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A Novel Phage Display Vector for Easy Monitoring of Expressed Proteins

Young Chul Shin, Young Eun Kim and Tae-Ju Cho*

Division of Life Sciences, College of Natural Sciences and Research Institute of Genetic Engineering, Chungbuk National University, Cheongju 361-763, Korea

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Phage display of proteins is a powerful tool for protein engineering since a vast library of sequences can be rapidly screened for a specific property. In this study, we developed a new phage display vector that was derived from a pET-25b(+) vector. The pET-25b(+) was modified in order that the expressed protein would have a T7-tag at the amino terminus and Gp8 (a major coat protein of M13 phage) at the carboxyl terminus. Another vector without the gp8 gene was also constructed. The newly developed phagemid vectors have several advantageous features. First, it is easy to examine whether or not the target proteins are functional and faithfully transported into the periplasmic space. This feature is due to the fact that recombinant proteins are produced abundantly in the pET system. Second, the T7-tag makes it possible to detect any target proteins that are displayed on the surface of filamentous bacteriophage. To verify the utility of the vector, the clones containing the glutathione S-transferase (GST) gene as a target were examined. The result showed that the GST produced from the recombinant vector was successfully transported into the periplasmic space and had the anticipated enzyme activity. Western blot analysis using a T7-tag antibody also showed the presence of the target protein displayed on the surface of the phage. The phages prepared from the recombinant clones were able to bind to glutathione-Sepharose and then eluted with glutathione. These results showed that the new vectors developed in this study are useful for the phage display of proteins.

Keywords: Phage display, Phagemid vector, T7-tag.

Introduction

Phage display utilizes filamentous bacteriophage as a vehicle for displaying target peptides, or proteins on their surface. Since it was first developed by Smith (1985) phage display has been a powerful technique to screen for the proteins, or peptides with specific properties (Hoess, 1993; Makowski, 1994; Winter, 1994). In principle, the gene of interest is inserted into the N-terminus of either the major coat protein, Gp8 (ca. 2800 copies per phage), or a minor tip protein, Gp3 (ca. 5 copies per phage), of filamentous phage. Then, the target protein, or the peptide, is displayed as a fusion protein with Gp3 or Gp8 on the surface of filamentous phages. The phage display is advantageous as a vast number of variants can be rapidly screened for a specific protein, or a peptide. A phage library is enriched for the variants that bind to an immobilized ligand. This enrichment is accomplished either by biopanning, or by affinity chromatography (Jackson *et al.*, 1992; Lowman *et al.*, 1991).

In our laboratory, efforts were made to apply the phage display technique to protein engineering. Initially, a pCANTAB5E vector, which was developed by Cambridge Antibody Engineering and Pharmacia (Jackson *et al.*, 1992), was used for this purpose. This vector is designed for use in selecting an antibody and a receptor, which have a high affinity to ligands. Since the target gene inserted in the pCANTAB5E vector is expressed rather weakly, there is a good possibility that the target protein is displayed in a monovalent fashion. However, the very feature of the pCANTAB5E vector was found to be unsuitable for protein engineering, since most of proteins including enzymes do not have such a high affinity to a ligand. In this study, we developed a new vector that is better suited for displaying proteins that do not show high-affinity interactions with ligands. The newly developed vector was based on a pET vector in order that the target gene cloned into the vector would be expressed strongly. The vector has also been designed in such a way that the target protein has a tag at its amino terminus. The peptide tag provides a tool for easy monitoring of the target proteins expressed in the vector.

Materials and Methods

Materials The pCANTAB5E vector was purchased from Pharmacia. The BL21(DE3), Nova-blue(DE3), pET-25b(+) and

*To whom correspondence should be addressed.
Tel: 82-431-261-2309; Fax: 82-431-267-2306
E-mail: tjcho@cbucc.chungbuk.ac.kr

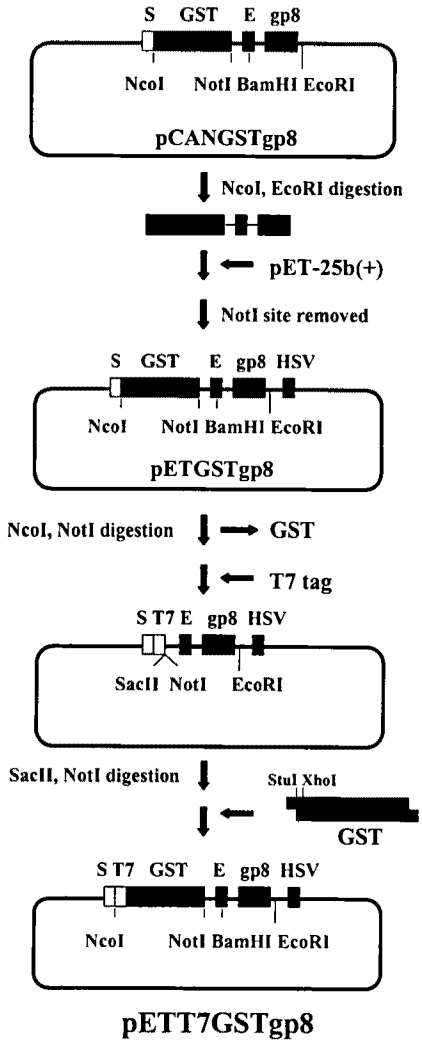

Fig. 1. Construction of a novel phage display vector. The pET-25b(+) was modified to a phage display vector by inserting the gp8 coding sequence of M13 phage. T7-tag sequence was also inserted for easy detection of target proteins. The details are described in Materials and Methods. E, E-tag; HSV, HSV-tag; S, signal peptide and T7, T7-tag.

T7-tag antibody HRP conjugate were from Novagen. Glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were from Sigma. The *Taq* DNA polymerase and restriction enzymes, including *Not*I, *Nco*I, *Eco*RI, *Bam*HI, *Sac*II, were purchased from Bioneer, Promega, Boeringer Mannheim and Takara. The ECL kit was obtained from Amersham. The GSH-Sepharose was kindly provided by Dr. Chung at Chungbuk National University. Centricon 500K was from Amicon.

Construction of a phage display vector The overall procedure for the construction of the pET-based phage display vector is depicted in Fig. 1. The oligonucleotides used for this study are shown in Table 1. The polymerase chain reaction (PCR), as well as the other DNA manipulations, were done as described by Sambrook *et al.* (1989). The *E. coli* GST gene (Genbank accession # D38497) was amplified by PCR using the primers EC-GST-F and EC-GST-R. The PCR product was digested with *Nco*I and *Not*I and cloned into the *Nco*I/*Not*I site of pCANTAB5E. The gp3 gene in the pCANTAB5E was removed by digestion with *Bam*HI and *Eco*RI and replaced with the gp8 sequence that was PCR-amplified with GP8PRF1 and GP8PRR1 primers using M13mp18 DNA as a template. The resulting plasmid construct was designated as pCANGSTgp8. The *Nco*I-*Eco*RI fragment containing the GST and gp8 genes was isolated from the pCANGSTgp8 and cloned into the *Nco*I/*Eco*RI sites of the pET-25b(+) vector. The original *Not*I site in the pET vector was removed by self-ligation of the vector DNA after it was digested with *Sal*I and *Xho*I, and purified by gel electrophoresis. The resulting construct was named pETGSTgp8. Then, the DNA fragment containing the GST gene was deleted by digestion with *Nco*I and *Not*I. The linker containing the T7-tag sequence was inserted into the *Nco*I/*Not*I sites. The T7 linker was prepared using two oligonucleotides, NSN-42-1 and NSN-42-2. The resulting plasmid was then digested with SacII and *Not*I and ligated with the DNA containing the GST gene obtained by PCR amplification using SSX EC-GST-F and EC-GST-R as primers. In this construct there is a stop codon TAG right ahead of the gp8 coding sequence. To modify the stop codon to a Gln codon (CAG) the DNA, including the stop codon and gp8, was replaced with the DNA amplified by PCR with GP8PRF2 and GP8PRR1 primers. The resulting construct was designated as pETT7GSTgp8. We made another construct that had no gp8 sequence. The *Nco*I-*Not*I fragment containing T7-tag and GST sequences was taken out of the pETT7GSTgp8 and cloned into the *Nco*I/*Not*I sites of pET-25b(+). This plasmid construct was called pETT7GST.

Analysis of proteins The periplasmic extracts were prepared by the osmotic shock method, as described by Cha and Kim (1999). The BL21(DE3) cells harboring a recombinant pET vector were grown at 37°C in 100 ml LB broth containing 100 μg/ml ampicillin and 2% glucose. When the optical density at 600 nm reached to 0.7, protein expression was induced by adding IPTG (final concentration: 1 mM) and incubated for an additional 3 h. After centrifuging at 7000 × g for 20 min, the bacterial pellet was resuspended in a 8 ml sucrose solution (30 mM Tris, pH 8.0, 20% sucrose). After adding 32 μl of 0.5 M EDTA the suspension was incubated at 30°C for 10 min. The suspension was centrifuged again as above, and the resulting pellet was resuspended in 3.6 ml of ice-cold sterile distilled water. After incubation on ice for 10 min, the suspension was centrifuged. To the supernatant was added 0.4 ml of 200 mM potassium phosphate buffer, pH 7.0 containing 1 mM EDTA. The resulting periplamsic extracts were analyzed by 12% SDS-polyacrylamide gel electrophoresis and Coomassie staining. For Western blot analysis the proteins in the gel were electroblotted onto a nitrocellulose membrane. The proteins on the blotted membrane were detected using T7-tag antibody HRP conjugate and ECL kit. The GST activity was assayed using GSH and CDNB as substrates, as described by Park *et al.* (1998).

Analysis of the phages displaying GST For analysis of the proteins displayed on phages, the Nova-blue(DE3) cells harboring a recombinant pET vector were incubated in a 2X YT broth containing 100 μg/ml ampicillin and 2% glucose. The phagemid

Table 1. Oligonucleotides used in this study.

Name	Sequence
	*Nco*I
EC-GST-F	5'-AATTCCATGGCGATGAAATTGTTCTACAAACCGG-3'
	*Not*I
EC-GST-R	5'-AATTCGCGGCCGCCTTTAAGCCTTCCGCTGAC-3'
	*Sac*II *Stu*I *Xho*I
SSX EC-GST-F	5'-AGTTCCGCGGAGGCCTCTCGAGCATGAAATTGTTCTACAAACC-3'
	*Bam*HI Stop
GP8PRF1	5'-GCCGGATCCGCTGGAACCGCGTGCCGCATAGGCTGAGGGTGACGATCCC-3'
	*Bam*HI Gln
GP8PRF2	5'-GCCGGATCCGCTGGAACCGCGTGCCGCACAGGCTGAAGGGTGCGATCCC-3'
	*Eco*RI
GP8PRR1	5'-GGGAATTCTTATTAGCTTGCTTTCGAGGTGAATT-3'
	T7-tag *Sac*II
NSN-42-1	5' CATGGCTAGCATGACTGGTGGACAGCAAATGGGCCGCGGTGC 3'
NSN-42-2	3' CGATCGTACTGACCACCTGTCGTTTACCCGGCGCCACGCCGG 5'

particles were then rescued by superinfection with a M13K07 helper phage in the presence of 1 mM IPTG as described by Jackson *et al.* (1992). The phages were precipitated in the presence of 5% PEG, 0.5 M NaCl. The phage pellet was suspended in a 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. Multiple rounds of ultrafiltration using Centricon 500K were carried out to remove small soluble proteins in the phage preparation. The GST activity of the phage preparation was assayed as described above. The GST activity was also examined by performing affinity chromatography. The phage preparation (10^6-10^7 cfu (colony forming unit)) in 20 mM potassium phosphate buffer was mixed with 50 µl of GSH-Sepharose and incubated for 3 h. The GSH-resin was then washed several times with the same potassium phosphate buffer containing 0.5% Tween-20. The bound phages were eluted with 50 mM Tris, 15 mM GSH, pH 9.6, and used to infect XL1-blue MRF'.

Results and Discussion

Construction of a new phage display vector In the past few years, we tried to display several proteins, including 5-enolpyruvylshikimate 3-phosphate synthase and glutathione S-transferase (GST), on filamentous phages using pCANTAB5E as a vector. Since it was anticipated that the target proteins would be transported into a periplasmic space, the periplasmic extracts from recombinant clones were prepared and examined by a SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Unfortunately, the result showed no clear indication that the target protein was expressed and transported into periplasmic space. Maybe this is because the pCANTAB5E is designed to express a target gene rather weakly for the purpose of displaying target proteins in a monovalent fashion, as described earlier. To get a strong expression of target genes, we decided to construct a new phage display vector based on a pET vector. The pET system has been used as a powerful system for the cloning and expression of recombinant proteins in *E. coli* (Rhim *et al.*, 1999; Lee and Kim, 1999), taking advantage of the strong T7 promoter and active T7 RNA polymerase (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990). The pET-25b(+) has a feature that is suitable for a phage display vector, except for the absence of a coat protein gene of filamentous phage. The pelB leader sequence can direct the target protein to periplasmic space. The vector also has a f1 ori so that the phagemid DNA can be packaged into phage particles in the presence of an appropriate helper phage.

The procedure of constructing a new phage display vector is summarized in Fig. 1. In order to modify the pET-25b(+) to a phage display vector, insertion of a coat protein gene of filamentous phage was necessary. As a source of the coat protein gene we used the pCANGST, where the *E. coli* GST gene was inserted into the pCANTAB5E. We modified the construct by replacing the gp3 gene with a gp8 gene in the hope of displaying more target proteins on the phage surface. The last 4-5 residues at the amino terminus of Gp8 form a flexible arm that appears to extend out away from the phage particle (Colnago *et al.*, 1987). Therefore, this arm has served as the site to insert target proteins or peptides (Markland *et al.*, 1991; Kang *et al.*, 1991; Iannolo *et al.*, 1995). Since Gp8 is the major coat protein, more target proteins can be displayed on the phage surfaces. The plasmid construct, where the gp3 gene had been replaced with the gp8 gene, was designated as pCANGSTgp8 (Fig. 1).

The *Nco*I-*Eco*RI fragment of the pCANGSTgp8 was then cloned into pET-25b(+) in order to make the pET vector a phage display vector, as shown in Fig. 1. The resulting plasmid construct had two *Not*I sites, one of which was present in the multiple cloning site of pET-25b(+). The other *Not*I site was introduced when the *Nco*I-*Eco*RI fragment of

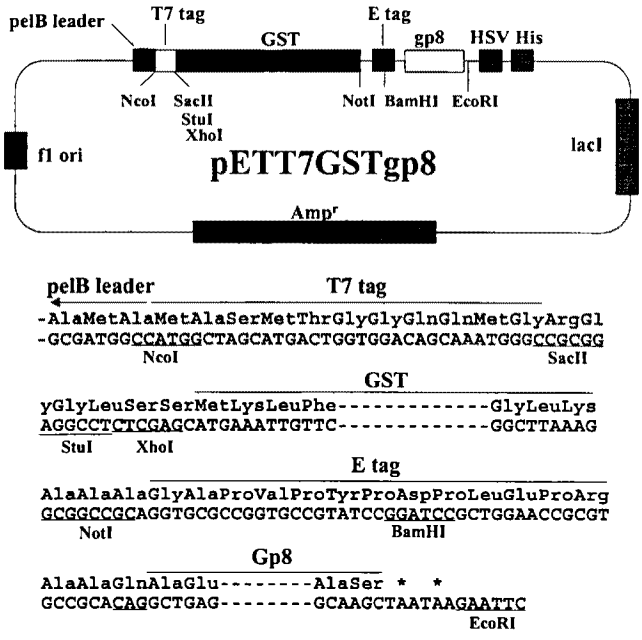

Fig. 2. pETT7GSTgp8 clone. In this construct, the DNA encoding T7-tag is present between the pelB leader sequence and the GST gene so that expressed proteins would have T7-tag peptide at their amino termini. Right after the T7-tag sequence, there are *Sac*II, *Stu*I, and *Xho*I sites into which a target gene can be cloned. At the bottom, the DNA and amino acid sequences for the signal peptide, T7-tag, GST, and Gp8 are represented.

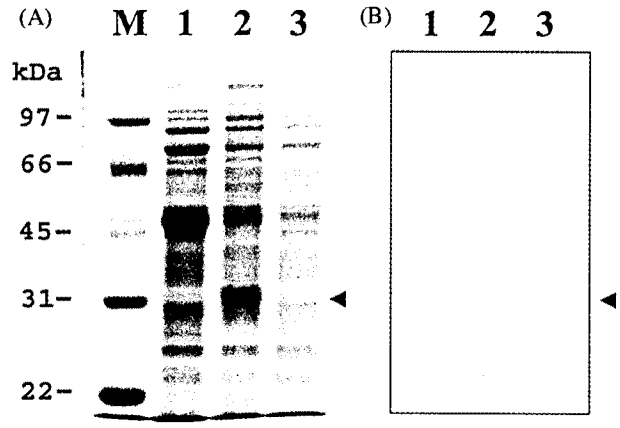

Fig. 3. Analysis of periplasmic extracts. A) SDS-polyacrylamide gel electrophoresis. The proteins in the periplasmic extracts from the BL21(DE3) bacteria harboring either the pET25b vector (1), pETT7GST (2), or pETT7GSTgp8 (3) were analyzed by 12% SDS-polyacrylamide gel electrophoresis and Coomassie staining. The bacteria were grown and induced to express GST as described in Materials and Methods. Lane M represents a molecular weight standard. B) Western blot analysis. The proteins in the gel were electroblotted onto nitrocellulose membrane, and analyzed using T7-tag antibody HRP conjugate as a probe. Arrowhead indicates the recombinant GST.

pCANGSTgp8 was inserted into the pET vector. Because the presence of two *Not*I sites made them inappropriate for a cloning site, and since the *Not*I site right behind the GST gene was a good place for the cloning target genes, we removed the *Not*I site that was present between the gp8 and HSV-tag sequences. The resulting construct was named pETGSTgp8. Then, the T7-tag sequence was inserted into the construct to make the target proteins detectable with ease. The T7-tag peptide (MetAlaSerMetThrGlyGlyGlnGlnMetGly) is the natural amino terminal end of the T7 major capsid protein. The T7-tag can also be used to follow target proteins by immunological procedures, as shown by Joneson *et al.* (1996). The T7-tag sequence was inserted between the pelB leader and GST sequences, and additional restriction sites for cloning were created right behind the T7-tag sequence, as described in Materials and Methods. This was done by first inserting a DNA linker that contained the T7-tag sequence, after removing the GST sequence out of the construct. Then, a DNA containing *Stu*I/*Xho*I cloning sites and the GST sequence was inserted.

The pCANGSTgp8 construct has a stop codon juxtaposed between the E-tag and gp8 sequences. The stop codon was originally introduced so that either phage-displayed, or soluble target proteins could be produced without subcloning (Lowman *et al.*, 1991). When a suppressor strain, such as Noba-blue (DE3), is used, translation can continue through the amber codon, and the target protein is displayed on phage surfaces. In non-suppressor strains, protein synthesis terminates at the stop codon and the proteins are produced as soluble proteins. Though convenient, the presence of a stop codon would result in a low yield of the displayed proteins, since the read-through across the stop codon is only 10-20%. In this context, the stop codon was modified to a Gln codon CAG by replacing the gp8 sequence with the DNA that was PCR-amplified using GP8PRF2 and GP8PRR1 as primers. The resulting construct was designated as pETT7GSTgp8. Thus, in this construct, the target protein is expressed exclusively as a fusion protein with Gp8.

As shown in Fig. 2, the pelB leader would direct the target protein to the periplasmic space. After the pelB leader is cleaved the T7-tag would be the N-terminus of the target protein. This would make any displayed protein detectable with a T7-tag antibody. Foreign genes can be inserted into the *Sac*II, *Stu*I, *Xho*I and *Not*I sites of the pETT7GSTgp8 after taking the GST gene out of the construct. Since *Stu*I digestion yields blunt-ended DNA, DNAs cleaved with other blunt-end producers, such as *Eco*RV and *Hinc*II, can also be inserted into the *Stu*I site. The E-tag serves as a flexible linker between the target protein and the Gp8. Protein synthesis terminates at the two stop codons located right after the gp8 sequence. Therefore, the HSV-tag and His-tag sequences are not expressed in this vector. An expression vector without a gp8 sequence was also constructed by inserting the *Nco*I-*Not*I fragment containing T7-tag and GST sequences into pET-25b(+). The construct was designated as pETT7GST. In the

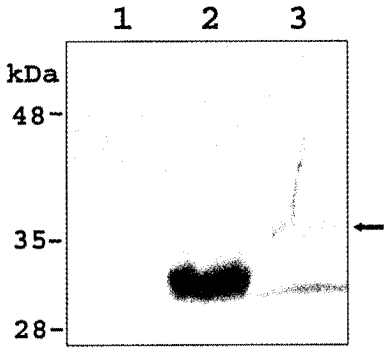

Fig. 4. Western blot analysis of phage. The phage samples (10^{10}-10^{11} cfu) obtained by phage rescue were examined by Western blot analysis using T7-tag antibody HRP conjugate after electrophoresis on 12% polyacrylamide gel containing 3 M urea and 0.1% SDS. Lane 1, phage samples from the bacteria containing pETT7GST. Lane 2, periplasmic extracts from the pETT7GST clone. Lane 3, phage samples from the pETT7GSTgp8 clone. Arrow indicates the GST-Gp8 fusion protein.

pETT7GST, target proteins produced would have T7-tag at the N-terminus, and HSV-tag and His-tag at the C-terminus. This makes purification and detection far easier.

Analysis of the proteins produced by pETT7GST and pETT7GSTgp8 For the success of the phage display, it is required that the proteins produced be transported to a periplasmic space and correctly folded. When the periplasmic extracts were prepared and analyzed by SDS-PAGE, a prominent 31 kDa band appeared in the case of the pETT7GST clone. In the case of pETT7GSTgp8, however, there was no such band (Fig. 3A). The 31 kDa band in pETT7GST corresponds to the recombinant GST, as confirmed by Western blot analysis (Fig. 3B). Prior to assembly of the filamentous phage, the major coat protein Gp8 is present in the inner host cell membrane as a transmembrane protein with its amino terminus outside the membrane and its carboxyl terminus inside the cell (Okahawa and Webster, 1981). Thus, it was expected that the fusion protein would also be embedded in the inner membrane. This explains why the recombinant GST band is absent in the periplasmic extracts, which are a collection of soluble proteins, of pETT7GSTgp8. Enzyme assay also showed that the periplasmic extract of pETT7GST had a high GST activity, whereas, pETT7GSTgp8 had negligible activity (data not shown). The periplasmic extract from the pCANGSTgp8 clone was also examined. As described earlier, most of the proteins produced from the clone would be in a soluble form, although the fusion protein with Gp8 was also produced. The result of the SDS-PAGE analysis showed that the protein band corresponding to GST was not evident. Enzyme assay also showed that the periplasmic extract of the pCANGSTgp8 contained only about 1/100 GST activity compared to that of pETT7GST (data not shown).

Table 2. Binding of display phage to GSH-resin.

	Eluted Phage[a] (% of input[b])	Relative Binding[c]
pET-25b(+)	5.8×10^{-3}	1.0
pETT7GSTgp8	2.5	431

[a]Phages were mixed with 50 μl of GSH-Sepharose and incubated for 3 h. Then, the resin was washed three times with 1 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.5% Tween-20. The bound phages were eluted with 1 ml of 50 mM Tris, pH 9.6 containing 15 mM GSH. The phages liberated were used to infect XL1-Blue MRF' and plated on LB agar containing ampicillin. The titer of the phages used was adjusted to 2.4×10^6 cfu (colony forming unit).
[b]Total cfu eluted from the GSH-resin, expressed as % of the starting number of phage.
[c]Binding of the GST display phage relative to that of the non-display phage, pET25b.

Analysis of phagemid particles To see if GST was faithfully displayed on the surface of phages, phagemid particles were obtained by infecting the bacterial clones harboring pETT7GST, or pETT7GSTgp8 with the M13KO7 helper phage. When SDS-PAGE and Western blot analysis were performed to examine the phages obtained by phage rescue, it was shown that the phages from pETT7GSTgp8 had the protein that can be detected by a T7-tag antibody (Fig. 4). This implies that the recombinant GST is displayed on phages. The detected protein band is about 35 kDa in size. Considering the fact that the size of the Gp8 is 5 kDa, 35 kDa then is assumed to be the correct size for the GST-Gp8 fusion protein. The phages prepared from pETT7GST did not show the band corresponding to the fusion protein. The band intensity of the GST-Gp8 fusion protein (lane 3) is much fainter than that of periplasmic GST (lane 2). This implies that only a small fraction of the fusion protein produced in the bacterial cells is incorporated into phagemid particles. One reason for the low incorporation could be a low yield of phagemid particles; the titer of phagemid particles is in general two orders of magnitude lower than that of the M13 phages. The low incorporation rate may also be caused by the competition between the GST-Gp8 fusion protein and the wildtype Gp8 provided by the helper phage M13K07.

To examine whether or not the GST displayed on phages was functional, an enzyme assay was performed. The result showed that the phages prepared from the pETT7GSTgp8 clone contained the GST activity. By contrast, the phages produced by pETT7GST had negligible GST activity (data not shown). To further confirm that the GST was faithfully displayed on the phages, an affinity chromatography on GSH-Sepharose was carried out. As shown in Table 2, the phages from pETT7GSTgp8 were able to bind to the GSH-Sepharose resin, and then eluted with GSH. This clearly indicates that the GST displayed on the phages is functional.

pETT7GST and pETT7GSTgp8 as cloning vectors for phage display The newly developed vectors are very useful as the cloning procedure in the phage display can be easily monitored using these vectors. When a target protein is to be displayed on phages, one can use the pETT7 (pETT7GST) vector as a first step to examine whether or not the proteins expressed are transported to the periplasmic space. This can be examined by simply performing a SDS-PAGE analysis since the recombinant proteins are produced abundantly. The target protein expression can be further confirmed by Western blot analysis using a T7-tag antibody as a probe. The biological activity of the target protein can also be examined with ease due to the massive production of the proteins. This preliminary test would be an important step before displaying target proteins on the phage surface. Presently, PCR amplification is a fast and convenient method for cloning genes. Though convenient, PCR amplification has an inherent problem as the PCR-amplified genes may have unwanted mutations, since misincorporation is frequent during PCR amplification. Thus, it might be wise to examine the clones obtained by PCR amplification for biological activity. Once it is confirmed that the proteins are faithfully expressed, the target gene in the pETT7 vector can be taken out and cloned into the pETT7gp8 vector for phage display. In this step, once again, the T7-tag antibody would be a useful tool to examine whether or not the proteins are displayed on phages. There has been difficulty in detecting the target proteins displayed on phage surfaces unless the antibody against the target protein is available. Using the pETT7gp8 vecter, however, any displayed target proteins can be detected with the T7-tag antibody that is commercially available. Thus, pETT7 and pETT7gp8 would be a pair of vectors that can be very useful in phage display.

Due to the fact that the Gp8 is a major coat protein (ca. 2800 copies per phage) of filamentous phage, the target protein can be displayed in a multivalent fashion. Besides the advantage of the high copy number display, the display of a target protein as a fusion protein with Gp8 has another advantage. There should be no infectivity reduction, as observed in the multivalent display of a target-Gp3 fusion protein (Smith, 1985; Parmley and Smith, 1988). The decrease of infectivity can be explained by the fact that the first step in infection by M13 is the binding of the phage via Gp3 to the end of the bacterial F pilus (Makowski, 1994). Although the pETT7gp8 vector is designed for displaying the proteins with low affinity to ligands, the vector can also be used for the proteins with high affinity. In the pETT7gp8 vector, the target proteins' expression level can be controlled by the concentration of the inducer, IPTG. Thus, the vector can also be used to display target proteins in a monovalent fashion simply by lowering the IPTG concentration during phage rescue. A controlled expression of target proteins by adjusting the IPTG concentration can also alleviate the problem of an inclusion body formation that occurs frequently when foreign proteins are abundantly expressed.

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