

Prokaryotic Expression and Allergenicity Assessment of Hygromycin B Phosphotransferase Protein Derived from Genetically Modified Plants

Y. LU, W. XU, A. KANG, Y. LUO, F. GUO, R. YANG, J. ZHANG, AND K. HUANG

ABSTRACT: The *hygromycin B phosphotransferase gene (hpt)* has been widely used in the process of plant genetic engineering to produce plants that can secrete the HPT protein. As part of a safety assessment, sufficient quantities of the protein were produced in *Escherichia coli* to conduct *in vitro* digestibility and animal studies. Western blotting analysis showed that the HPT protein was digested by simulated gastric fluid within 40 s. ELISA demonstrated that the protein did not induce detectable levels of specific IgE antibodies or histamine in test animals. Alignment of the amino acid sequence of HPT with those of known allergens did not produce evidence of sequence similarities between these allergens and the HPT protein. We conclude that HPT has a low probability to induce allergenicity.

Keywords: allergenicity, animal model, HPT protein, *in vitro* digestibility, safety assessment

Introduction

The *hygromycin B phosphotransferase gene (hpt)*, derived from aminoglycoside 4-phosphotransferases encoded by *aph (4)-Ta* of *Streptomyces hygroscopicus*, is a useful selectable marker to screen for transformants in gene transfer experiments involving plants (Linda 1983). The gene codes for an enzyme, which detoxifies hygromycin B through phosphorylation. Hygromycin B is an aminocyclitol antibiotic that inhibits protein synthesis in both prokaryotes and eucaryotes by interfering with both ribosomal translocation and aminoacyl-tRNA recognition (Waldron and others 1985).

The gene is found in several transgenic plants such as rape, rice, corn, and so on (Li and Narayana 1991; Sawahel 2003). However, before these plants can be commercialized, it is necessary to conduct safety assessments (FAO/WHO 2001).

Since the level of HPT in these plants is low, HPT was produced in *E. coli* to obtain sufficient HPT protein for *in vitro* digestion and animal studies.

Materials and Methods

Materials

The *E. coli* strains *DH5 α* and *BL21 (DE3)* (reserved in lab at -80°C) were used for cloning and protein expression, respectively. The plasmid pET30a was used for preparing the His₆-Tag expression vector. Restriction enzymes and other enzymes were purchased, respectively, from Promega (Promega Co. Beijing, China) and TaKaRa (TaKaRa Biotechnology Co., Ltd., Dalian, China). Simulated gastric fluid and intestinal fluid digestion were pur-

chased from Sigma (Sigma Co., St. Louis, Mo., U.S.A.). Mouse antirat IgE serum and HRP-goat antimouse IgG serum were purchased from PharMigen (PharMigen Bioscience Co., Calif., U.S.A.). His₆-Tag monoclonal antibody and Ni-NTA metal-affinity column were purchased from Novagen (Merck Co., Darmstadt, Germany). O-phenylene diamine was purchased from Sigma. Rat histamine kit was purchased from RapidBio Calabas, Calif., U.S.A. The biochemicals and kit were used according to the recommendations of the supplier. All other common reagents were of analytical grade.

Comparison of the HPT amino acid sequence with that of other proteins

The sequence of HPT was analyzed by the procedure formulated by the FAO/WHO Consultation on Assessment of the Allergenicity of Genetically Modified Food (FAO/WHO 2002). The sequence of HPT protein was aligned with the complete entries in the Protein Information Resource (PIR database <http://pir.georgetown.edu>) using FASTA (version 2.4, 2002) (Gendel 1999).

Construction of protein expression system in prokaryotic cell

The *hpt* gene (GenBank Accession Number: E00287) was cloned from the genome of genetically modified plants by polymerase chain reaction. Two oligonucleotide primers were designed (5'CGC GGA TCC GCG ATG AAA AAG CCT GAA3' and 5'CCC AAG CTT GGG TCT ATT TCT TTG CCC TC3'), which defined the sequence of *hpt* gene and introduced *Bam*H I and *Hind*III at the 5' and 3' end of the polymerase chain reaction product, respectively. Polymerase chain reaction conditions were as follows: preincubation at 95°C for 5 min, 35 cycles consisting of denaturing at 94°C for 30 s, annealing at 58°C for 60 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. The polymerase chain reaction product was gel purified and digested with *Bam*H I and *Hind*III, then ligated at 15°C overnight into *Bam*HI/*Hind*III-digested pET30a(+) through a standard T4 DNA ligase procedure (according to the instruction). After transformation into *E. coli* strain *DH5 α* cells, the recombinant plasmids were screened by digestion with *Bam*H I and *Hind*III, then confirmed

MS 20060611 Submitted 11/10/2006, Accepted 6/12/2007. Authors Xu and Huang are with College of Food Science and Nutritional Engineering, China Agricultural Univ., Beijing 100083, China. Authors Lu, Xu, Luo, Guo, Yang, Zhang, and Huang are with The Supervision, Inspection & Testing Center of Genetically Modified Organisms, Ministry of Agriculture, Beijing 100083, China. Author Kang is with Dept. of Laboratory Animal Science, Peking Univ. Health Science Center, Peking Univ., Beijing 100053, China. Direct inquiries to author Huang (E-mail: hkl009@163.com.)

Authors Lu, Xu, and Kang contributed equally.

by DNA sequencing. The constructed plasmid was designated as pET30a-*hpt*.

The sequence of the *hpt* gene was independently verified by Shanghai Co. Ltd., Shanghai, China, to ensure exact construction. The encoding sequence was compared with the sequence reported by GenBank (GenBank Accession Number: E00287).

Expression and purification of HPT protein

Plasmid pET30a-*hpt* was introduced into *E. coli* BL21. The expressions of the HPT fusion protein were done in 200 mL cultures of Luria-Bertain (LB) liquid medium at 37 °C. The cells were grown until an OD (600 nm) of 0.6 to 1.0 was reached, 0.6 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added, and the culture was incubated for 3 h at 37 °C. Cells were collected by centrifugation and resuspended in binding buffer (20 mM Tris-HCl, 0.2 M NaCl, 5 mM iminazole pH 8.0). Then they were treated with lysozyme (2 KU of lysozyme per gram cell paste) and sonicated (40% to 50% power) (Xu 2005). The lysate was subjected to centrifugation and the supernatant was analyzed for HPT content by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The purification of HPT was performed by Ni-NTA metal-affinity chromatography. Purified protein was obtained first by binding to a Ni-NTA-agarose column followed by removal of the His6-Tag with an enterokinase (EK) for 18 h at 37 °C (Xu 2005).

Identification of the recombinant HPT protein

The purified HPT protein was identified by SDS-PAGE and Western blotting. Proteins were transferred to a nitrocellulose (NC) membrane (Millipore Co., Bedford, Mass., U.S.A.), which was blocked with 1% BSA for 2 h and washed 3 times with TBST (2 mM Tris-HCl, 15 mM NaCl, pH 7.5, 0.05% Tween-20). His₆-Tag monoclonal antibody (Novagen), diluted in TBS (with 0.1% BSA), was added and the membrane was incubated for 1 h. The membrane was washed with TBST 6 times, and was exposed to peroxidase-conjugated goat antimouse IgG secondary antibody (Sigma), washed 6 times, and the substrate, 4 chloro-1-naphthol was added. After color appeared, the membrane was washed twice with distilled water (Sambrook and Russell 1999a).

Polyclonal antibodies to the HPT protein were produced by immunization of New Zealand White rabbits receiving 1 mg/mL emulsified HPT protein with intradermic injections 5 times. Antiserum was obtained from blood samples after ammonium sulfate precipitation (Ba 1998) and purification with a protein A-Sepharose 4B column (Zhao 2003). ELISA was used to test the sensitivity and specificity of the antiserum. And total proteins were extracted from nongenetically modified plants as the reported methods (Xu 2005).

In vitro digestion of HPT protein

HPT protein was added into simulated gastric fluid (SGF; porcine pepsin in 0.03 M NaCl at pH 1.2) (Sigma Chemical Co.) to a final concentration of 2 μ g/mL, incubated at 37 °C for 40 s and then adjusted to pH 8.0 with 100 mM Tris-HCl, pH 9.5, 2 mM CaCl₂ for intestinal fluid digestion (IFD; porcine trypsin and bovine chymotrypsin) (Sigma Chemical Co.) (US Pharmacopeia 1990; Roesler and Rao 2001) at 1:100 ratio (digestive enzyme: protein). The reaction was stopped with a buffer solution (50 mM Tris-HCl, 100 mM NaCl, pH 9.5). The samples were removed at different times, mixed with protein denaturing 2 $\times$ SDS-PAGE sample buffer, boiled, subjected to SDS-PAGE, and then transferred to membranes for Western blotting as described by Sambrook and Russell (1999b). The experiments were also done for positive control (OVA) and negative control (bovine serum albumin) as the method above.

Allergenicity evaluation of HPT protein in brown Norway (BN) rats

Three groups of 4-wk-old male brown Norway (BN) rats ($n = 6$) were used. Ovalbumin (OVA), a potent respiratory and food allergen, was used as positive control (Knippels and others 1998; Kimber and Dearman 2001). Rats received 1-mg OVA or 5-mg pure HPT protein both dissolved in 1-mL water, without the use of adjuvants, by daily gavage for 42 d. The 3rd group received only 1-mL water as the negative control.

Measurement of IgE, IgG, and histamine

The ELISA techniques were used to measure total and specifically induced serum IgE antibodies for OVA and the recombinant HPT protein. Ninety-six-well microliter plates (Dynex, Technologies, Va., U.S.A.) were coated overnight at 4 °C with a 10 μ g/mL solution of OVA (SigmaA-5503) or with a 100 μ g/mL solution of HPT protein in carbonate buffer (50 mM carbonate/bicarbonate, pH 9.6). The plates were washed 3 times with 200 μ L/well of PBST (containing 0.1% Tween-20). This was followed by addition of 150 μ L/well of blocking buffer (PBS containing 5% skim milk). After 1 h of incubation at 37 °C, the plates were washed, and 1:10 dilutions of rat serum in blocking buffer were added to the well and incubated for 1 h at 37 °C. After washing, 100 μ L/well of mouse anti-rat IgE serum diluted 1:200 (PharMingen Bioscience) with blocking buffer were added. After incubation for 1 h at 37 °C, the plates were washed again, and 100 μ L/well of HRP-goat antimouse IgG serum (PharMingen Bioscience) diluted 1:5000 with blocking buffer were added and incubated at 37 °C for 1 h. The plates were washed 6 times, and 100 μ L/well of fresh substrate O-phenylene diamine (Sigma) was added. The plates were developed at 37 °C for 30 min. Finally, the reaction was stopped with 50 μ L/well of 2 M sulfuric acid. Optical density was read spectrophotometrically at 492 nm with an ELISA plate reader (Finland LABSYSTEMS). IgG was analyzed in the similar way as above. And histamine was tested with a rat histamine kit (RapidBio).

Results and Discussion

With the development of genetically modified crops, more and more interest has been focused on the potential allergenicity of novel gene products. To ensure that a novel protein is not a potential allergen, certain criteria must be met (Taylor and others 2002). If a protein has not been consumed before or its source is not known, that is, no definitive test can be used to evaluate the allergic response, its allergenicity must be assessed using amino acid sequences analysis and *in vitro* digestibility (ILSI HESI 2001). An animal model also can be used (FAO/WHO 2001). All potential characteristics of the protein must be considered to evaluate its potential allergenicity.

Sequence similarity analysis

At present, about 200 kinds of main allergen, including their amino acid sequences, are known (Lin and Ni 2001). In general, if at least 8 continuous amino acids are identical to those in the sequences of known allergens, the similarity is considered significant. The reason is that the shortest peptide chains to bind to T cell to trigger allergic reaction include 8 or 9 amino acids (Zhang 2004). Also, antigenic determinant binding to IgE requires much longer peptide chains. So, it is a reliable way to determine similarity with a standard of 8 amino acid residues (Rothbart and Gefter 1991). The HPT protein sequence was compared with each member sequence in databases containing known allergen sequences (Gendel 1999). The protein had no significant similarity with known allergy proteins (PIR database, NCBI, PubMed, EBI, and

CBI: www.cbi.pku.edu.cn). This negative result indicates that the protein is unlikely to be cross-reactive to antibodies of known allergens. But sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. Also, they have limitations in the ability to detect noncontiguous epitopes capable of folding themselves in space and binding specifically to IgE antibody.

Construction of protein expression system in prokaryotic cell

The *hpt* gene (GenBank Accession Number: E00287) was amplified by polymerase chain reaction and correctly inserted into the pET30 (+) vectors at the *Bam*H I site and *Hind* III site. DNA sequencing results (data not shown) further confirmed that there was no error or unwanted mutation in the inserted sequence and that the *hpt* gene was correctly inserted into the expression vector pET30 (+) in-frame with the His₆-Tag gene.

Expression, purification, and identification of HPT protein

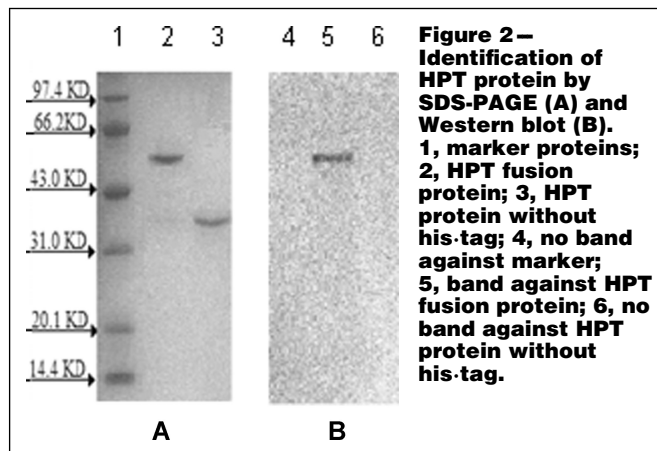
E. coli BL21 (DE3) containing the *hpt* gene was used to produce HPT protein instead of extracting it directly from genetically modified plants. Tests for evaluating equivalence between the bacteria- and the plant-derived forms were carried out and the results demonstrated that the protein produced by *E. coli* was an appropriate substitute for that extracted directly from genetically modified plants (data not shown). The HPT fusion protein was highly expressed in soluble form (Figure 1). The HPT fusion protein was about 45 kDa, and homogeneous HPT protein of about 38 kDa was obtained when the His₆-Tag was cleaved by EK (Figure 2A).

To elucidate whether the expressed protein was the recombinant HPT His₆-Tag fusion protein, SDS-PAGE and Western blot were performed. The results indicated that HPT fusion proteins were expressed with the right size of 45 kDa. Reactions occurred between His₆-Tag monoclonal antibody and fusion proteins, but not with proteins without tag (Figure 2 A and 2B).

The antiserum reached a very high degree of sensitivity to recombinant HPT protein. The cross-reactivity determined experimentally showed a high degree of specificity for the antiserum used, because values were all less than 0.1%.

In vitro digestibility of the HPT protein

With the exception of pollen protein, all allergenic proteins have high stability to digestive enzymes (Astwood 1996). Therefore this stability can be used as an indicator of the potential of a protein being an allergen (Astwood and others 1996). The digestibility of HPT



protein was confirmed by using SGF and IGE. SDS-PAGE stained gel and immunoblotting of the protein samples (Figure 3A and 3B) indicated that high molecular weight proteins were completely digested within 40 s. The positive control protein was affected differently in accordance with previous studies (Astwood and others 1996). OVA showed persistent resistance to hydrolysis at $t > 40$ min, while bovine serum albumin underwent rapid hydrolysis ($t < 20$ min) (data not shown).

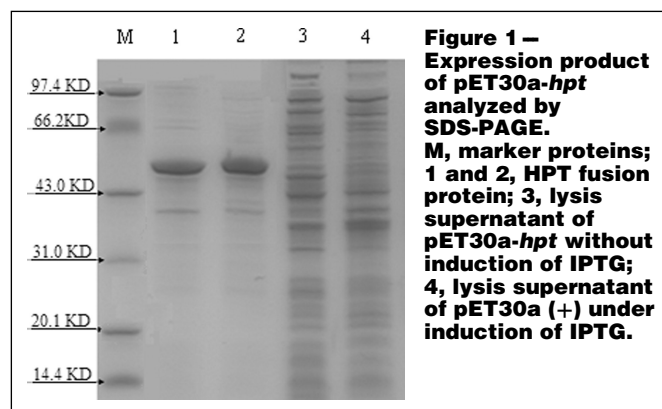
It has been reported that proteins that are rapidly hydrolyzed to peptides and single amino acids smaller than 3.5 kDa by pepsin can be considered less likely to be allergenic (Fu and others 2002). And if protein allergens are to reach and pass through the intestine mucosa to elicit an allergic response, they must be able to resist peptic and tryptic digestion and the acid conditions of the digestive system. The HPT protein was degraded rapidly by digestive enzymes as assessed by both SDS-PAGE and Western blot. In addition, the HPT protein was unstable in heat, especially at temperatures above 100 °C that are used for processing of most crops (Cai and others 2006). These results suggest, but do not prove, that the protein has no allergenic activity, since the correlation between resistance to proteolysis and allergenic activity is not absolute (Fu and others 2002).

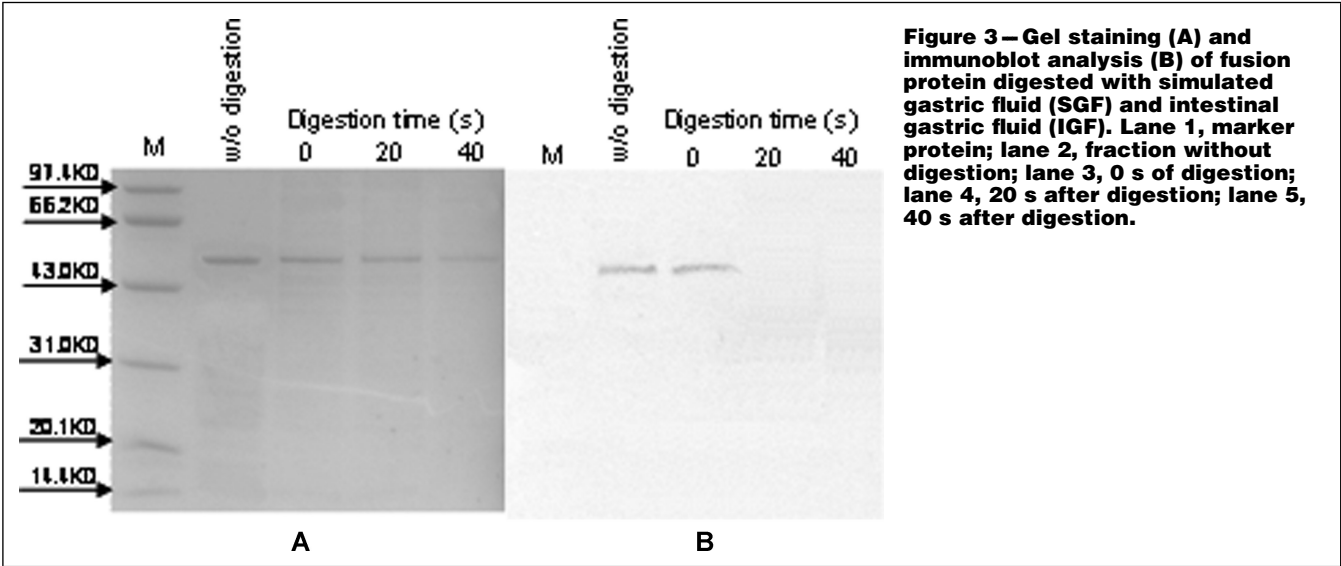
Antigen-specific IgG response

The positive group of BN rats treated by gavage with OVA showed significant immunoreaction and their sera, which were diluted by 32-fold, had a much higher level of specific-IgG antibody than that of the negative group ($P/N > 2$, Guo 2001). In contrast, the test group's immune response was basically equivalent to that of the negative group (Table 1). This result indicates that OVA triggered an obvious immunology reaction, but HPT protein did not.

Evaluation of IgE response

Food allergies are adverse reactions to an otherwise harmless food or food component that involves an abnormal response of the body's immune system to specific proteins in foods. True food allergies may involve several types of immunological responses, mainly including IgE-mediated reactions and cell-mediated reactions. The most common type is the former, also known as immediate hypersensitivity reactions because symptoms occur within minutes to a few hours after ingestion of the offending food (Zhang 2004). Therefore, when fed allergic proteins, the immune system of animals could produce specific IgE antibodies, and ELISA was carried out to test their production. The OD values of sera for the OVA-group were higher than those of the water group, and they were significantly different ($P/N < 2$, Guo 2001). In contrast, the OD values





corresponding to the HPT protein were similar to those of the negative group. The result showed that the HPT protein did not evoke specific IgE antibodies, whereas high levels of specific anti-OVA IgE levels were found.

Results of histamine test

From Table 2, we can see that the level of histamine of the test group was not significantly different from that of the negative group, while that of the positive was 2-times higher than and differed significantly from that of the test group ($P < 0.05$).

FAO/WHO has proposed the decision tree to assess the potential allergenicity of novel gene products. If the introduced gene encodes a known allergen, the recombinant protein may be tested for allergenicity by the classical *in vitro* tests using sera. If the source of the transgene has no history of allergenicity, alternative approaches are required based on the structure and stability of the foreign protein. These approaches must be critically appraised and applied on a case-by-case basis. The HPT protein belonged to the latter group, so it was assessed mainly by *in vitro* gastrointestinal digestion and by an animal model. At present, digestion *in vitro* is an accepted model system, but good (oral) animal models to study food allergy and allergenicity of food proteins are not (yet) available. Several strains of rodent have been developed, but are still under discussion. At present, most studies have been done with BN rats receiving the

potential allergen via gavage without the use of adjuvant (Kimber and Dearman 2001; Knippels and André 2005).

We adopted the 3R International Principle that proposes that the number of test animals should not exceed 6 and that healthy conditions should be maintained (Zheng 2005). We demonstrated that under these conditions, the BN strains could be a good allergy model.

Conclusions

This research successfully constructed the HPT protein expression system in *E. coli* and evaluated the potential allergenicity of the recombinant protein. *In vitro* digestions in SGF and animal tests indicated that the HTP protein has likely a low allergenic potential. Further trials still need to be done, for the safety assessment system has its shortcomings (Astwood 1996; Cockbrun 2002; Hileman 2002; Fu and others 2002). And methods still need to be developed for more comprehensive and exact evaluation of such introduced new protein by biotechnology.

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Table 1 – IgG and IgE antibody production following oral exposure of brown Norway rats to OVA, HPT protein, and tap water

Treatment	Number of responders			
	IgG		IgE	
	28th d	42nd d	28th d	42nd d
OVA (l mg)	5/6	4/6	4/6	5/6
HPT	0	0	0	0
Tap water	0	0	0	0

Table 2 – Effects of OVA, HPT protein, and tap water by oral administration on plasma histamine level

Groups	28th d	42nd d
OVA (l mg)	40.4 ± 9.7	41.3 ± 9.8
HPT protein (5 mg)	20.7 ± 7.8	16.7 ± 6.1
Tap water	20.3 ± 9.8	18.0 ± 7.8

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