rounds of immunization with 6His-HPT, two rabbits were all generated high titer antiserum (1:15 000), which was measured by ELISA. Western blot analysis showed that there was a specific Ag-Ab binding band in the relative molecular weight 37KD position between antiserum to the purified 6His-HPT protein, the expressed product (bacterial protein) and the whole proteins of GMOs (Fig. 6). We consider that the HPT protein was thought to be identified by the 6His-HPT pAb.

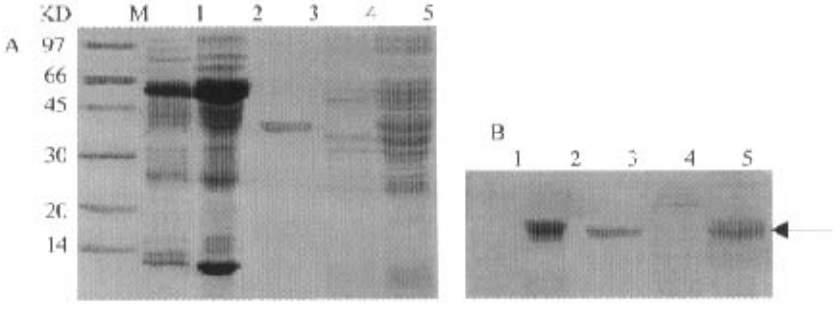

FIG. 6. Western Bolt analysis of HPT
A. SDS-PAGE B. Western Blot
1. whole lamina proteins of non-transgenic rice; 2. whole lamina proteins of transgenic rice; 3. purified 6His-HPT protein; 4. Cell lysate of *E.coli* DH5α (PBV222-HPT) before induction; 5. Cell lysate of *E.coli* DH5α (PBV222-HPT) after induction.

DISCUSSION

In the bio-safety assessment of the GMOs, the safety of the expression product (proteins) of the introduced foreign genes is an issue of major concern. However, the quantity of these kinds of product is known to be quite small in the GMOs and it is not easy for us to get them in a large amount in a directly way to evaluate their safety. So we turned to produce these proteins in *E.coli* expression system at first, and then to immunize some animals with the purified protein to generate antibodies against them. In return, we can use them to prepare the specific affinity column and gain the corresponding plant proteins finally. According to the "Substantially Equivalent" concept[9], if we can demonstrate that the microbe produced protein is equal to the plant expression protein, then we can use the former one to carry out a series of safety assessment experiments.

At the beginning of this work, the HPT gene was cloned downstream of GST of prokaryotic expression vector PGEX4T-3 and transferred into an *E.coli* strain BL21. By Glutathione Sephrose 4B affinity chromatography, the fusion protein GST-HPT was obtained with the purity of 90% and with high immunity activity. In order to remove the GST tag, we inoculated the fusion protein with Thrombin protease for 6 h and detected the eluted liquid by SDS-PAGE. No 37 KD (the molecular weight of HPT) protein could be found, for there was some kind of Thrombin recognition site in the sequence of HPT. We considered that the fusion with GST tag and the refolding of recombinant protein could not cover the site.

As a consequence we changed to express HPT in the PBV222 vector, another kind of prokaryotic expression vector with the high efficiency P_RP_L promoters and a His-6 sequence in the carboxyl terminus. Shifting the culture temperature from 30℃ to 42℃ led to a high level expression of 6His-HPT in the form of inclusion body. His-6-labelled proteins can be purified substantially in a single step by metal chelate affinity chromatography (IMAC). This protocol has become popular for it is both high effective and relatively insensitive to proper protein folding, ionic strength, chaotropes and detergents. Moreover, the purification can be performed under denaturing conditions (e.g., in buffers containing 8 mol/L urea and 6 mol/L guanidine hydrochloride), so it is quite fit for the purification of inclusion body[10]. On the other hand, His-6 sequence is a small tag, and has little effect on the structure and function of the target protein, so it is quite convenient for the purification of Ag protein for preparing the corresponding Ab.

In our study, 37KD of 6His-HPT fusion protein band appeared on SDS-PAGE after induction. This expression was further confirmed with Western blot. The recombinant proteins were eluted from Ni-NTA affinity column using a series of buffers of decreasing pH. The pure 6His-HPT was used to immunize rabbits and generated specific polyclonal antibody at a high titer. All these findings are quite important for both further bio-safety evaluation experiments and the application of HPT to wider varieties of GMOs.

REFERENCES

1. Dale, E. C. and Ow, D. W. (1991). Gene transfers with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA.* **88**, 10558-10562.
2. Waldron, C., Murphy, E.B., Roberts, J.L., Gustafson, G.D., Armour, S.L., and Malcolm, S.K. (1985). Resistance to hygromycin B. *Plant Molecular Biology* **5**, 103-108.
3. Hausner, T.P., Geigenmuller, U., and Nierhaus, K.H. (1988). The allosteric three-site model for the ribosomal

elongation cycle. New insights into the inhibition mechanisms of aminoglycosides, thiostrepton, and viomycin. *J. Biol. Chem.* **263** (26), 13103-13111.

4. Rao, R.N., Allen, N.E., Hobbs, J.N., Alborm, W.E., Kirst, H.A., and Paschal, J.W. (1983). Genetic and enzymatic basis of hygromycin B resistance in Escherichia coli. *Anitimicrob Agents Xhemoth.* **24**, 689-695.5. Gritz, L. and Davies, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyxes cerevisiae*. *Gene* **25**, 179-188.
6. Zheng, Z., Li, Z., Hayashimoto, A., and Murai, N. (1991). Hygromycin resistance gene cassettes for effective selection of transformed rice protoplasts. *Plant Physiology* **97**, 832-835.
7. The QIA expressionist. (January 2000). A handbook for high-level expression and purification of 6 His-tagged proteins. QIAGEN.
8. Van Regenmortel, M.H. (1988). Which value of antigenic valency should be used in antibody avidity calculations with multivalent antigens? *Mol. Immunol.* **25**(6), 565-567.
9. OECD. Safety evaluation of foods produced by modern biotechnology: Concepts and principles. OECD, Paris, 1993.
10. JosepH. S. and David, W. (2001). Purification of Histidine-tagged proteins by immobilized Ni^{2+} absorption chromatograpy. New York: Cold Spring Harbor Laboratory Press, **15**, 44-48.

(*Received December* 28, 2002 *Accepted March* 11, 2003)