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A Strategy for Improved Expression of Permuted Myoglobins

Anna Buser

Western Washington University

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A STRATEGY FOR IMPROVED EXPRESSION OF PERMUTED MYOGLOBINS

by
Anna Buser

**A Thesis Presented
to Dr. Spencer Anthony-Cahill and Dr. Thomas Moore
of Western Washington University**

**Submitted in Partial Fulfillment
of the Requirements for
Graduation from the Honors Program**

**Supervised by Professor Spencer Anthony-Cahill
Department of Chemistry
The College of Arts and Sciences
Western Washington University
June 2001**



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ABSTRACT

Our research is aimed at generating and characterizing topological mutants of sperm whale myoglobin (swMb). The long-term experimental design involves the production of circular permuteins from swMb genes fused by a linker and determination of the effect of linker sequence on protein stability. We have expressed circularly permuted sperm whale myoglobins in *E. coli* including variants that start at the C helix and end with the B helix (CBLx), and others beginning at the H helix and ending with the G helix (HGLx). Expression yields for mutant myoglobins have been shown to correlate with stabilities of the mutants (Hargrove *et al.*, *Biochemistry*, 1994, 33, 11767). We have observed reduced levels of expression for our permuted myoglobin mutants. In order to improve the expression yields for destabilized topological mutants of myoglobin, we are attempting the expression of Glutathione S-transferase (GST)-permutein fusion proteins. The GST fusion system has been shown to be useful in expression, purification, and detection of proteins (Smith, *Methods Mol. Cell. Biol.*, 1993, 4, 220). We hope this will provide a general strategy for increased expression of our permuteins.

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INTRODUCTION

Circular Permutation

Our group is focused on the generation and study of myoglobin and hemoglobin permutins. Permutins are proteins that have had the topological structure rearranged. We can create circular permutins by linking the original N and C termini of a protein and creating new N and C termini within the protein (Figure 1). The primary, and therefore secondary structure, is then in a different order. This can be accomplished by rearranging the gene that codes for the protein.

The circular permutins allow us to study the effects of topological rearrangement on thermodynamic stability and folding pathways. This information is important for directing studies of the topological mutation of proteins with similar structure such as hemoglobin. Furthermore, understanding principles of topological rearrangement is important for the design of novel proteins and for the creation of functional fusion proteins that have different connectivities.

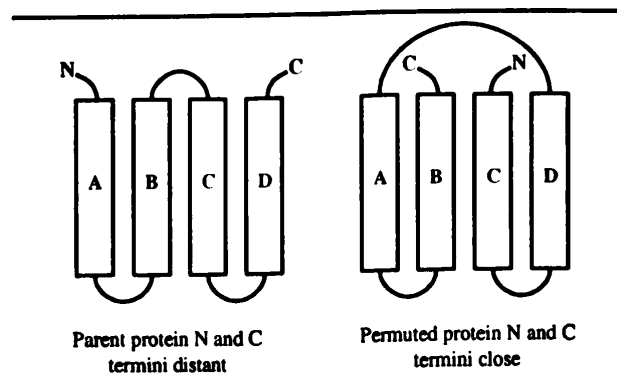


Figure 1. A diagram of circular permutation. The original N- and C- termini are fused by a linker and new termini are present in the permuted protein.

Circular permutins can be used for many different purposes. Kreitman *et al.* (1994) used circular permutation as a means to increase the biotherapeutic activity of a fusion protein. They fused a *Pseudomonas* exotoxin (PE) to the IL-4 protein that binds to receptors that are overexpressed

on the surface of cancer cells in order to make a cytotoxic drug specific for cancer (Figure 2). However, linking the cytotoxin to the original C-terminus of IL-4 interfered with binding to the IL-4 receptor, so they only recovered a 1.1% binding affinity. However, when they created new termini far from the binding site and linked the toxin there, they were able to recover up to 13% of the relative receptor binding affinity. The circular permutation helped to make this a better cancer cell specific toxin.

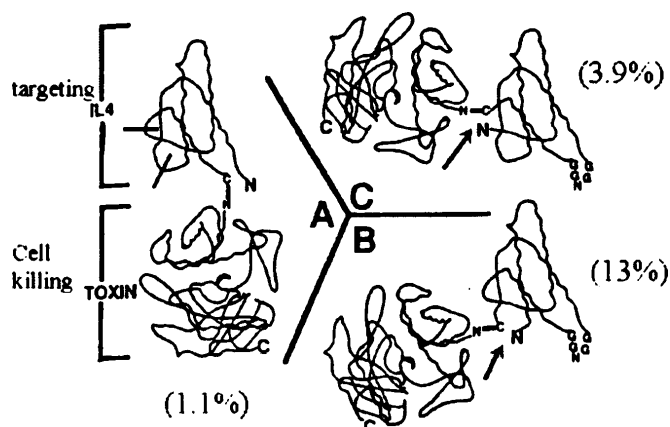


Figure 2. A diagram of the different binding affinities (shown in parentheses) of wild-type vs. permutein bound to IL-4.

Another use of permuteins involves the systematic circular permutation of a protein in order to reveal folding elements (Iwakura *et al.*, 2000, Hennecke *et al.*, 1999). Folding elements are areas of the protein that are required for proper folding. This information is important in understanding how primary structure can effect tertiary structure. A circular permutein is not meant to disrupt the tertiary structure of the protein. However, by definition, it changes the order of amino acids from which the protein is synthesized.

Viguera *et al.* (1995) studied the effect of permutation of alpha-spectrin SH3 domain on its structure and folding kinetics. They found that the permuteins had very similar structure to wild type protein. However, the kinetics of folding were very different. They found that the refolding rate was slower for some permuteins and faster for others. Characterization of the transition state for the

folding reaction of the permutants showed that the transition state structure varied with different mutants.

Sperm Whale Myoglobin

Myoglobin is a 17kD protein that is found in muscle tissue. It contains a prosthetic heme group that binds oxygen for muscle tissue use. Its globular structure is composed primarily of eight α -helices designated A-H. Its structure has been solved by X-ray crystallography (Kuriyan *et al.* 1986) (Figure 3). The myoglobin we work with has been optimized for expression in *E. coli* (Springer and Sligar 1987).

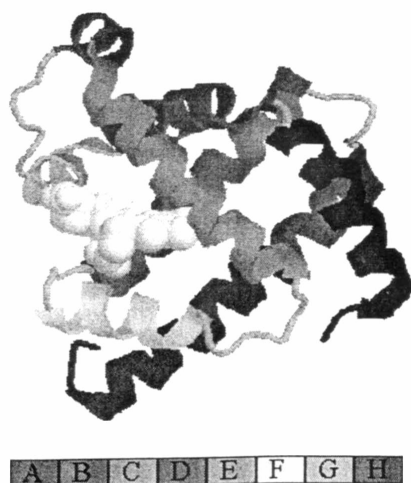


Figure 3. A Rasmol structure of sperm whale myoglobin. The eight helices are labeled A-H and color coded as shown.

The folding pathway for swMb has also been studied (Jennings and Wright 1993). Intermediates have been found in the pathway and data shows that the A, G, and H helices and a region of the B helix fold first in the pathway.

We have chosen to study sperm whale myoglobin for many reasons. First, it is a highly studied and characterized protein so we can easily compare our data to that in the literature (Gupta *et al.* 1996, Puett 1973, Ramos *et al.* 1999). Second, it has characteristic absorbances due to the heme

group that allow for easy purification and study. And finally, because it has similar structure to other proteins such as hemoglobin and the cytochromes, we hope to use the information we gain from studying myoglobin and apply it to these other proteins.

Experimental Design

Thus far, our group has focused on the generation of myoglobin permuteins that have new N and C termini in the loop regions using a rational approach. Many other groups have shown that new termini can be stable in loop regions as well as in secondary structural elements (Hennecke *et al.* 1999, Iwakura *et al.* 2000). In the future, using a random approach may help us better understand the best place to create new termini in myoglobin.

To generate several permuteins of myoglobin we have constructed a tandem gene template (Figure 4). Using this construct, we can easily make many different permuteins by just changing the site-specific DNA primers we use in the Polymerase Chain Reaction (PCR). Our tandem gene construct allows for easy generation of permuteins using restriction sites that are recognized by restriction enzymes, and site specific DNA primers used in the PCR. Simply, the PCR allows us to amplify the gene of interest.

As discussed above, our scheme for making permuteins requires a linker between the original N and C termini (Figure 5). The linker we choose to connect the two myoglobin genes is expected to have effects on the stability of the permuteins. We want something long enough to connect the termini and go around any existing structural features, but also short enough to not introduce a lot of destabilizing entropy to the system. The original termini are 20 Ångstroms apart. The linkers used in this project are 16 and 20 amino acids long and are rich in glycine and serine. Glycine provides flexibility, while serine makes the linker more hydrophilic.

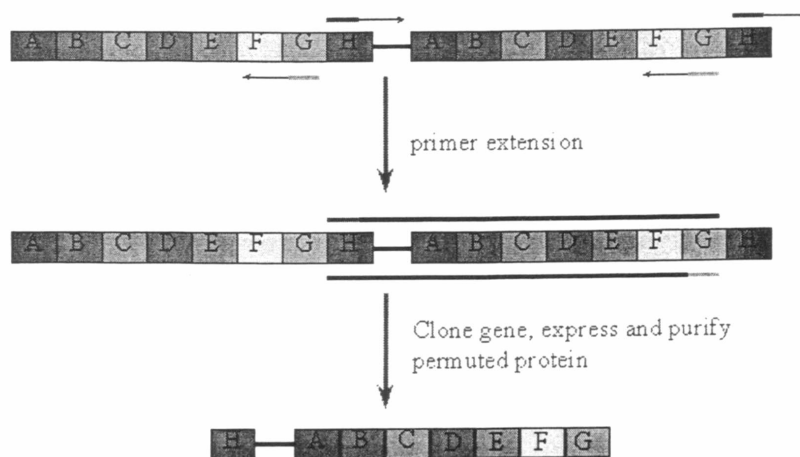


Figure 4. A diagram of our tandem gene construct. The two myoglobin genes are color coded by helices A-H. The DNA linker connecting them is shown in black. Restriction sites are engineered into this construct for easy substitution. Permuteins are amplified using site specific DNA primers in the PCR.

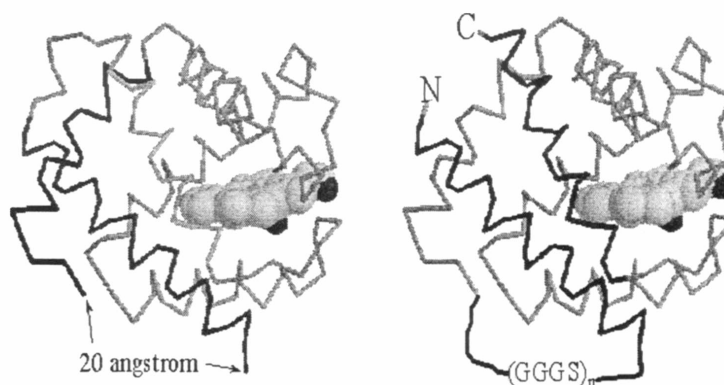


Figure 5. Rasmol diagram of myoglobin on the left showing the distance between the termini. On the right, the permutein with original termini connected by flexible linker and new termini between the H and G helices.

Background

We have expressed circularly permuted sperm whale myoglobins in *E. coli* including variants that start at the C helix and end with the B helix (CBLx), and others beginning at the H helix and ending with the G helix (HGLx). They are structurally similar to wild type as shown by circular dichroism and have similar functions as shown by binding kinetics data. However, we have observed reduced levels of expression for our permuted myoglobin mutants. Expression yields for mutant

myoglobins have been shown to correlate with stabilities of the mutants (Hargrove *et al.* 1994). Furthermore, Betton *et al.* (1998) have shown that slow folding and misfolding may result in the formation of inclusion bodies and even degradation.

GST Fusion System

In order to improve the expression yields for destabilized topological mutants of myoglobin, we are attempting the expression of glutathione S-transferase (GST)-permutin fusion proteins (Figure 6). We are able to make these fusion proteins easily using the pGEX vector system (Figure 7). The GST fusion system has been shown to be useful in expression, purification, and detection of proteins (Smith 1993, Weiss *et al.* 1995). In addition, Waldo *et al.* (1999) showed that having a highly stable protein as the N-terminal protein in a fusion system can help to stabilize the protein that is C-terminal in the fusion. The GST protein will be fused to the N-terminus of the permutin, thereby shielding it from degradation. We hope this will provide a general strategy for improving the expression of our permuteins.

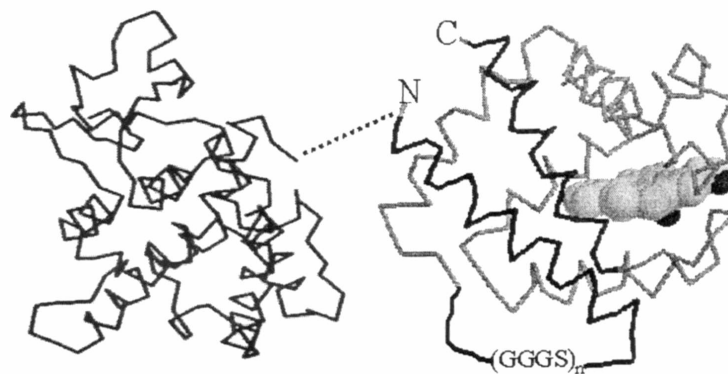


Figure 6. Rasmol diagram of GST protein fused to HG permutin by Factor Xa protease recognition site. This sequence will allow for easy cleavage of permutin from GST after expression and purification.

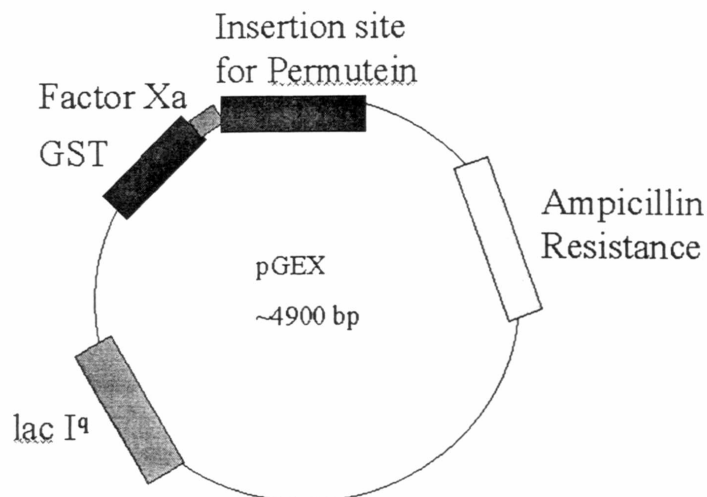


Figure 7. Diagram of pGEX vector fusion system including GST gene, factor Xa recognition sequence and insertion site for permutein gene.

DISCUSSION AND RESULTS

PCR of Circular Permuteins

PCR primers were designed by Anna Fishburn (Mowry) to generate different permuteins to be ligated into pTrc99a or pGEX5x-1 (Table 1). The HG, CB, and GF L16 pGEX primers were used in PCR reactions to generate permutein genes of the expected size (Figure 8). The HG and CB L20 pTrc primers were used to create similar permuteins that would not be fused to GST and would have a 20 amino acid linker (Figure 9). All PCR reactions were fairly clean and gave the expected bands.

Digestion of Vector and Insert

Restriction digests were performed on all inserts and vectors using the appropriate enzymes (Table 1). This step in the process of subcloning was problematic due to incompletely cut vectors. Enzyme efficiency was improved using sequential digestions and more concentrated DNA. After several attempts, the double digested vector was recovered for the ligation reaction (Figure 10). The digestion of the pTrc vector was also efficient once contaminants were removed by the phenol-chloroform extraction (Figure 11).

Lane 1: HiLo Marker
 Lane 2: uncut pTRC
 Lane 3: single digest pTRC with PstI
 Lane 4: single digest pTRC with NcoI
 Lane 5: double digest pTRC with AflIII
 Lane 6: double digest pTRC with PstI and NcoI

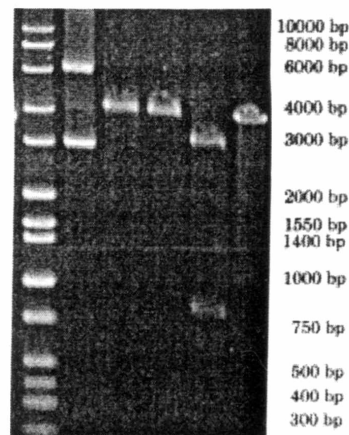


Figure 11. 1% agarose gel showing digestion of pTrc99a vector to prepare for ligation to insert.

Ligation and Analysis of Subcloning

The permutein genes and desired vectors were ligated together after digestion. The ligated plasmid DNA was then electroporated into *E.coli*. Plasmid DNA was recovered using Promega Wizard Miniprep® kits. The DNA was then redigested and, in some cases used in a PCR reaction, to verify that the insert had been subcloned into the vector. In many experiments, the redigestion gave inexplicable results, and so the ligation was repeated with either less concentrated or more concentrated DNA to try to get just the right amount of vector and insert. Finally, through redigestion and PCR, the L16 pGEX and L20 pTRC constructs were tentatively confirmed (Figures 12 and 13, respectively). Sequencing must be performed for definite confirmation once we get efficient sequencing primers.

Expression of Protein

Expression experiments with the fusion proteins resulted in reddish pellets after centrifugation of overnight culture. SDS-PAGE was performed on 1 ml samples of cell culture of HG-GST, CB-GST, and GF-GST L16 constructs in pGEX (Figure 14). The gel shows that the HG-GST and CB-GST proteins are expressed. However, the GF-GST protein is not expressed or it is rapidly degraded; only a band corresponding to GST alone appears in that lane. It could be that the fusion protein is degraded as it is made because it is highly unstable, or that there is a mutation in the

gene that is stopping expression. Sequencing the DNA would allow us to rule out the possibility of a mutation. Initial Western Blots (not shown) also show that both myoglobin and GST antibodies bind the HG-GST and CB-GST constructs, but only GST antibodies bind the protein in the GF-GST lane. This data suggests that the HG and CB fusions are being expressed, but that GF fusion is not. Initial data (not shown) also suggests that the GST fusion system does increase expression because we are able to grow the fusion proteins overnight and get larger quantities, whereas the HGL16 alone can only be grown for 4.5 hours. However, when the CB-GST fusion protein was purified on a Glutathione Sepharose Column, no color was observed. The proteins may not be folding properly and further research must be done in this area.

Figure 12. 1% agarose gel showing band at expected 500bp size.

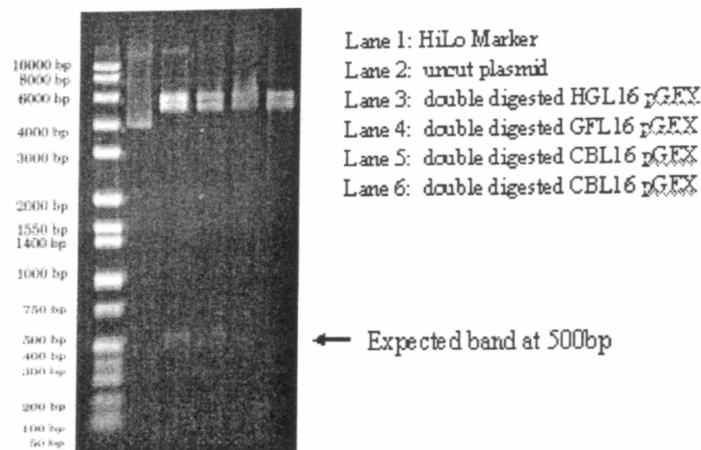
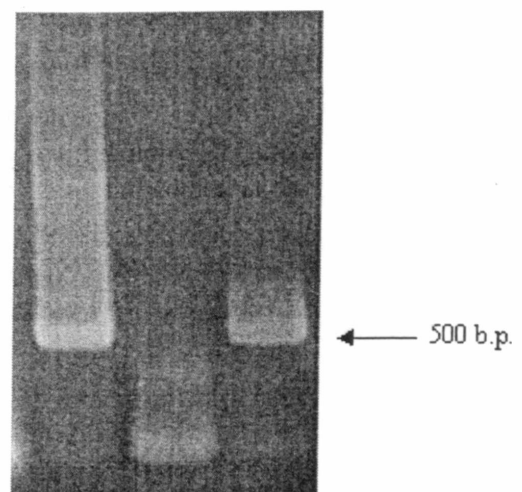


Figure 13. 1% agarose gel of PCR with colony DNA as template to detect for insert. Insert apparent for lanes 1 and 3.

Lane 1: PCR HGL20 insert
 Lane 2: no insert
 Lane 3: PCR CBL20 insert



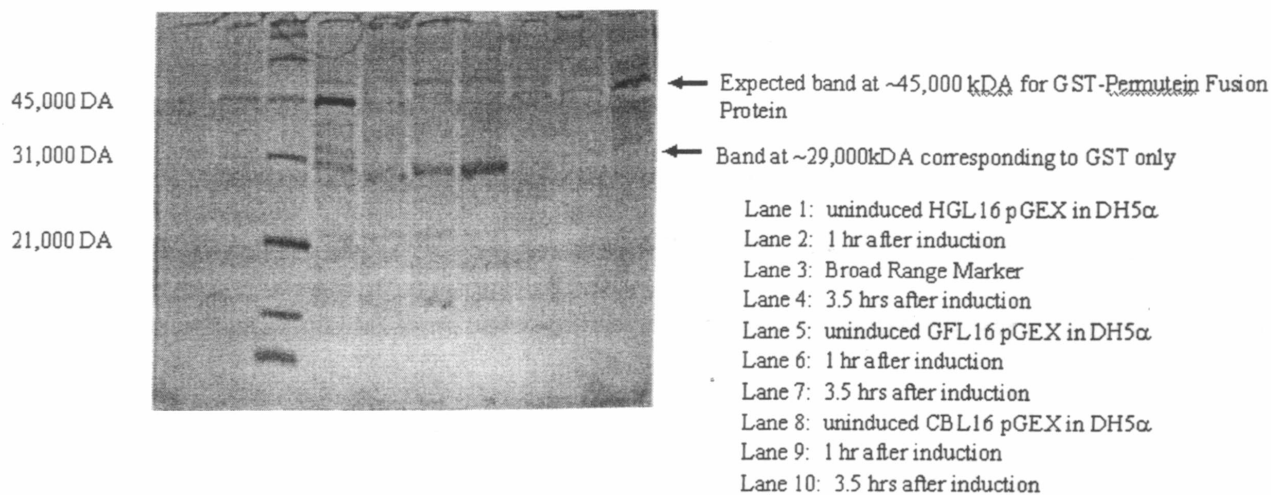


Figure 14. SDS-PAGE gel of HG, CB, and GF 16 constructs. Notice expected bands for HG and CB, but not for GF.

The HG and CB L20 constructs in pTRC have been verified just recently. The HG construct has been grown up to harvest but twice at the first spin the cells did not pellet well. Both proteins need to be run out on a SDS-PAGE gel to confirm that a myoglobin permuterin is being expressed.

CONCLUSION

From the bands on the protein gel and appearance of bands on Western Blots (not shown), we can conclude that the fusion proteins GST-HGL1 and GST-CBL1 are being expressed. There is an insert for the GFL1 permuterin as shown by the DNA gel, but the protein gel shows that only GST is being expressed. Recent data (not shown) also suggest that the fusion proteins are more stable than the permuterins expressed alone. Further research needs to be done to figure out why GF-GST is not being expressed. In addition, comparison of the yields of fusion proteins to proteins expressed alone should be carried out.

The HG and CB L20 constructs in pTRC have been made. The next step will be to do expression experiments.

METHODS

Sperm Whale Myoglobin

The myoglobin gene used to generate these proteins has been optimized for high level expression in *E. coli* (Springer and Sligar 1987). They incorporated an efficient ribosome binding site, appropriate initiation and termination sequences, restriction enzyme sites for future cloning, and codons optimized for expression in *E. coli*.

Tandem Gene Construct

All permuteins of myoglobin were made from the tandem wild-type genes with the nucleotide sequence encoding the desired linker connecting the C-terminus of the 5' gene to the N-terminus of the 3' gene. Anna Fishburn constructed TanL16 using a tandem gene with a 20 amino acid linker, TanL20, inserted in the plasmid pUC19. TanL20¹, the original tandem myoglobin gene, was made by Sam Schaefer-Joel, a former student in the Anthony-Cahill group. The 20 amino acid linker in TanL20 was removed using BstXI and SpeI restriction enzymes. Four oligonucleotides encoding L16² were annealed together and ligated into the digested tandem gene and pUC19 vector. In the work described herein, the pGEX 5x-1 permuteins have the 16 amino acid linker, while the pTrc99a permuteins have the 20 amino acid linker.

Polymerase Chain Reaction

The Polymerase Chain Reactions (PCR) were performed using a Stratagene Robocycler with a hot top. 50 ul reactions were performed with 100 ng DNA template, 2mM dNTP's, 1pM of each primer, 1mM MgSO₄, 1x buffer supplied by New England Biolabs (NEB), and 4 units of Deep Vent Polymerase. Each cycle consisted of a 30 second melting stage at 95 °C, one minute annealing stage at various annealing temperatures followed by a 30 second elongation stage at 72 °C.

¹ The L20 sequence encodes a (SGGG)₅ linker and the 5' sequence of the myoglobin gene. The amino acid sequence for the L20 cassette is: SGGGSGGGSGGGSGGGSGGGVLSEGEWQ

² The L16 sequence encodes a (SGGG)₄ linker and the 5' sequence of the myoglobin gene. The amino acid sequence for the L16 cassette is: SGGGSGGGSGGGSGGGVLSEGEWQ

Using the tandem gene template and primers (Table 1) in the PCR reaction, we were able to create different permuteins with different linkers.

Primer	Sequence	Restriction Site	Use
HGpGEXB	CGCGGATCCCAGGTGACTTCGGTGCTGACGCTCAGG	BamHI	Fusion Permutedin Construct
HGpGEXX	CTAACCCGGGTCATCAATGTCTAGAATGCAGAACATGGATG AT	XmaI	Fusion Permutedin Construct
CBpGEXB	CGCGGATCCATCCGGAAACTCTGGAAAAATTCG	BamHI	Fusion Permutedin Construct
CBpGEXX	CTAACCCGGGTCATCAAGATTTGAACAGTCGAATCAAGATG TC	XmaI	Fusion Permutedin Construct
GFpGEXB	CGCGGATCCCGATCAAATACCTGGAATTCATCTCT	BamHI	Fusion Permutedin Construct
GFpGEXX	CTAACCCGGGTCATCAATGTTTAGTAGCATGCGATTGCGCAA G	XmaI	Fusion Permutedin Construct
CB3btm pTRC	ATAACTGCAGTCATCATTTGAACAGTCGAATCAAGATGTC	PstI	L20 Permutedin construction
CB2top pTRC	CCCACATGTCTCATCCGGAAACTCTGGAAA	AflIII	L20 Permutedin construction
HG2top pTRC	ATAACTGCAGTTATCACTCGAGTTTGAACAGTCGAATCAAG AT	NcoI	L20 Permutedin construction
HG3btm pTRC	ATAACTGCAGTCATCATGGATGTCTAGAATGCAGAACAT	PstI	L20 Permutedin construction

DNA Visualization

DNA visualization was possible using 1% agarose gels run at 80V in modified TAE buffer for 1.5 hours. Gels were stained in an ethidium bromide solution (10 mg/mL) for 10 minutes, then destained in deionized water for 10 minutes. DNA was visualized on an UV transilluminator and a Polaroid camera was used to record data.

DNA Purification

DNA purification was done using Promega kits, ethanol precipitation, and phenol-chloroform extractions. PCR fragments and small fragments purified on agarose gels were purified using the PCR-prep kits. Larger DNA fragments were purified using the DNA Clean-up kits. Plasmid DNA was extracted from *E.coli* cells using the Mini-prep kit.

Phenol-chloroform extractions were performed by mixing the DNA solution with an equal volume of phenol saturated with Tris (pH=8), vortexing, followed by a 20 second spin and removal of the aqueous upper layer. This was followed by extraction with phenol-chloroform (1:1) using the same procedure. Finally, an equal volume of chloroform was used with the same procedure to remove traces of phenol.

Ethanol precipitations were performed following phenol-chloroform extractions. First, NaOAc was added to 0.3M followed by 2X absolute ethanol at 4°C. The solution was incubated on ice for 1 hour and then spun at 14,000 x g for 15 minutes. The supernatant was poured off and the pellet was covered with 100ul 70% ice-cold ethanol and spun immediately at 14,000 x g for 5 minutes. The supernatant was poured off and the pellet was dried at 37°C. The DNA was then resuspended in 30-50ul of water.

Restriction Digests

Restriction digests were performed using appropriate enzymes (1 uL), 1X buffers, and standard protocols from New England Biolabs. Sequential digests were done when enzymes used in double digests had incompatible buffers.

Ligation

After digestion of the vector and insert, they could be ligated together using 1X ligase buffer, and 400 units T4 DNA ligase. 1:1 ratios of vector to insert were used. The estimated concentrations were based on visual estimation from the agarose gels. This reaction was incubated at 16°C overnight. Incubating for 20 minutes at 60°C killed the ligase. Ligation of the HGL16, CBL16, and GFL16 inserts into the pGEX plasmid resulted in a GST-permuterin fusion protein. HGL20 and CBL20 were ligated into the pTrc99a plasmid to yield permuteins without extra N-terminal amino acids.

Electroporation

Electroporation was performed by adding 5uL heat killed ligation to 100uL of XL1-Blue or DH5 α electrocompetent cells, which were prepared as described in the appendix. Electroporations used a Biorad Gene Pulser II set to a resistance of 250 ohm, 1.25 kV/mm with 1mm cuvetts, and a capacitance of 25uF. Cells were incubated for 1 hour in SOC medium at 37°C and were then plated on ampicillin (100ug/mL) LB agar plates. The plates were then incubated overnight at 37°C. Potential colonies containing plasmid were selected and grown overnight for mini-prepping.

Protein Expression

Expression experiments were carried out after confirmation by restriction analysis and PCR that the insert was in fact in the plasmid. All permuteins were expressed in 1 L bacterial cultures in 2.8 L Fernbach flasks at 37 °C with shaking of about 200 revolutions/min in a Lab Line Incubator-Shaker. Cells were grown in LB and ampicillin at 100ug/mL. Protein expression was induced by the addition of .1mM isopropylthiogalactoside (IPTG) to cells at mid log phase ($OD_{600}=0.6$). The cultures were allowed to grow for an additional 3-5 hours then harvested by centrifugation (4000 g for 10 minutes). For the pGEX L16 constructs, 1mL samples were taken prior to induction (uninduced) and then at 1 hours and 3.5 hours after induction. The cells were harvested at 3.5 hours. For the pTrc99a L20 constructs, 1 ml samples were taken at induction and cells were harvested at 4.5 hours.

The harvested cells were resuspended in approximately 30 mL lysis buffer, then lysed by sonication, using a Branson Instruments, Inc. Sonifier at approximately 5 amperes. Each sample was allowed to sonicate for 10-12 minutes or until lysis was complete. The samples were then centrifuged at 21,000 g for 20 minutes and the supernatant retained. The pH of the lysate was adjusted to 8.0-8.5 and zinc acetate was added to a final concentration of 1 mM. The lysate was centrifuged at 40,000 g for 20 minutes then filtered through a 0.45 um filter. The filtered lysate of

the GST-permutin fusion proteins was loaded onto a Glutathione Sepharose Column (see Appendix).

SDS-PAGE

The 1 mL culture samples were prepared for SDS-PAGE (sodium-dodecyl-sulfate polyacrylamide gel electrophoresis) by boiling 20 uL of cell culture with 20 uL of loading buffer for 15 minutes. SDS-PAGE gels were 15% acrylamide (w/v) and the polymerization reaction was induced with 100 uL ammonium persulfate (APS) and 20uL TEMED. These gels were run at 200mA for 2 hours. Gels to be stained with Coomssie Blue were microwaved in the dye for 1.5 minutes and then incubated at room temperature with gentle swirling for one half-hour. The gels were then destained until bands were of desired intensity (overnight).

Western Blot

For Western Blot analysis, the proteins were separated on a SDS-PAGE, then transferred onto nitrocellulose paper using a standard sandwich setup in transfer buffer at 250mA for one hour. The membrane was then incubated in blocking solution made with 6% (w/v) powdered milk in 40mL TBS for 30 minutes. Next, the membrane was incubated for one hour with 42uL rabbit anti-human Mb or goat anti-GST in 14 mL blocking solution and 38 mL TBS. The membrane was washed for 5 min 3 times in 40 mL TTBS. Then 14 uL of the secondary antibody goat anti-rabbit or anti-goat conjugated to horseradish peroxidase was incubated with the membrane in 14 mL blocking solution and 38 mL TBS. Finally, it was washed 3 times for 5 minutes with 40 mL TTBS. Color development solution was made by dissolving 24 mg 4-chloro-1-naphthol (HRP reagent) in 8 mL cold methanol and mixing with 40 MI premixed cold TBS and 14 uL H₂O₂. The membrane was then incubated in the solution for 30 minutes. Rinsing in water for 10 minutes stopped the development.

Strains and Vectors

E. coli strains DH5 α and XL1-Blue were used in experiments. Diluting overnight culture 1:1 with 40% glycerol made seed stocks of bacteria carrying the gene constructs.

Vectors used for cloning and expression include pTrc99a and pGEX5x-1. Both vectors use an IPTG inducible lac promoter for expression. Both vectors also carry an ampicillin resistance gene for selection with ampicillin up to 100 ug/mL.

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Appendix

Preparation of Electrocompetent Cells

1. 1 L of rich broth (e.g. LB medium, in a 2.8 L Fernbach flask) was inoculated with at least 10 mL of overnight culture.
2. The culture was incubated with shaking at 37°C until the A_{600} is 0.5.
3. The flask was chilled on ice for 15-30 min.
4. The culture was then centrifuged at 4000 x g for 10 min in a pre-chilled GA-3 rotor. The supernatant was poured off, including any cellular debris.
5. The cells were gently resuspended in 1L ice-cold, autoclaved, nanopure water by gentle swirling or by pipetting using cold, sterile-pipets.
6. The culture was then spun at 4,000 x g for 10 min, and the supernatant was decanted.
7. The cells were resuspended in 0.5 L ice-cold, autoclaved, nanopure water.
8. The culture was then spun at 4,000 x g for 10 min, and the supernatant was decanted.
9. The cells were next resuspended in 20 mL ice-cold 10% glycerol (prepared with nanopure water and autoclaved).
10. The culture was then spun at 4,000 x g for 10 min in a pre-chilled SS-34 rotor, and the supernatant was decanted.
11. The cells were resuspended in 2.5 mL of ice-cold 10% glycerol.
12. The cells were then aliquoted by 100 μ L into pre-chilled microfuge tubes (0.5 mL) using pre-chilled pipet tips.
13. The aliquots were then flash frozen in liquid N_2 and were immediately stored in the -80°C freezer.

Glutathione Sepharose Column

1. Wash the column with 10-20 mL of PBS (150 mM NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , pH=7.3 to remove the preservative.
2. Equilibrate the gel bed with 6 mL PBS + 1% triton X-100
3. Load sample
4. Wash the column with 2x10 mL of PBS

5. Elute the protein with 10 mL of elution buffer (5 mM Glutathione in 50 mM Tris-HCl pH=8.0) and collect 1-2 ml fractions.
6. Regenerate by washing with high salt buffer (PBS+ 3M NaCl) For longer storage, wash the column with 2x5 bed volumes of PBS and then 2X5 bed volumes of 20% ethanol. Store at 4°C.