FUNCTIONAL DISPLAY OF IMMUNOGLOBULIN BINDING DOMAINS ON THE SURFACE OF BIOPOLYMER BEADS

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Kishor Rajendran
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ABSTRACT

Protein A, G and L are immunoglobulin binding proteins isolated from the cell wall of certain gram positive bacteria. The interaction between the binding domain of these proteins and the immunoglobulin molecule occur without affecting the functional Fc region of the antibody, thus making them an ideal tool for antibody purification. The capacity of protein A to bind IgG with such high affinity is the driving motivation for its industrial scale use for immunoglobulin purification such as in chromatography resins. A major disadvantage however, is the inability of protein A to bind certain subclasses of IgG as well as IgG from certain species; a pitfall that can be overcome with the display of either protein G in combination with protein A, or with protein L which binds a range of immunoglobulin based on light chain interactions. Here we display both the binding domain of protein A with the binding domain of protein G on a single platform; the surface of polyhydroxyalkanoate (PHA) biopolyester granules. We also produce a PHA granule displaying the binding domain of protein L. This was achieved via fusion and expression of the genes for these immunoglobulin binding bacterial proteins and the *phaC* gene on a single plasmid construct. The *phaC* gene codes for polyhydroxyalkanoate synthase (PhaC), a critical enzyme involved in PHA granule production in the bacterial host and which remains covalently attached to the surface of the PHA granule. When transformed into an *E.coli* strain engineered for polyhydroxyalkanoate (PHA) bead production, the functional PhaC allows for the self-assembly of intracellular PHA beads with the immunoglobulin binding proteins expressed on their surface. Based on the results of this study, these novel beads provide us with added functionalities and significantly increased immunoglobulin binding efficiency when compared to commercial standards, which could lead to an up-scaled production of novel bio polyester beads to serve as an ideal tool for immunoglobulin purification.

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"Vi veri universum vivus vici"

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Abbreviations

˚C	**Degree Celsius**
3HB	**3-hydroxybutyrate**
AGE	**Agarose Gel Electrophoresis**
BSA	**Bovine Serum Albumin**
DMSO	**Dimethyl sulfoxide**
FM	**Fluorescent microscopy**
GC/MS	**Gas chromatography mass spectrometry**
Ig	**Immunoglobulin**
kDa	**Kilo Daltons**
Maldi-TOF/MS	**Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry**
PBS	**Phosphate buffered saline**
PHA	**Polyhydroxyalkanoate**
PhaC	**PHA synthase**
PHB	**Polyhydroxybutyrate**
SDS-PAGE	**Sodium dodecyl sulfate gel electrophoresis**
WT	**Wild-type**

CHAPTER 1: IMMUNOGLOBULIN BINDING PROTEINS

1.1 Introduction

Biotechnologically produced antibodies are extremely high-value diagnostic tools and therapeutic agents used in purifying cytokines, blood-clotting factors and various other pharmaceutically significant procedures. As of 2007, global sales of commercially produced antibodies reached USD$ 26 billion; with over 22 monoclonal antibodies being approved by the FDA. Production of even single classes of antibody (IgG for example) exceeds 10 tons annually, with the total amount of immunoglobulin produced exceeding 40 tons per year according to a report by Biopharm International in 2002. (Chen, 2009) For use in the medical industry, immunoglobulin has to be thoroughly purified to ensure zero contamination. This usually involves affinity chromatography using proteins with a high adsorption capacity for a particular immunoglobulin. Many different bacterial surface proteins have the capability of binding to the constant Fc region of the immunoglobulin molecule, a mechanism which benefits the organism in terms of virulence and pathogenicity, allowing it to evade opsonisation. These bacterial Fc-receptors have been highly characterized and are critical tools in immunoglobulin purification. (Huse *et al.*, 2002) In today's industry, several different bacterial proteins are used in antibody purification; primarily Staphylococcal Protein A, Streptococcal Protein G and Peptococcal Protein L. While our knowledge of the binding interactions between these proteins and specific immunoglobulin date back to before the 1990s, the extent of manipulations and applications these proteins are capable of are constantly being explored. While the most significant of these applications is affinity chromatography, the optimization of bacterial protein based affinity chromatography has led to novel research leading to advancements in fusion protein technology, as well as immobilization of proteins onto solid surfaces, the immunoglobulin molecule, and current biotechnological applications in the medical and pharmaceutical industry.

Species	Antibody Class	Protein A	Protein G	Protein A/G	Protein L*
Human	Total IgG	S	S	S	S*
	IgG_1	S	S	S	S*
	IgG_2	S	S	S	S*
	IgG_3	W	S	S	S*
	IgG_4	S	S	S	S*
	IgM	W	NB	W	S*
	IgD	NB	NB	NB	S*
	IgA	W	NB	W	S*
	Fab	W	W	W	S*
	ScFv	W	NB	W	S*
Mouse	Total IgG	S	S	S	S*
	IgM	NB	NB	NB	S*
	IgG_1	W	M	M	S*
	IgG_{2a}	S	S	S	S*
	IgG_{2b}	S	S	S	S*
	IgG_3	S	S	S	S*
Rat	Total IgG	W	M	M	S*
	IgG_1	W	M	M	S*
	IgG_{2a}	NB	S	S	S*
	IgG_{2b}	NB	W	W	S*
	IgG_{2c}	S	S	S	S*
Cow	Total IgG	W	S	S	NB
	IgG1	W	S	S	NB
	IgG2	S	S	S	NB
Goat	Total IgG	W	S	S	NB
	IgG_1	W	S	S	NB
	IgG_2	S	S	S	NB
Sheep	Total IgG	W	S	S	NB
	IgG_1	W	S	S	NB
	IgG_2	S	S	S	NB
Horse	Total IgG	W	S	S	?
	IgG(ab)	W	NB	W	?
	IgG(c)	W	NB	W	?
	IgG(T)	NB	S	S	?
Rabbit	Total IgG	S	S	S	W*
Guinea Pig	Total IgG	S	W	S	?
Pig	Total IgG	S	W	S	S*
Dog	Total IgG	S	W	S	?
Cat	Total IgG	S	W	S	?
Chicken	Total IgY	NB	NB	NB	NB

Legend:	W = weak binding	M = medium binding S = strong binding	NB = no binding ? = information not available

*Binding to Protein L will occur only if the immunoglobulin has the appropriate kappa light chains. The stated binding affinity refers only to species and subtypes with appropriate kappa light chains. Lambda light chains and some kappa light chains will not bind. For more information, see instructions for Protein L (Product No. 21189).

Table 1: Binding characteristics of antibody-binding proteins. (from Thermo-Scientific 2008)

1.2 Protein A

Protein A is a surface protein adhesin from *Staphylococcus aureus.* (Forsgren, 1970) The protein is covalently linked to the peptidoglycan layer of the cell wall of *S. aureus*. (Hjelm *et al.*, 1972) Studies linking protein A to *S. aureus* pathogenicity have dated as far back as the 1970's, however the exact mechanism explaining why the protein is produced in pathogenic strains had yet to be explained. In order for *S. aureus* to colonize and cause infection, the organism has to first attach to the endovascular layer. In a study done by Jörg Hartleib and colleagues, evidence was presented that there was a link between protein A and the Von Willebrand factor (vWF), a glycoprotein that mediates platelet adhesion; in causing successful endothelial damage and adhesion. While most of the experimental procedure was performed *in vivo*, the results obtained, paired with evidence that isolates from patients suffering from *S. aureus* related infections confirmed a relationship between protein A expression and pathogenicity. (Hartleib *et al.*, 2000)

Protein A is capable of binding to immunoglobulin G via 5 different binding domains on the protein molecule; E, D, A, B and C. In short, every domain of the protein has an IgG binding region. These interactions may account for increased levels of inflammation that occurs during *S. aureus* infections. (Hjelm et al., 1972, Forsgren & Sjoquist, 1966) Of particular interest however, is the B domain. The triple-helical B domain is capable of binding to the constant Fc region of IgG, via an interaction between 9 amino acid residues in the Fc domain and 11 amino acid residues in the B domain. The exact conformational change to the helices of the protein domain has yet to be confirmed. Considering only 2 helices are involved in the binding interaction, it was previously accepted that helix unwinding of the 3rd helix was involved in IgG binding, however in a 1996 study, it was suggested that a small tilt in 2 of the interacting helices mediated the interaction (Jendeberg *et al.*, 1996). Whilst not capable of binding certain classes of human IgG and all classes of IgG from certain animal species;

protein A is the most commonly used protein in bacterial Fc receptor affinity chromatography due to the fact that it may be produced in large amounts from recombinant bacteria. Another advantage is that the presence of protein A in elution fractions does not lead to any known adverse effects (Huse et al., 2002).

A commonly used analogue of the B domain of protein A is the Z domain. The Z domain was engineered as an affinity-purification handle for fusion protein production, and contains 2 amino acid substitutions relative to the B domain. It has identical binding affinities and interacts with the Fc region of the IgG molecule exactly the same way the B domain does, the only modification being the replacement of a glycine residue with an alanine residue in the first helix of the Fc-binding domain. It was later found that a tandem repeat of the domain; ZZ, produced significantly higher binding capacities. It was suggested that this was due to interactions between uncomplexed Z domains in the ZZ molecule interacting with complexed Z domain or the Fc region of the immunoglobulin. (Tashiro *et al.*, 1997) The significance and applications of this synthetically produced protein domain will be discussed later in this thesis.

1.3 Protein G

A major drawback in the use of protein A for antibody purification is that the protein is unable to interact with monoclonal IgG class 3 and certain animal IgG, thus limiting its applications. In 1984, Lars Bjorck and Goran Kronvall published a paper characterizing the advantages of protein G as an IgG binding reagent as compared to the more widely used protein A. Protein G is a bacterial surface protein, found covalently attached to the cell wall of group G Streptococcus. (Bjorck & Kronvall, 1984) Similar to protein A, protein G binds to the Fc region of the IgG molecule. This combined with protein G's ability to bind other

serum components may allow the organism to mask its antigenicity from the host defence mechanism. (Fahnestock, 1987) However, the protein is not as biologically significant as a determining virulence factor when compared to other Streptococcal surface proteins (like M protein). (Caparon & Scott, 1987)

The primary advantage of protein G is its affinity for classes of IgG which protein A cannot interact with. The specificity of protein G for IgG is maintained even with this additional binding capacity. Protein G is also capable of retaining IgG reactivity even after being treated with proteolytic enzymes used in solubilisation. (Bjorck & Kronvall, 1984, Huse et al., 2002)

Table 1

Immunoglobulin binding specificities

Immunoglobulin	Type III receptor Protein G, FcRc	Type I receptor Protein A
Rabbit	++	++
Goat	++	–
Mouse	++	++
Rat	+	+/–
Bovine	++	++
Chicken	–	–
Human IgG1	++	++
IgG2	++	++
IgG3	++	–
IgG4	++	++
Human IgA	–	+
IgM	–	+
IgD	–	–

++, strong binding; +, weak binding; –, no binding. Compiled from Refs 1–5.

Table 2: Comparison between immunoglobulin-binding specificities of the type III Fc receptor protein G and the type I receptor protein A (from Fahnestock, 1987).

One limitation of protein G is that it exhibits binding sites for albumin and α2-macroglobulin; which would result in contamination of antibodies post-affinity purification. To overcome this problem, genetic modifications to protein G have been performed, as well as the generation of cloned genes allowing for large scale production in recombinant *E.coli*. This also overcomes the problem faced with purifying protein G for commercial use. Previously,

there was great difficulty in isolating the protein from *Streptococcus* itself, as the organism did not naturally produce much of the protein. (Huse et al., 2002, Fahnestock, 1987) With recent research resulting in the generation of new cloned genes, further characterization of the binding domains through methods such as NMR analysis, and optimization of the interaction that occurs between the protein and the immunoglobulin molecule, researchers in the field are optimistic that the production of protein G in recombinant strains will result in the supplementation or replacement of protein A by protein G in many immunochemical applications (Fahnestock, 1987).

1.4 Protein L

The isolation of protein L was first detailed in 1988, in Sweden. (Bjorck, 1988) Protein L is a bacterial surface protein belonging to some strains *Peptococcus magnus*, gram-positive cocci. *Peptococcus magnus* is a known human pathogen, commonly associated with urinary tract infections, infections among diabetics, wound infections, and device-associated infections. (Bourgault *et al.*, 1980) Protein L has long been a suspected virulence determinant in Peptococcal infections, due to the strong correlation between protein expression and successful infections. While protein L and protein L expressing bacteria are known to stimulate histamine release by the host defence mechanism and may be a virulence factor; the binding mechanism of protein L to immunoglobulin allows for exposed Fc regions, thus leading to coating and eventual uptake by macrophages. Unlike protein A and G however, intensive characterization of protein L and successful animal models of Peptococcal infections have yet to be established; therefore the exact role protein L plays in Peptococcal infections has yet to be detailed. (Enokizono *et al.*, 1997, Kastern *et al.*, 1990)

In contrast with protein A and G; protein L binds to κ light chains of the immunoglobulin molecule. The protein is also capable of binding all classes of Ig. The capability of protein L to bind to light (L) chains of Ig led to its naming. Many studies have been done on characterizing the binding interactions that occur between protein L and the immunoglobulin light chains; probably one of the first of these studies was published in 1992. (Kastern *et al.*, 1992) The study involved the sequencing of the gene that codes for protein L; the first gene of *P.magnus* to ever have been sequenced. While sharing similarities to other cell wall proteins, protein L's light chain binding property was related to its B domain repeats, which sequence shared no homology with other Fc-binding proteins like protein A and G. (Kastern et al., 1992) The folds of this domain consisted of a single α-helix against a four-stranded β-sheet, as determined by NMR analysis. NMR analysis further details that the 2^{nd} β-strand preferentially binds to the variable region of the Ig light chain. The binding occurs on the outer/framework region of the variable light chain region, thus allowing the immunoglobulin to retain its ability to bind to antigens while being bound to protein L. (Enokizono et al., 1997, Kastern et al., 1992)

The ability of protein L to bind to all classes of immunoglobulin makes it an extremely useful tool in polyclonal antibody purification. Besides being able to bind to Ig light chains, the protein is also capable of binding to the Fab region of IgG, IgM, as well as IgA. The protein is capable of binding to immunoglobulin from common laboratory animal species; mice, rat and rabbit immunoglobulin may all be purified using protein L. Protein L does have limitations however; the light chain interaction does not extend to κII and λ light chains. This prevents the binding of the protein with bovine, goat and sheep immunoglobulin. (Enokizono et al., 1997, Bjorck, 1988) Continued research in characterizing the protein and its interactions is crucial; not only in understanding the usefulness of the protein as an

immunoglobulin binding tool, but also to understand the host-parasite relationship in *P.magnus* infections.

1.5 Affinity chromatography using bacterial proteins

The method of affinity chromatography was first detailed by Pedro Cuatrecasas, Meir Wilchek, and Christian B. Anfinsen in 1968. The published paper illustrated the method of purifying proteins using affinity adsorption to Sepharose columns and subsequent elution of the protein using changes in pH, salt, or competitive inhibitors. (Cuatreca.P *et al.*, 1968) Today, affinity purification of is the most commonly used method in isolating antibodies, a powerful tool in obtaining targeted antibodies rapidly for later use. Such uses include passive immunization, radio-labelled antibodies in treating tumours, and the manipulation of antigens in complex environments like blood. (Huse *et al.*, 2002)

Affinity purification relies on the effectiveness of several components that make up the method. Of critical importance is the specificity of the antibody for the affinity adsorbent (i.e. an antigen or protein). This adsorbent is immobilized on the surface of a matrix. Serum is then passed through the matrix, and the resulting bound antibody is eluted using an elution buffer. With this in mind, the most significant advantage of affinity chromatography using bacterial proteins is the ability to bind antibody while retaining the capability of the immunoglobulin to bind to antigen. While weak-binding as well as cross-reacting antibodies still need to be purified using specific antigens or anti-antibodies; bacterial protein affinity chromatography continues to remain the method of choice for antibody purification. Characterization of these proteins, optimization of the binding domains and the genes involved in the synthesis of the protein; combined with the ability to transform recombinant

production bacterial strains such as *E.coli*, have made the large scale production of immunoglobulin binding bacterial proteins possible. (Huse et al., 2002, Hober *et al.,* 2007)

Limitations do however, exist. Protein A remains limited to certain subclasses of IgG. Protein G binds non-specifically to certain components in serum such as macroglobulins. Some proteins may leak into the elution fraction during the elution process, resulting in contamination. In addition, the elution process in bacterial protein based affinity chromatography requires the use of a very low pH elution buffer, in which the conformation of the immunoglobulin chains may be altered. The isolation of these bacterial proteins from even recombinant bacteria still continues to remain expensive. Current research continues to move in the direction of further optimization and diversifying the proteins used in this method (Hober et al., 2007, Huse et al., 2002).

1.6 Immobilization of proteins on PHA

As previously mentioned, the affinity adsorbent (bacterial protein) and the matrix to which it is immobilized to both play key roles in the efficacy of antibody purification using bacterial proteins. Several different protein sorbents have been developed for commercial purification of antibodies. Ideally, a dynamic matrix would maintain binding capacity while withstanding increased flow velocity and harsh chemical conditions for elution, as well as not leaking ligand into the elution. Currently, Sepharose and agarose-based matrices with protein A ligands immobilized on their surface are the most widely used affinity media. However, Sepharose beads and resins are both expensive to produce and are subject to the complication of chemical cross-linking and exhibit signs of leakiness. (Huse *et al.* 2002) While current evaluation of methods used to immobilize bacterial proteins onto surfaces continue, novel ideas have surfaced in terms of improving cost effectiveness while maintaining or even

exceeding the binding capacity that commercial beads are capable of. Among these is the approach of producing fusion proteins in excess using genetically engineered bacteria, creating a microbe factory. However, the overproduction of a protein might be toxic to the bacterial host and the isolation and subsequent purification of the protein might be difficult and time consuming. These challenges are overcome by the use of PHA granules which are engineered to display the fusion protein of interest on their surface.

Polyhydroxyalkanoates (PHA) are produced as insoluble, spherical inclusions and a wide range of both gram-positive and gram-negative bacteria are able to form these storage materials (Rehm, 2003) They are comprised of a polyester core surrounded by phospholipids and proteins. (Rehm, 2006) One of the most commonly isolated biopolymers is poly(3-hydroxybutyric acid) or PHB (Mifune *et al.*, 2009). PHB was the first identified bacterially produced polymer and was synthesized from the precursor molecule 3-hydroxybutyrate (Lütke-Eversloh *et al.* 2002). The biopolyester is biodegradable, biocompatible and elastomeric; generating great interest in its applications in recent years from being used as a material for tissue engineering (Chen and Wu 2005) to serving as a platform for antigen display and vaccine delivery (Parlane et al. 2009) . The granules range from a size of 50-500 nm and therefore provide a much greater surface area to volume ratio as compared to commercially produced Sepharose beads which are much larger in size (Invitrogen 2007). The production of the fusion protein and its subsequent immobilization and display on the PHA granule is part of a self-assembly process within the production host. Purification of the protein of interest would only require isolating the PHA granules which is a relatively simple and cost effective process.

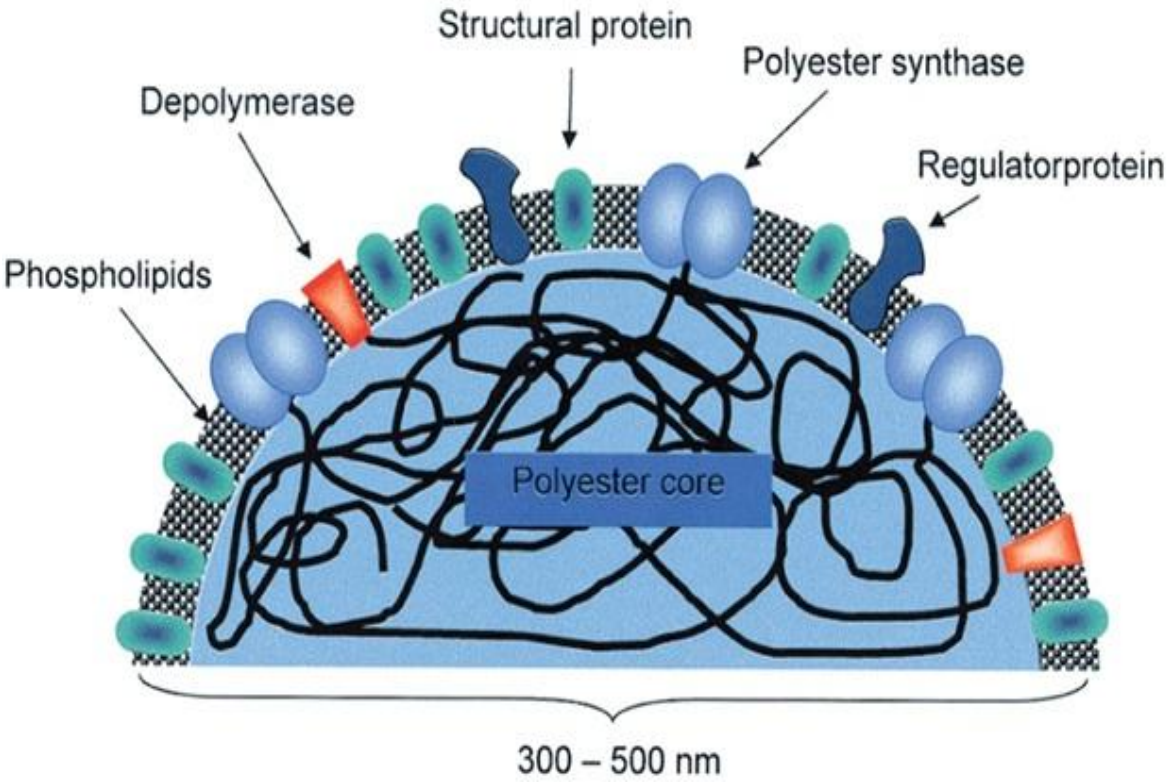


Figure 1: Schematic diagram visualizing the PHA granule and associated proteins, including the polyester synthase or PhaC. (from Rehm, 2003)

The production of this biopolymer in bacteria is catalysed by three crucial enzymes; PhaA, PhaB and PhaC or PHA synthase, (Mifune et al., 2009, Steinbuchel *et al.*, 1998) PhaA is a β-ketothiolase, condensing two acetyl-CoA molecules to acetoacetyl-CoA. This is then reduced by the reductase PhaB into *R*-3-hydroxybutyryl-CoA. PHA synthase or PhaC then polymerizes this compound into a linear chain, releasing CoA simultaneously (Peters & Rehm, 2006). These polyester chains aggregate to form a spherical granule 50-500nm in diameter with a PHA polyester core and the PHA synthase remaining covalently attached on the surface of the granule. The display of PHA synthase on the surface of these granules

provides an ideal target for immobilizing functional proteins. The PhaC polymerase is capable of tolerating N and C-terminal fusions (Peters & Rehm, 2005, Peters & Rehm 2006). Targeting the displayed PHA synthase has previously allowed for the immobilization of green fluorescent protein (GFP) to the surface of PHA granules (Peters & Rehm, 2005).

PHA synthase engineering has also enabled the display of the immunoglobulin G binding ZZ domain of protein A on the bio polyester granule surface, providing an efficient, particle-based IgG purification system (Brockelbank et al., 2006). In 2006, a paper published by Jane A. Brockelbank, Verena Peters, and Bernd H. A. Rehm detailed the production of polyhydroxyalkanoate beads with the ZZ binding domain of protein A immobilized to their surface (Brockelbank et al., 2006). The ZZ-displaying functional beads detailed in Brockelbank's study showed equal capacity to that of commercially produced protein A Sepharose beads in terms of both purity and eluted immunoglobulin yield; maintaining this standard through repeated purification cycles (Figure 2). The success of these beads suggests that great potential exists for the economical production of an affinity adsorbent and matrix in the same system by recombinant bacteria. Combined with recent studies on successful hybrid molecules and protein fusion technology, (Kihlberg *et al.*, 1992), PHA synthase engineering may result in more complete binding properties, allowing for fusions of protein A and protein G binding domains to be expressed on the same bead via covalent linking to the PHA synthase. This would overcome the pitfalls of affinity adsorbents using individual proteins, allowing a wider array of immunoglobulin classes to be purified using the same affinity adsorbent.

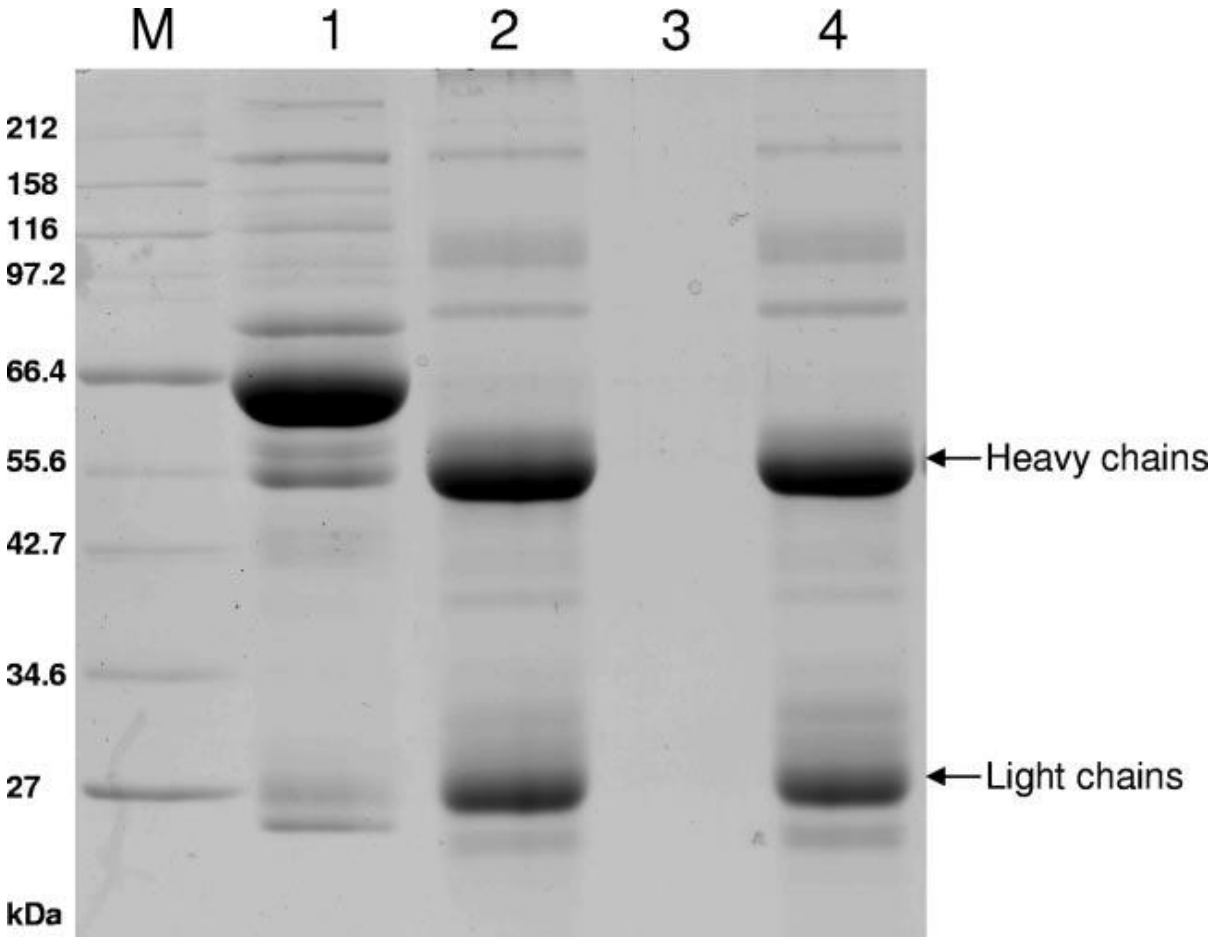


Figure 2: SDS-PAGE gel analysis of proteins bound to ZZ-PHA granules or protein A-Sepharose after elution. Lanes: molecular weight standard; 1, human serum; 2, proteins eluted from protein A-Sepharose beads; 3, proteins eluted from wild-type PHA granules; 4, proteins eluted from ZZ-PHA granules displaying the ZZ domain. Heavy and light chains of IgG are indicated. (From Brockelbank et al., 2006)

1.7 Aim of this study

The aim of this study was to generate novel PHA bio-beads with the immunoglobulin binding domains of protein A, protein G and protein L displayed on their surface, providing an efficient and cost effective tool for immunoglobulin purification.

We hypothesize that a combination of both proteins A and G on a single platform would overcome the disadvantages faced by a purification system using a single protein. PHA synthase engineering would provide this ideal platform via N and C-terminus fusion of PhaC with the protein A and G binding domains. This novel bead should outperform commercial, single functionality beads in terms of IgG binding capacity, while remaining cost effective.

We also aim to create a bio-bead displaying protein L. We know that protein L is capable of binding a wide range of immunoglobulin, making it particularly useful in polyclonal antibody purification. Producing a bio-bead with the κ light chain binding region of protein L displayed on its surface would provide a novel tool for immunoglobulin purification suitable for use on a wide range serums, including supernatant obtained from hybridomas.

CHAPTER 2: MATERIALS AND METHOD

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Ajax Finechem, or Merck

2.1 Bacterial strains and plasmids

Table 3: Bacterial strains used in this study

Strain	Relevant characteristics*	References
E.coli		
BL21 (DE3)	F^- *dcm ompT hsdS*(r_B^- m_B^-) gal λ (DE3)	Stratagene
XL1-Blue	*recA1 endA1 gyra96 thi-1 hsdR17 supE44 Rel31 lac* [F' *proAB* $lacI^qZ\Delta M15$ *Tn10* (Tet^r)]	Stratagene

Table 4: Plasmids used in this study

Plasmid name	Characteristics	Reference
pET14b	Ap^r; T7 promoter	Novagen
pET14b-ZZ (-)phaC	pET14b containing XbaI /BamHI fragment comprising gene *zzphaC* without the signal sequence-encoding region	(Brockelbank et al. 2006)
pET-14b-phaC-GFP	pET-14b derivative containing *gfp* directly fused to the 3' end of *phaC*	(Jahns & Rehm 2009)
pET-14b-ZZ-phaC-GFP	pET-14b PhaC-GFP derivative containing the ZZ sequence 5' of *phaC*	(Jahns & Rehm 2009)
Polybind-Z™	pET-14b derivative containing ZZ sequence fused to the 3' end of *phaC* via a linker sequence	Polybatics Ltd.
Polybind-G™	pET-14b derivative containing the $GB1_3$ sequence directly fused to the 3' end of *phaC*	Polybatics Ltd.
pET14b-ZZ-linker-ZZ-phaC-$GB1_3$	pET-14b derivative containing $GB1_3$ fused to the 3' end of *phaC*	This study
pET-14b-phaC-L	pET-14b derivative containing *L-domain* sequence directly fused to the 3' end of *phaC*	This study

pET14b-ZZ-linker-ZZ-phaC-L	pET-14b PhaC-L derivative containing the ZZ sequence 5' of *phaC*.	This study
pUC57	2,710 bp vector and isolated from *E. coli* strain DH5α by standard procedures.	Genscript
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* co-linear to *lac* promoter	(Amara & Rehm 2003)

2.2 Liquid Media

Luria-Bertani (LB) media (Invitrogen) was prepared as per manufacturer instructions by adding 20 g per litre of Milli-Q water. Media was autoclaved at 121˚C for 20 minutes.

2.3 Solid Media

1.5% (w/v) agar (Oxoid) was added to liquid media and autoclaved at 121˚C for 20 minutes.

2.4 Antibiotic stock solutions and final concentrations

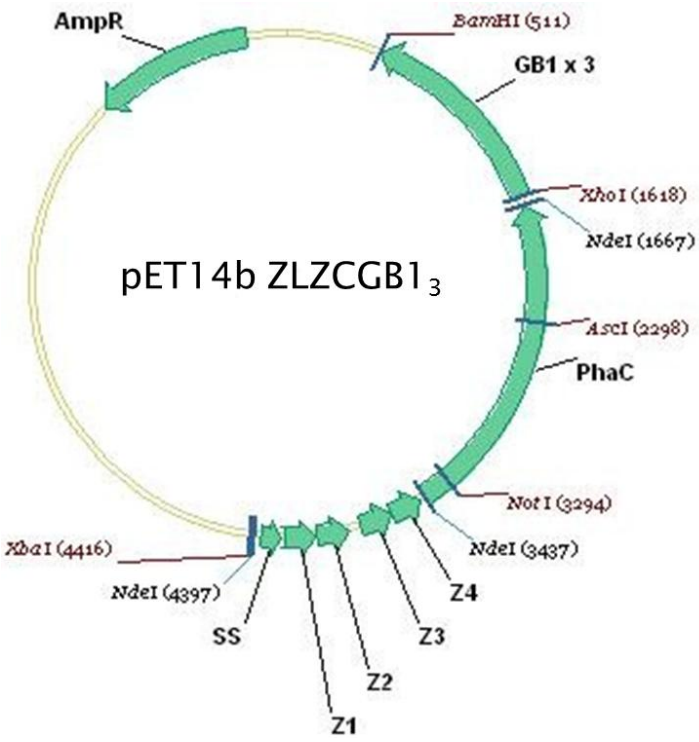
Antibiotic solutions were prepared as listed below. Sterilization was achieved by filtration through a 0.22 µm filter then after which the solution was aliquoted into 1ml sterile tubes and stored at -20˚C until required. All autoclaved media was cooled to 50˚C before addition of antibiotic solutions.

Table 5: Antibiotic stock solutions and final concentrations.

Antibiotic	**Stock solution (mg/ml)**	**Final concentration (µg/ml)**
Ampicillin (Na-salt)	75 in H_2O	75
Chloramphenicol	50 in EtOH (95%)	50

2.5 Cloning Method

The synthetic, extended *gb1* gene coding for the protein G IgG binding regions was ordered from GenScript using the OptimumGene Codon Optimization Analysis (Genscript, USA). This allowed for the addition of *Xho*I and *BamH*I cleavage sites on either end of the gene during the design process. Upon arrival, the vector plasmid pUC57 containing the synthesized gene was used to transform the *E.coli* strain XL1 Blue. Plasmid isolation was performed and isolated plasmid was digested with *BamH*I and *Xho*I. The pre-existing Polybind-Z^{TM} construct containing the synthetic gene *ZZ* was also digested using the restriction enzyme *Xho*I and *BamH*I. This excised the linker-*ZZ* region fused to the 3' end of phaC, and linearized the plasmid. The linearized backbone was then ligated with the synthetic, extended $gb1_3$ gene designed with *Xho*I and *BamH*I flanking regions. As a control, the Polybind-G^{TM} construct was prepared by fusing the synthesized $gb1_3$ gene with the PHA synthase gene without the protein A binding regions (ZZ) present. This was achieved by excising the *gfp* fragment of the previously constructed PhaC-gfp plasmid by *Xho*I and *BamH*I digest (Jahns & Rehm 2009). Similarly, the synthetic *L* gene designed with *Xho*I and *BamH*I flanking regions encoding the immunoglobulin-binding domain of protein L was fused to the 3' end of phaC to produce the ZZ-linker-ZZ-phaC-L and phaC-L constructs. All plasmid construction utilized the vector pET14b as a backbone, which contains a T7 polymerase for gene expression and overproduction of the recombinant protein of interest when transformed into *E.coli* XL1 Blue.



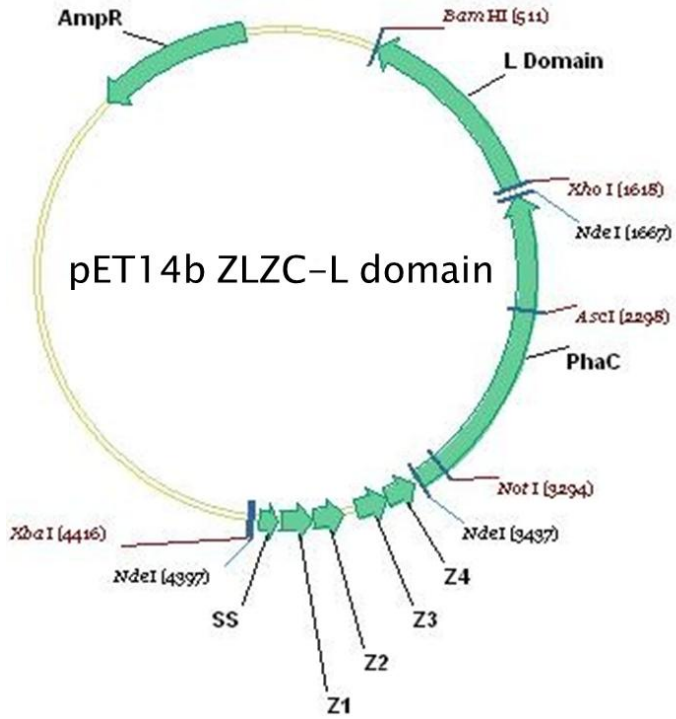


Figure 3: Vector map of the ZZ-linker-ZZ-phaC-GB1$_3$ and ZZ-linker-ZZ-phaC-L constructs.

2.6 PHA bead production in *E.coli*

The recombinant pET14b expression vectors were transformed into the *E.coli* strain BL21 (DE3) (Stratagene) containing the plasmid pMCS69 containing the *phaA* and *phaB* of *C. necator* necessary for bead production. BL21 (DE3) primary cultures were inoculated with 1% (v/v) fully grown overnight culture and cultivation was performed at 37˚C in LB medium containing 75 µg/ml ampicillin, 50µg/ml chloramphenicol and 4% glucose. IPTG was added at a concentration of 1 mM to induce the cultures once an OD_{600} of 0.5 was reached (approximately 3 hours). Cultures were then grown at 25 ˚C for 72 hours.

2.7 Long term storage and revival of bacterial strains

Strains were grown overnight in LB liquid media containing the appropriate antibiotics at 37˚C on an orbital shaker. 1 ml of the culture was then mixed with 60 µl of sterile dimethylsulfoxide (DMSO) in a 2 ml cryovial tube giving a final concentration of 7.5% (v/v) and stored at -80 ˚C. Revival of a stored strain was performed by isolating a small chip of the frozen stock using a sterile pipette tip and using the obtained chip to inoculate 150 ml of antibiotic-containing liquid media contained in a conical flask. The inoculated media was then incubated overnight at 37 ˚C on an orbital shaker.

2.8 Preparation of competent *E.coli* cells. (Hanahan 1983; Hanahan 1985)

50 ml LB media was inoculated with 1% inoculum of overnight culture. This was cultivated at 37 ˚C until OD_{600} reached 0.3. Cell culture was then stored on ice for 15 minutes and harvested by centrifugation at 8,000 x *g* (Heraeus Multifuge 1 S-R, Sorvall). Cells were then resuspended in 18ml RF1 solution and incubated on ice for a further 30 minutes. Cells were then harvested again by centrifugation at 2250 x g (Heraeus Multifuge 1 S-R, Sorvall) and

resuspended in 4ml of RF2 solution. These competent cells were then aliquoted into 1.5ml microfuge tubes in volumes of 200 µl per tube and stored at -80˚C.

RF1 solution

100 mM	Rbcl
50 mM	$MnCl_2$
30 mM	Potassium acetate
10 mM	$CaCl_2.6H_2O$

Adjust to pH 5.8 with acetic acid

RF2 solution

10 mM	RbCl
10 mM	MOPS
75 mM	$CaCl_2.6H_2O$
15% (v/v)	Glycerol

Adjust to pH 5.8 with NaOH

Solutions were filter sterilized (0.22µm) and stored at -20˚C

2.9 Transformation of *E.coli* cells

200µl of competent cells prepared using the method described above were thawed on ice for 30 minutes and mixed thoroughly with 1-3 µl of plasmid DNA and incubated on ice for 30 mins to allow for the adsorption of the DNA to the cell surface. To ensure uptake of adsorbed DNA, the *E.coli* cells were heat-shocked at 42 ˚C for 90 seconds and then put immediately

back on ice for 5 minutes. To promote regeneration of the cells and expression of plasmid-encoded antibiotic resistance, 800 µl of LB was added and the cells were incubated at 37˚C for one hour. For the selection and isolation of recombinant clones, 100 µl of the cells were plated on solid LB-agar medium containing the appropriate antibiotic and the remaining cells were pelleted by centrifugation for 2 minutes at 6000 x g (Heraeus Pico 17, Thermo Scientific), resuspended in 100 µl LB and plated onto fresh solid LB-agar medium. The plates were then incubated overnight at 37 ˚C.

2.10 DNA isolation and manipulation

2.10.1 Plasmid isolation and concentration

Plasmid isolation from recombinant *E.coli* was done using the High Pure Plasmid isolation kit (Roche, Switzerland) and was performed according to manufacturer's instructions. When isolated DNA concentrations (measured using the methods described below) were low or for dirty plasmid preparations, the Clean and Conentrator kit (Zymo Research, USA) was used to generate a plasmid preparation suitable for cloning and sequencing purposes.

2.10.2 DNA hydrolysis with restriction endonucleases

Plasmid DNA was digested with restriction endonucleases (RE) to obtain the desired fragments based on available restriction sites. Restriction enzymes were purchased from New England Biolabs (NEB) or Roche and were used as per manufacturer's instructions. DNA hydrolysis involving the use of two RE were performed via an intermediate isopropanol precipitation step or using compatible buffers (100% activity for both enzymes) in a single reaction.

2.10.3 Determination of DNA fragment size and concentration

DNA fragment size was estimated using comparison with fragments of known size on agarose gel electrophoresis. Phage lambda DNA previously digested with *Pst*I to fragments with known size was used as a size standard or marker. (Sambrook *et al.*, 1989) DNA concentration was measured using several separate methods to ensure that plasmid preparations were of suitable concentration both for cloning and to meet sequencing requirements. These include agarose gel electrophoresis, measuring the absorption of the plasmid preparation at 260 nm and 280 nm using the Nanodrop 1000 (Thermo Scientific, USA) and/or using the Qubit™ fluorometer (Invitrogen, USA) which is used in conjunction with the Quant-iT DNA Broad Range (BR) assay kit (Invitrogen, USA). The Qubit™ system is accurate for measuring DNA concentrations between 2 – 1000 ng and was used according to manufacturer's instructions.

2.10.4 Agarose gel electrophoresis (AGE)

DNA fragments were separated using agarose gel electrophoresis on horizontal gel slabs. Analysis of a DNA sample typically involved the use of 1-2% agarose gels made in TBE buffer. (1% gels for fragments > 1000 bp and 2% gels for fragments < 1000 bp). Stop-mix or loading dye was added to DNA samples before loading them into the wells of the gel. Typical electrophoresis conditions used in this study ranged between 100-120 V for 30-50 minutes; depending on the dimensions of the gel electrophoresis chamber and the degree of separation required between DNA fragments of interest. Gels were then stained in ethidium bromide (EtBr) solution for 15-20 minutes, destained for 1-2 minutes in distilled H_2O and visualised using a UV transilluminator at 254 nm (Bio-Rad, Gel Doc 2000). Where DNA fragments were required after electrophoresis for cloning purposes rather than analysis, SYBR safe

DNA gel stain was used in the place of EtBr. The gel was then visualized using a blue light screen as per manufacturer's instructions (Invitrogen, USA)

TBE Buffer

50 mM Tris/HCl

50 mM Boric Acid

2.5 mM EDTA

Adjust to pH 8.5 using HCl

Stop-Mix (6x)

4 M Urea

50 mM EDTA

50% (v/v) Sucrose

0.1% Bromophenol Blue

2.10.5 DNA fragment recovery from agarose gels

Recovery of DNA fragments of interest after agarose gel electrophoresis was performed using the Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, USA) as per manufacturer's instructions.

2.10.6 DNA ligation

The ligation of DNA fragments into a suitable expression vector was performed using T4 DNA ligase (Invitrogen, USA). After DNA hydrolysis with the use of restriction endonuclease(s) followed by separation via gel electrophoresis and subsequent purification

using the method described above, the fragments of interest were used in a ligation reaction performed in a 1.5 ml microfuge tube and prepared according to manufacturer's instructions. The reaction mix was incubated overnight in a floating water bath at 4˚C.

2.10.7 DNA sequencing

Sequencing of all recombinant plasmids were performed in house by Allan Wilson Centre Genome Service using a capillary AB13730 Genetic Analyzer (Applied Biosystems Inc.). Each 15 µl sequencing reaction was set up in a sterile 0.2ml thin-walled PCR tube (Axygen, USA) and consisted of 300 ng of DNA with suitable primers (added at a concentration of 3.2 pM). Results were provided in ABI format and analysed using Vector NTI version 10.

2.11 PHA extraction, preparation and analysis

2.11.1 Cell disruption

Cells were harvested from cultures by centrifugation at 6000 x rpm for 20 minutes in a Sorvall RC-5B (Du Pont Instruments). The resulting sediment or cell pellet was then re-suspended in 50mM potassium phosphate buffer (pH 7.5) and transferred into 50ml falcon tubes. Cells were then centrifuged again at 8000 x *g* for 15 minutes in a Heraeus Multifuge 1 S-R (Sorvall, Germany) and the supernatant was discarded. The cell sediment was then re-suspended in 50mM potassium phosphate buffer (pH 7.5) and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche, Switzerland) was added to 10ml buffer to ensure inhibition of any protease activity. DNaseI (10 µg ml^{-1}) and lysozyme (300µg ml^{-1}) was also added and the mixture was incubated on ice for 15 minutes. This crude cell extract was then subjected to 2 passes of mechanical cell disruption at 20 kPsi. Crude cell lysate was then centrifuged at 8000 x *g* for 20 minutes at 4˚C. To maximize the amount of PHA beads isolated, the density of the supernatant after this centrifugation step was observed. Should the

supernatant remain particularly cloudy at this point, the supernatant was then subjected to an additional centrifugation step at at 8000 x *g* for 20 minutes at 4˚C. This is to ensure that all beads, unlysed cells and large insoluble cellular debris are sedimented. The sediment was then re-suspended in a small amount (5ml for a typical harvest from 250 ml culture or 5-10 g wet cell mass) of 50 mM potassium phosphate buffer.

50 mM Potassium Phosphate Buffer

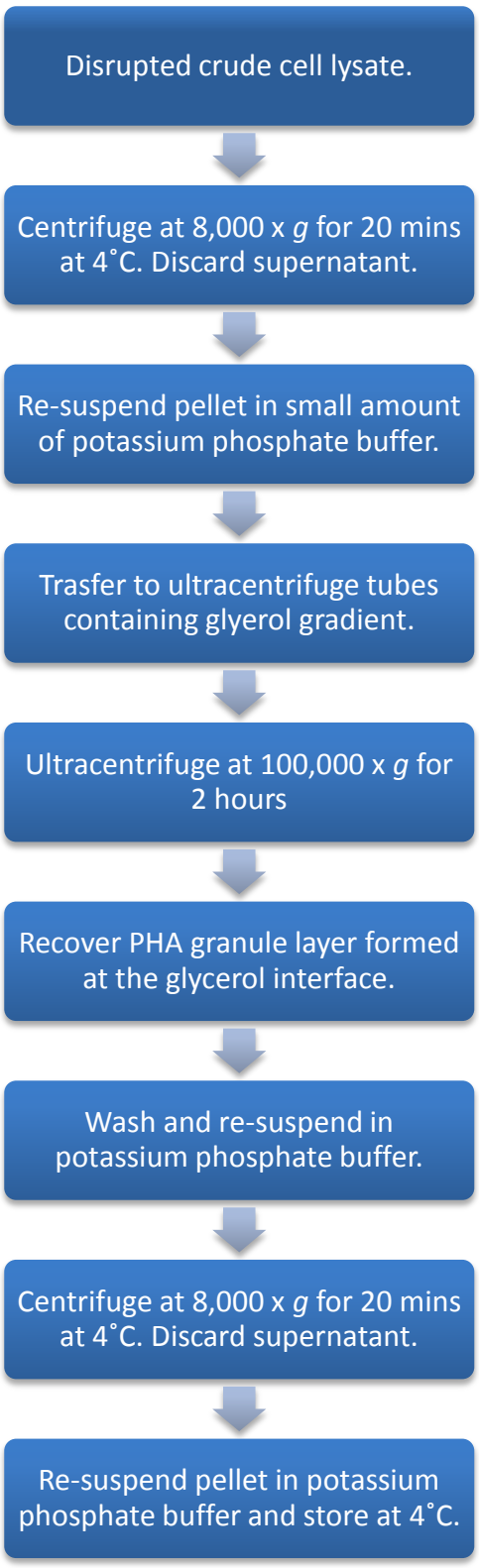
50 mM K_2HPO_4 (Base)

50 mM KH_2PO_4 (Acid)

The pH of K_2HPO_4 was adjusted to pH 7.5 using KH_2PO_4.

2.11.2 Isolation of PHA granules from crude cell lysate (Ultracentrifugation)

The cell lysate containing PHA beads obtained through the cell disruption method described above were purified and the desired PHA beads isolated through ultracentrifugation at 100,000 x *g* for 2 hours (Sorvall WX Ultra 80. Thermo Scientific) using a glycerol gradient containing a lower 88% glycerol layer and an upper 44% glycerol layer. During ultracentrifugation, the PHA beads would form a layer between the 44% and 88% glycerol interface while the remainder unlysed cells and cellular debris would sediment and thus be separated from the beads due to difference in density. The process is visualised in the figure below.



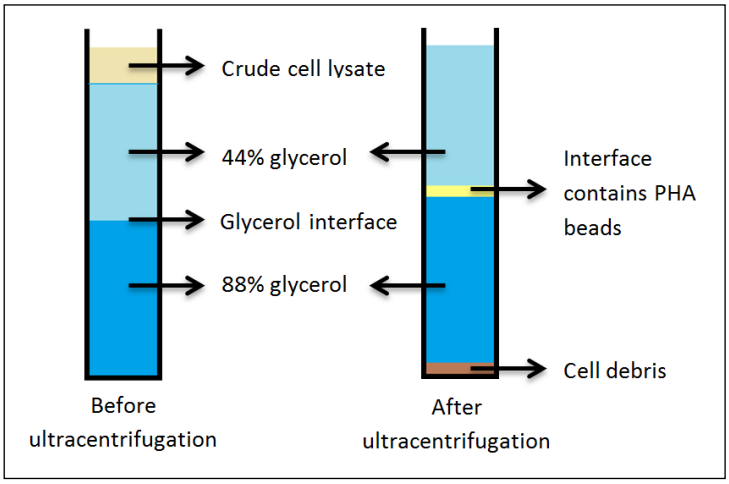


Figure 4: Diagram of the PHA bead extraction process from crude cell extract. Inset also shows a schematic of the density separation of PHA beads after the ultracentrifugation process.

2.11.3 Detection of PHA accumulating cells using Nile-red.

PHA accumulating cells grown in liquid media were routinely analysed using Nile-red staining. 1ml of culture was harvested by centrifugation (Hearaeus Pico 17, Thermo Scientific). 10 µl of Nile-red stock solution (0.25 mg/ml in DMSO) was added to the cell pellet and mixed. 1ml of 50mM potassium phosphate buffer (pH 7.5) was then added and the mix was left to incubate at room temperature for 15 minutes. Cells were then centrifuged and washed again with 1 ml of 50 mM potassium phosphate buffer (pH 7.5) and applied onto a microscope slide and covered with a cover slip. This was then examined using fluorescent microscopy (Olympus) and Magnafire imaging software was used to capture the images digitally for further analysis and confirmation of PHA accumulation.

2.11.4 Gas chromatography-mass spectrometry analysis (GC/MS)

10-20 mg of lyophilised cells were suspended in 2ml chloroform and subjected to lysis in 2ml methanol in the presence of 15% (v/v) sulphuric acid. The methanolysis procedure was done in a heated oil bath (100 ˚C) for 5 hours. Tubes were then removed and allowed to cool to room temperature before adding 2 ml of H_2O. The mix was then vortexed and left at room temperature to allow for phase separation. The resulting methyl esters of the corresponding fatty acid were recovered from the bottom phase and assayed by gas chromatography-mass spectrometry (GC/MS) for 3-hydroxyalkanoate methyl esters. All GC/MS anaylsis was performed at Plant and Food (Palmerston North).

2.12 General methods for protein analysis

2.12.1 Protein concentration measurement (Bradford 1976)

Bradford protein assay was used to determine the concentration of PHA bead associated proteins. 10µl serial dilutions of a sample were loaded into a 96-well flat bottom microtitre

plate (Greiner bio-one). Bovine serum albumin (BSA, Sigma-Aldrich, USA) or Immunoglobulin G (IgG, GE life sciences) of known concentrations were used as standards and also loaded. 200 µl of filtered 1x Bradford reagent (Bio-Rad laboratories, USA) was added to all plate wells and incubated at room temperature for 5 minutes to allow for colour development. The colour absorbance change was then measured at 595 nm by an ultra microplate reader ELx808 IU (BIO-TEK Instruments Inc, USA). A standard curve was prepared based on absorbance readings for the known concentrations of BSA (ranging from 0.05 mg/ml to 0.4 mg/ml) or IgG (0.05 mg/ml to 0.8 mg/ml).

2.12.2 Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

For the characterization and evaluation of proteins, SDS-PAGE was performed in a vertical slab gel electrophoresis apparatus (Laemmli *et al*, 1970). Each Tris-glycine gel consisted of a lower separating gel layer (8-10%, w/v, pH 8.9) and an upper stacking gel layer (4%, w/v, pH 6.8). The gel was prepared in an 8cm x 8cm pre-cast cassette (Invitrogen, USA) separated by a 1.5cm gap.

The separating gel mixture was prepared and degassed by the addition of a small amount of Na_2SO_3 to prevent air bubbles from forming. The polymerization reaction was started by adding 20 µl of N, N, N', N'-tetramethylethyl-endiamin (TEMED) and 40 µl ammonium persulfate (APS, 40% w/v) to the solution. The solution was then carefully poured into the pre-cast cassette and immediately layered with isopropanol. This was then left to polymerize for 1 hour.

Once the gel had set, the isopropanol was removed by washing with distilled H_2O. The stacking gel layer was then prepared. As was used in the separating gel, the gel mixture was degassed using Na_2SO_3, followed by starting the polymerization by the addition of 4.4µl TEMED and 8.8µl APS (40% w/v). This was then carefully poured into the pre-cast cassette

above the separating gel layer. A comb was inserted for the formation of wells, and the gel was left to polymerize for 30 minutes.

Seperating gel

8% (w/v)	**10% (w/v)**	
10.4ml	10.4ml	Seperating gel buffer
11.1ml	13.9ml	30% Acrylamide
20.1ml	17.3ml	Milli-Q water
41.6ml	41.6ml	Total volume

Stacking gel

4% (w/v)

3.12ml	Stacking gel buffer
1.88ml	30% Acrylamide
7.50ml	Milli-Q water
12.5ml	Total volume

Seperating gel buffer

54.45g	Tris/HCl
1.2g	SDS
300ml	Milli-Q water

Adjust to pH8.9 with HCl

Stacking gel buffer

18.14g Tris/HCl

1.2g SDS

300ml Milli-Q water

Adjust pH to 6.8 with HCl

Electrode buffer

28.8g Glycine

6.0g Tris

2g SDS

Make up to 2 L with distilled H_2O

Adjust to pH 8.5 with HCl

2.12.3 Preparation of protein samples for SDS-PAGE and electrophoresis conditions.

1-20µl of protein sample containing between 5 – 30 µg of protein was used in the preparation. 2 volumes of the protein solution were mixed in a 1.5ml microfuge tube with 1 volume of SDS denaturing buffer and incubated for 15 minutes at 95˚C. Denatured protein solution was then cooled on ice prior to loading into SDS-PAGE wells. The electrophoresis conditions were 15mA through the stacking gel layer and 26mA through the separating gel layer. Relative molecular weights of proteins were estimated by comparing the motility of the proteins with that of the molecular weight standard proteins. Protein Marker Broad Range (2-212 kDa, New England Biolabs, England) was used as this standard for protein size determination.

SDS denaturing buffer (3x)

8.0g	SDS
37.2mg	EDTA
40.0ml	Glycerol
20.0ml	β-mercaptoethanol
10.0mg	Bromothymol blue

Make up to 100ml with Tris/HCl (100mM, pH 6.8)

2.12.4 Protein staining with Coomassie Blue (Weber & Osborn, 1969)

The SDS-PAGE gel was removed from the cassette and carefully transferred into Coomassie brilliant blue staining solution and stained for 30-45 minutes on a tilt-shaker (Labnet International, USA) set to a low speed setting. After staining, the gel was washed with distilled H_2O and destained using destaining solution until protein bands were visible and the background colour was removed.

Coomassie blue staining solution

4g	Coomassie blue R 250
450ml	Ethanol
90ml	Acetic acid
460ml	Distilled H_2O

Destaining solution

600ml	Ethanol

200ml Acetic acid

1200ml Distilled H_2O

2.12.5 Maldi-TOF mass spectrometry (Maldi-TOF/MS)

Protein bands of interest (approximate size to the expected fusion protein) were cut from SDS-PAGE and subjected to mass spectrometric analysis. All sample preparation and Maldi-TOF/MS was performed by The Centre for Protein Research (Otago University). Excised protein bands were subjected to in-gel digestion with trypsin (Shevchenko *et al*., 1996). The eluted peptides were then dried using a centrifugal concentrator and then re-suspended in 30% (v/v) acetonitrile (ACN) and 0.1% (v/v) trifluoroacetic acid (TFA) in water. 1µl of the peptide solution was mixed with 2µl matrix (10mg/ml alpha cyano-4-hydroxycinnamic acid or CHCA, dissolved in 65% (v/v) acetonitrile containing 0.1% (v/v) TFA and 10mM ammonium dihydrogen phosphate). 0.8 µl of this peptide sample/matrix mixture was applied onto a Maldi sample plate (Opti-TOF 384 well plate, Applied Biosystems, USA) and air dried. The sample was then analysed on a 4800 Maldi tandem Time-of Flight Analyzer (Maldi-TOF/TOF, Applied Biosystems. USA). All readings were acquired in positive-ion mode with 800-1000 laser pulses per sample spot.15-20 of the strongest precursor ions for each sample spot were then selected for collision-induced dissociation (CID) mass spectrometry analysis. The CID spectra were acquired with 2000-4000 laser pulses per precursor using a 2 kV setting and air as the collision gas at 1*E-6 torr. Proteins were identified by comparison with the UniProt/SWISS-PROT amino acid sequence database (updated January 2006) using the Mascot search engine (Matrix Science, USA). This search allowed queries of full tryptic peptides with a maximum of 4 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine and pyroglutamate were included as

variable modifications. Precursor peptides had a mass tolerance threshold of 75ppm and a max fragment mass error of 0.4 Da.

2.13 Determination of fusion protein activity on PHA beads through IgG binding assay

The Immunoglobulin G (IgG) binding assay was used to determine the functionality of the fusion protein displayed on the surface of the PHA bead. The assay also assessed the maximum binding capacity of the recombinant PHA beads displaying protein A (ZZ binding domain) and protein G (GB1 binding domain) on their surface. 50mg (wet weight) PHA beads were measured per sample in a 1.5ml microfuge tube. 50mg of the commercial control beads (Immobilized Protein A or IPA300, cross-linked with 6% agarose, Repligen) was also measured in a 1.5ml microfuge tube. All samples and controls were re-suspended in 500µl chilled 1x PBS buffer (pH 7.5). 1-2 mg total protein of purified IgG from serum (Sigma-Aldrich, USA) was added to each sample and tubes were incubated at 25˚C for 30 minutes with agitation. The tubes were then centrifuged at 6000 x *g* for 4 minutes (Heraeus Pico 17, Thermo Scientific). Aliquots of supernatant were taken for analysis (unbound fraction) and the remaining supernatant was discarded. The pellet was then washed 3 times with 1ml 1x PBS buffer (pH 7.5), centrifuging at 6000 x *g* for 4 minutes and discarding the supernatant after each wash. Bound IgG from PHA beads and controls were then eluted by re-suspending in 1ml of low pH, 50mM Glycine (pH 2.7) and incubating at 25˚C for 5 minutes. All samples were then centrifuged at 16,200 x *g* for 5 minutes. The supernatant containing eluted IgG was then transferred into a new tube and the pH was neutralized to ~pH 7.0 using 1 M K_2HPO_4 and stored at -20˚C for further analysis.

2.14 Purification of IgM from hybridoma supernatant and half- strip lateral flow assay (Competitive inhibition assay)

This experiment was done as a collaboration project with IFS research spinout Science Haven Limited (SHL). 100 mg PhaC-L or Wild-type PHA beads was added to 1ml hybridoma supernatant (3.2mg/ml) in a 1.5ml microfuge tube. The tubes were then centrifuged at 6000 x *g* for 4 minutes (Heraeus Pico 17, Thermo Scientific). The supernatant was collected and stored at 4˚C for further analysis. The pellet was then washed 3 times with 1ml 1x PBS buffer (pH 7.5), centrifuging at 6000 x *g* for 4 minutes and discarding the supernatant after each wash. Bound IgM from PHA beads and controls were then eluted by re-suspending in 1 ml of low pH, 50 mM Glycine (pH 2.7) and incubating at 25˚C for 5 minutes. The supernatant containing eluted IgG was then transferred into a new tube and the pH was neutralized to ~pH 7.0 using 1M K_2HPO_4 and stored at 4˚C for further analysis.

The supernatant and elution fractions were then sent for analysis by half-strip lateral flow assay to Science Haven Limited (SHL). The competitive inhibition assay and subsequent analysis was performed by Jenness Guthrie (Institute of Fundamental Sciences, Massey University, New Zealand). The methodology is briefly described below. Detection reagent is added to the sample or is present at the tail-end of the strip; this reagent contains an anti-IgM antibody conjugated to gold particles that does not compete for the same binding region as the test strip antigen. The sample is then added to the strip, and passes through a test region containing immobilized IgM-specific antigen and captures IgM in the sample. The sample then passes through a control region that captures unbound detection reagent. A band appearing in control line indicates that test is complete. Intensity of the band formed on the test region is indicative of the concentration of IgM in a given sample. The band intensity or apparent absorption (Aapp) of both test and control lines were measured using a lateral flow strip reader. (Stripscan, USA).

CHAPTER 3: RESULTS

The results of this study are divided into two separate parts, the first pertains to PHA beads displaying protein G, and the second involves PHA beads displaying protein L.

3.1 Protein G-displaying PHA beads

The plasmids Polybind-Z ™, Polybind-G ™ and the production plasmid and strain were kindly provided by Polybatics Ltd. PHA beads displaying the binding domain of Protein G were made and isolated using the methods outlined in Chapter 2.PHA accumulation was confirmed via fluorescence microscopy (Figure 5) and GC/MS analysis.

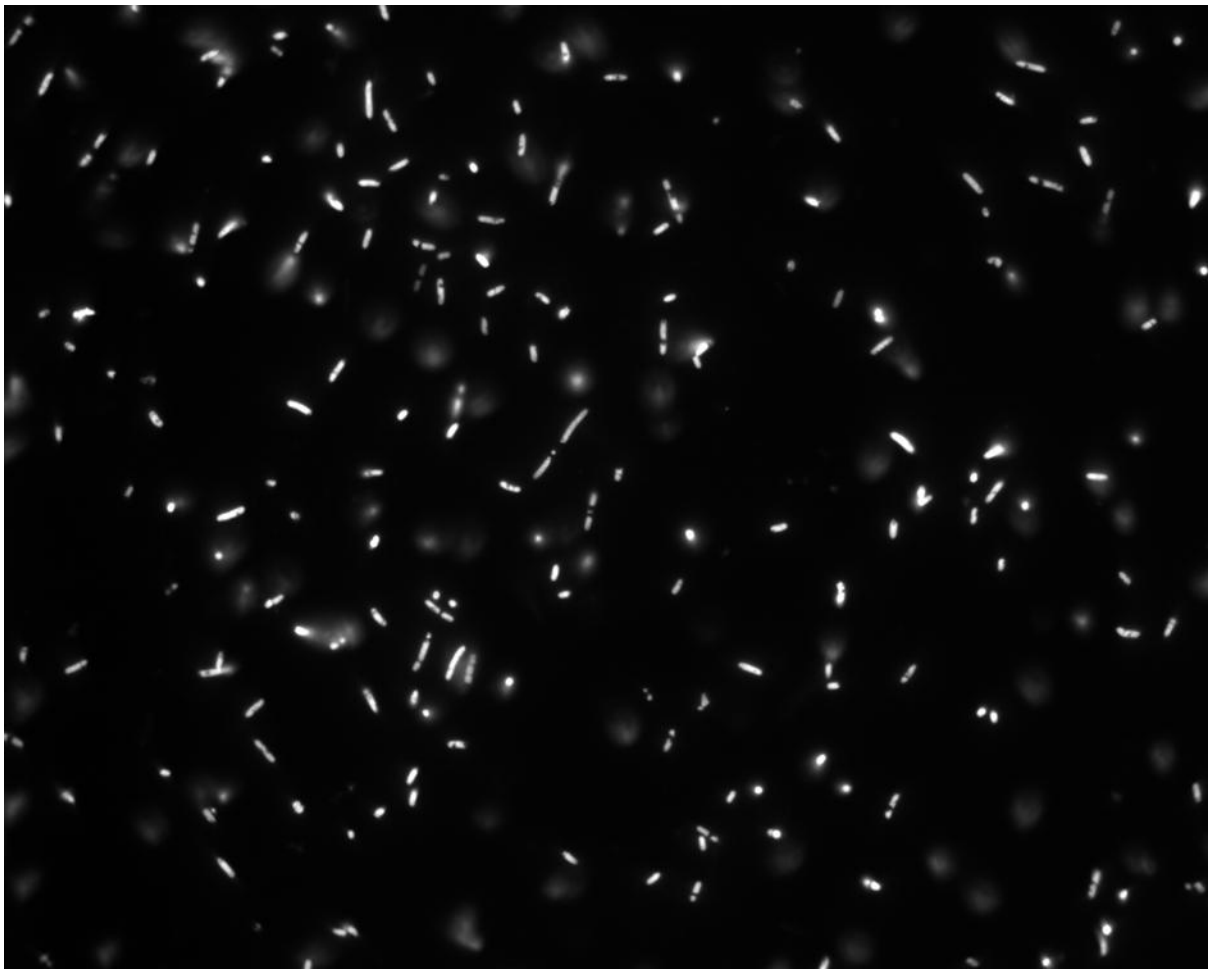


Figure 5: Fluorescence microscopy image of ZZ-linker-ZZ-PhaC-GB1$_3$ beads produced in *E. coli*.

E. coli BL21 (DE3) harbouring the plasmid containing the ZZ-linker-ZZ-PhaC-$GB1_3$ fragment was expressed under PHB accumulating conditions.

The fluorescent inclusions seen with *E. coli* samples under PHB accumulating conditions (Figure 5) suggests intracellular PHB accumulation and confirms PHA synthase activity. This was further confirmed by GC/MS analysis which gave a result of 57% PHB of dry cell weight for the ZZ-linker-ZZ-PhaC-$GB1_3$ PHA bead cultivation (Wild-type PhaC PHA bead cultivation gave 45% PHB of dry cell weight).

The isolated and purified ZZ-linker-ZZ-PhaC-$GB1_3$ PHA beads were then run on SDS-PAGE and the overexpressed presence of the fusion protein of interest was confirmed by a band at the expected molecular weight when compared with the protein marker. This band was excised and sent for MALDI-TOF/MS analysis, the results of which indicated high ion scores and ≥50% sequence coverage when compared to the expected protein sequence, thus further confirming the presence of the fusion protein.

3.1.1 Functional assessment of IgG binding domains

The functional activity of the protein A and protein G binding domains on the surface of the ZZ-linker-ZZ-PhaC-$GB1_3$ (AG) fusion beads was confirmed by performing IgG binding assays (2.13). The first assay assessed the overall purification capability of the novel AG-bead. The hypothesis was that the addition of the $GB1_3$ binding domain would contribute to an increase in overall IgG bound due to the ability of the AG-bead to also bind IgG subclass 3 from human serum as compared to the protein A-only bead. This would be indicated by an increase in detected protein concentration at an absorbance of 490 nm when the eluted fraction from the binding assay was analysed.

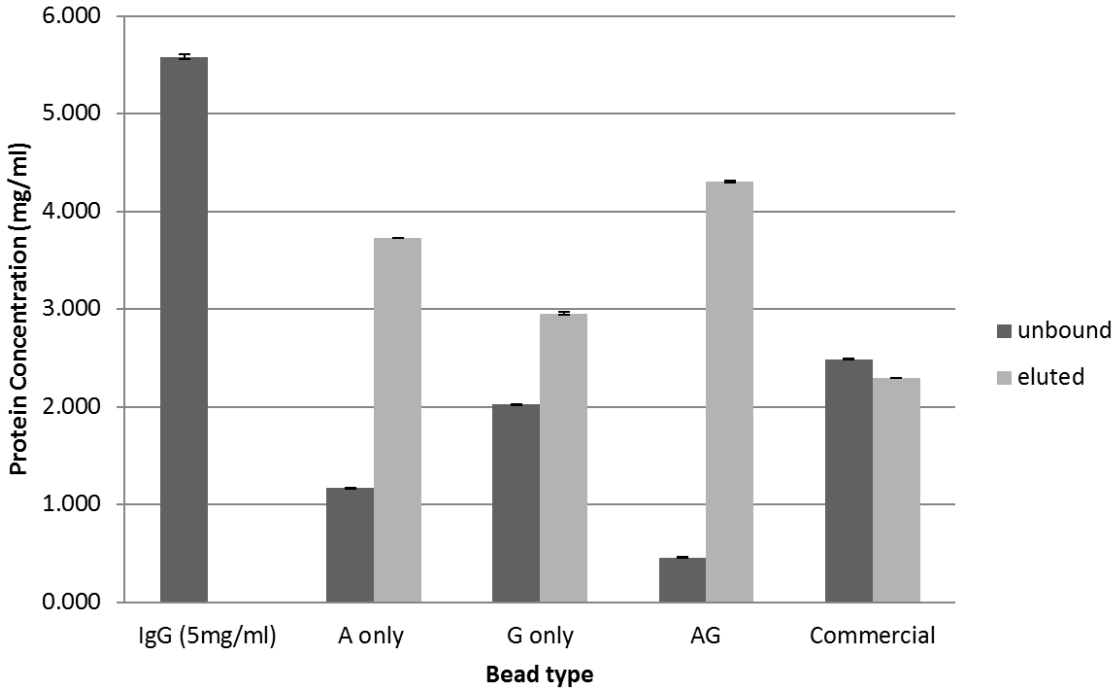


Figure 6: Binding experiment of PHA beads with IgG from human serum. Measured protein concentrations of the unbound and elution fractions obtained from the binding assay of 5 mg IgG with 50 mg of Polybind-Z^{TM} (A-only), Polybind-G^{TM} (G-only), ZZ-linker-ZZ-PhaC-GB1$_3$ (AG) and commercial 6% agarose cross-linked IPA300 beads. Concentration of 5 mg IgG from human serum as detected by the same Bradfords protein assay is also indicated (IgG, 5 mg/ml).

As demonstrated above (Figure 6), the ZZ-linker-ZZ-PhaC-GB1$_3$ (AG) beads bind a higher amount of IgG when the eluted fraction from the binding assay was compared with that of the eluted fraction from the Polybind-Z^{TM} (A-only) and Polybind-G^{TM} (G-only) PHA beads. The AG-bead also demonstrated higher binding capacities when compared to that of the commercial control Protein A bead (Repligen) by 42% (percentage based on concentration of eluted protein from total detectable protein in both fractions)

With this positive result, we sought to further assess the specific functionality of the $GB1_3$, which would be possible by performing a binding assay using IgG from goat serum. While some unspecific binding is expected for protein A-only beads (including the commercial control) (Table 1), it is expected that these A-only beads will bind very low amounts of goat IgG. This is because the type I receptor for protein A does not interact with goat IgG (Table 2). In contrast, the AG-fusion bead and G-only bead should demonstrate an increased amount of bound goat IgG due to the strong binding interaction between the $GB1_3$ domain and the goat immunoglobulin molecule.

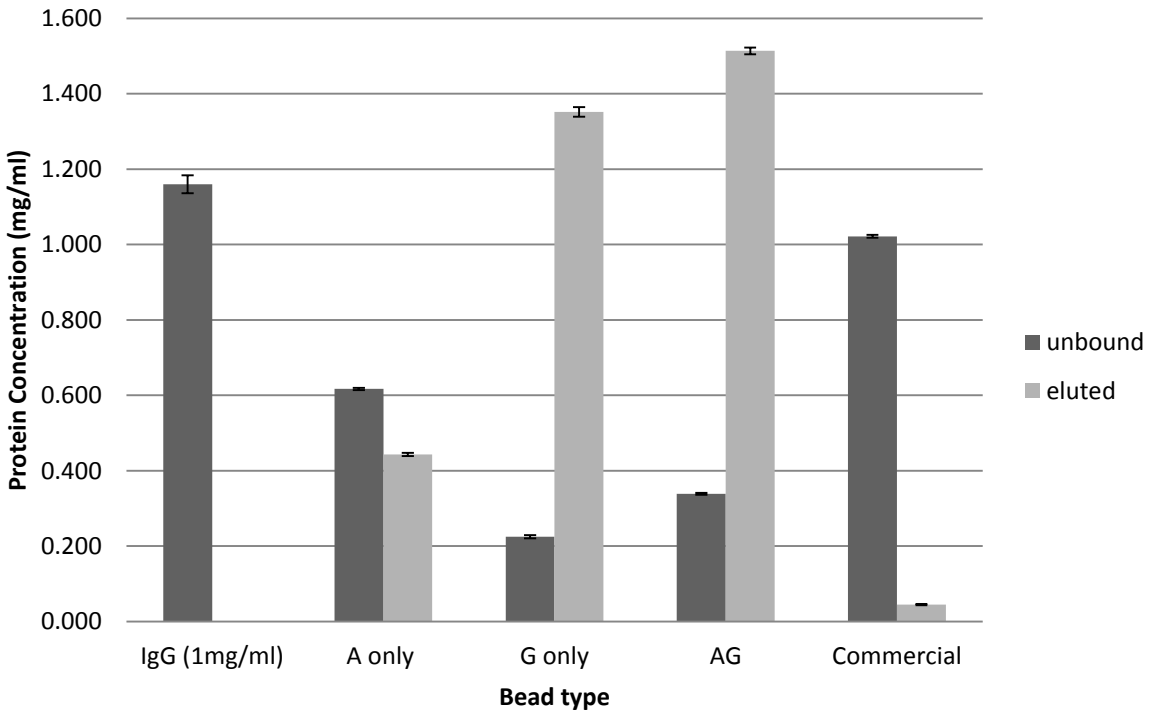


Figure 7: Binding experiment of PHA beads with IgG from goat serum. Measured protein concentrations of the unbound and elution fractions obtained from the binding assay of 5 mg IgG with 50 mg of Polybind-Z^{TM} (A-only), Polybind-G^{TM} (G-only), ZZ-linker-ZZ-PhaC-$GB1_3$ (AG) and commercial 6% agarose cross-linked IPA300 beads. Concentration of 1 mg IgG from goat serum as detected by the same Bradfords protein assay is also indicated (IgG, 1 mg/ml).

As shown in Figure 7, both the G-only and AG-fusion PHA beads bind a much greater amount of goat IgG as compared to that of the A-only PHA beads and the commercial control, providing clear confirmation of the functionality of the $GB1_3$ domain displayed on the novel G-only and AG-fusion PHA beads. The commercial control beads exhibited minimal efficiency in binding goat IgG as was expected, due to the inability of protein A to bind goat IgG. The elevated level of binding seen in the A-only PHA beads may be attributed to a small amount of unspecific attachment of the immunoglobulin molecules to other granule-associated proteins on the surface of the bead.

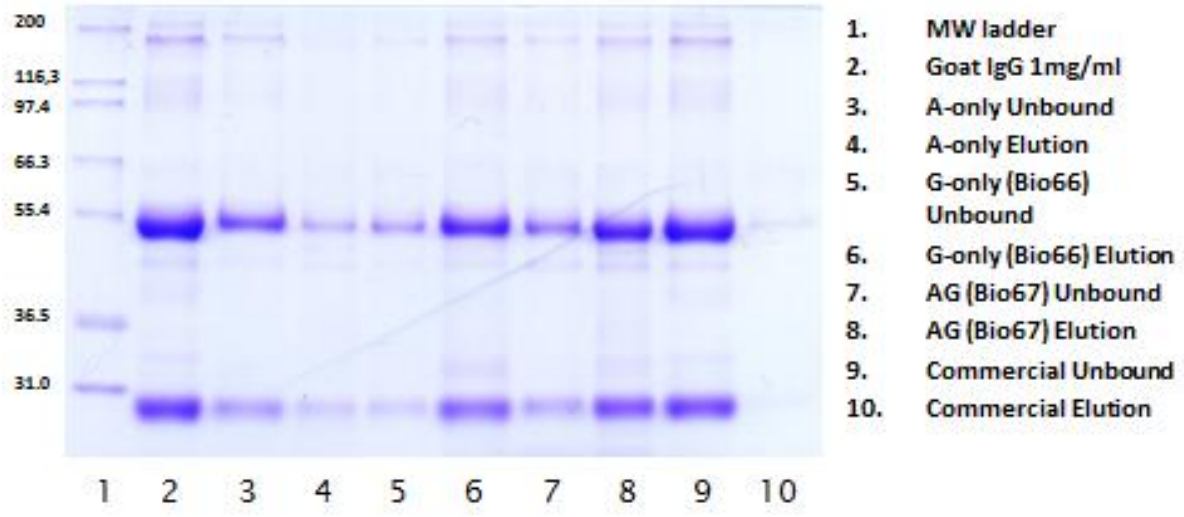


Figure 8: SDS-PAGE analysis of unbound and elution fractions of the goat IgG binding experiment. Resulting fractions from the IgG binding experiment run on SDS-PAGE. Lanes were loaded with the following samples: 1. Broad-range molecular weight marker, 2. 1mg/ml IgG from goat serum. 3. Unbound fraction from binding with Polybind-Z^{TM}, 4. Eluted fraction from binding with Polybind-Z^{TM}, 5. Unbound fraction from binding with Polybind-G^{TM}, 6. Eluted fraction from binding with Polybind-G^{TM}, 7. Unbound fraction from binding with ZZ-linker-ZZ-PhaC-$GB1_3$ (AG), 8. Eluted fraction from binding with ZZ-linker-ZZ-

PhaC-GB1$_3$ (AG), 9. Unbound fraction from binding with IPA300 commercial beads, 10. Eluted fraction from binding with IPA300 commercial beads.

The unbound and elution fractions from the above binding experiment were further analysed by SDS-PAGE. SDS-PAGE analysis of the eluted protein indicated a protein apparent at a molecular mass of 50kDa (representing the heavy chains of the immunoglobulin molecule), and a protein at 25kDa (representing the light chains) were both purified. Greater band intensity of these bands purified by both the G-only and AG-fusion bead as compared to that of the A-only and commercial control further confirmed that the increased protein concentrations seen in the Figure 7 was indeed due to an increase in bound IgG in the elution fraction.

3.2 Protein L-displaying PHA beads

As with the protein G and AG fusion beads, PHA beads displaying the Ig- binding L domain were made and isolated using the methods outlined in Chapter 2. PHA accumulation was confirmed via fluorescence microscopy (Figure 5) and GC/MS analysis. Similar to that of the protein G study, 2 separate bead types were produced, one with only the L domain displaying on the surface of the bead (PhaC-L) through covalent attachment with the PHA synthase, and the other would display both a fusion protein with the binding domains of both protein A and protein L (ZZ-linker-ZZ-PhaC-L).

While GC/MS and fluorescent microscopy confirmed that PHA beads were being produced within the cell, further analysis of the isolated and purified beads via SDS-PAGE revealed that the fusion protein of the expected size for the AL-fusion bead was not present. This unexpected result and subsequent troubleshooting is further elaborated in the discussion section (Chapter 4). Experimentation continued with the PhaC-L bead, the results of which

are presented here. The beads were analysed by SDS-PAGE, and an intense band confirms overexpression of the PhaC-L fusion protein at the expected size of 105 kDa (Figure 9)

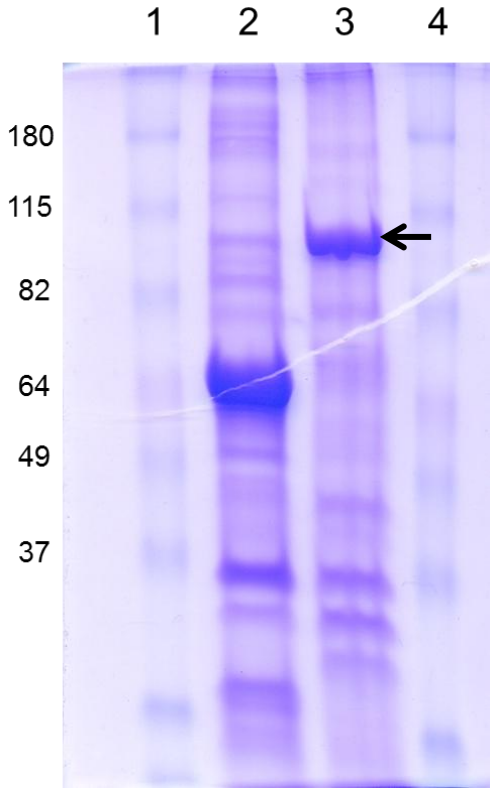


Figure 9: SDS-PAGE of isolated and purified PHA beads displaying the immunoglobulin binding domain of protein L via covalent attachment to the PHA synthase. Lanes are marked as follows: 1,4: Protein marker, 2: Wild-type PHA granules, 3: PHA granules PhaC-L. Arrow indicates overexpression of the PhaC-L fusion protein at 105 kDa.

3.2.1 Functional assessment of immunoglobulin binding domains

3.2.1.1 Binding experiment with IgG from rat serum.

As previously mentioned (1.4), protein L is capable of binding all classes of immunoglobulin through κ light chain interaction with the immunoglobulin molecule. The L domain shows a

particularly strong affinity for rat IgG, (Table 1) in comparison to the binding domains of protein A and G, which show an overall weak or medium affinity. Therefore, the functional activity of the L-domain on the surface of the PHA bead was then assessed by performing a binding assay with IgG from rat serum using the methodology explained in Chapter 2.

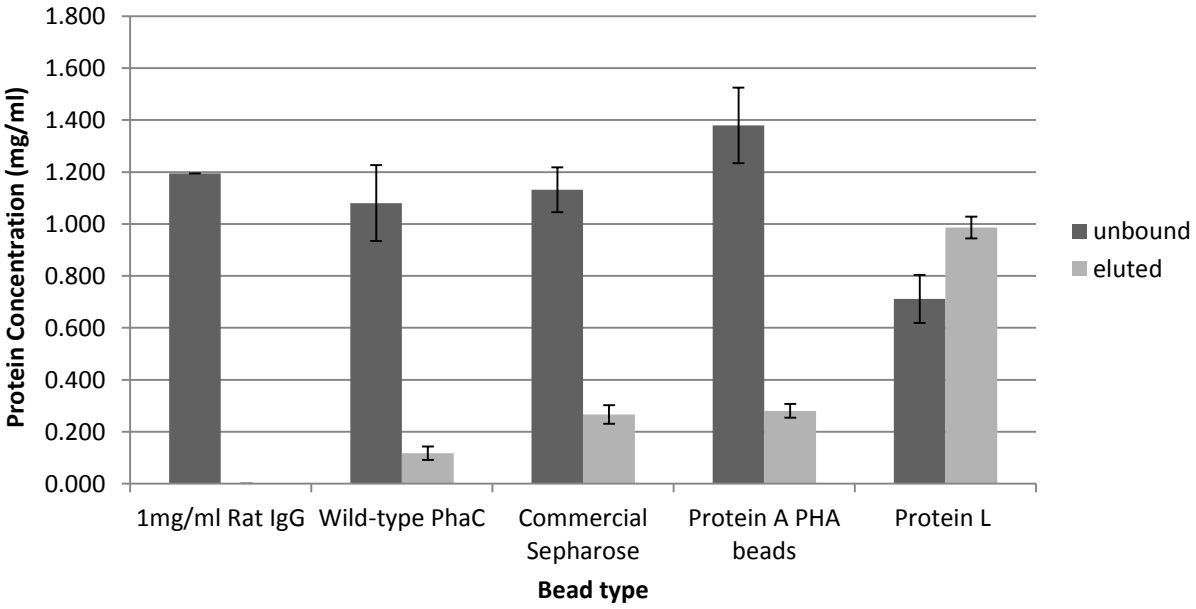


Figure 10: Binding experiment of 50 mg beads with 1mg IgG from rat serum. Measured protein concentrations of the unbound and elution fractions obtained from the binding assay of 5 mg IgG with 50 mg of Wild-type PhaC PHA beads, commercial 6% agarose cross-linked IPA300 beads, Polybind-Z^{TM} PHA beads (A only), and PhaC-L PHA beads (L domain). Concentration of 1 mg IgG from rat serum as detected by the same Bradfords protein assay is also indicated (Rat IgG, 1 mg/ml).

Very low concentrations of eluted IgG were detected in the wild-type PhaC, associated with unspecific binding to the PHA granule. Low concentrations of IgG were eluted from the binding experiment with the Protein A commercial sepharose and Protein A PHA beads (Polybind-Z^{TM}). This is indicative of the expected weak binding activity between protein A

and the rat IgG molecule. The protein L beads (PhaC-L) show a much greater concentration of protein in the elution fraction of the binding assay, indicative of the strong binding interaction with the rat IgG molecule. This confirmed that the PhaC-L bead was functional and suitable for immunoglobulin purification purposes.

3.2.1.2 Purification of IgM from hybridoma supernatant. (In collaboration with Science Haven Limited, NZ)

To further assess the capabilities of the PhaC-L beads in the purification of IgM, isolated and purified PHA beads were used to purify IgM from crude hybridoma supernatant as previously described (2.1.4). Using the protocol described, PHA beads were added to crude hybridoma supernatant provided by Science Haven in an attempt to bind and subsequently elute IgM. Protein concentration of the unbound and elution fractions were measured and analysed using Bradford protein assay.

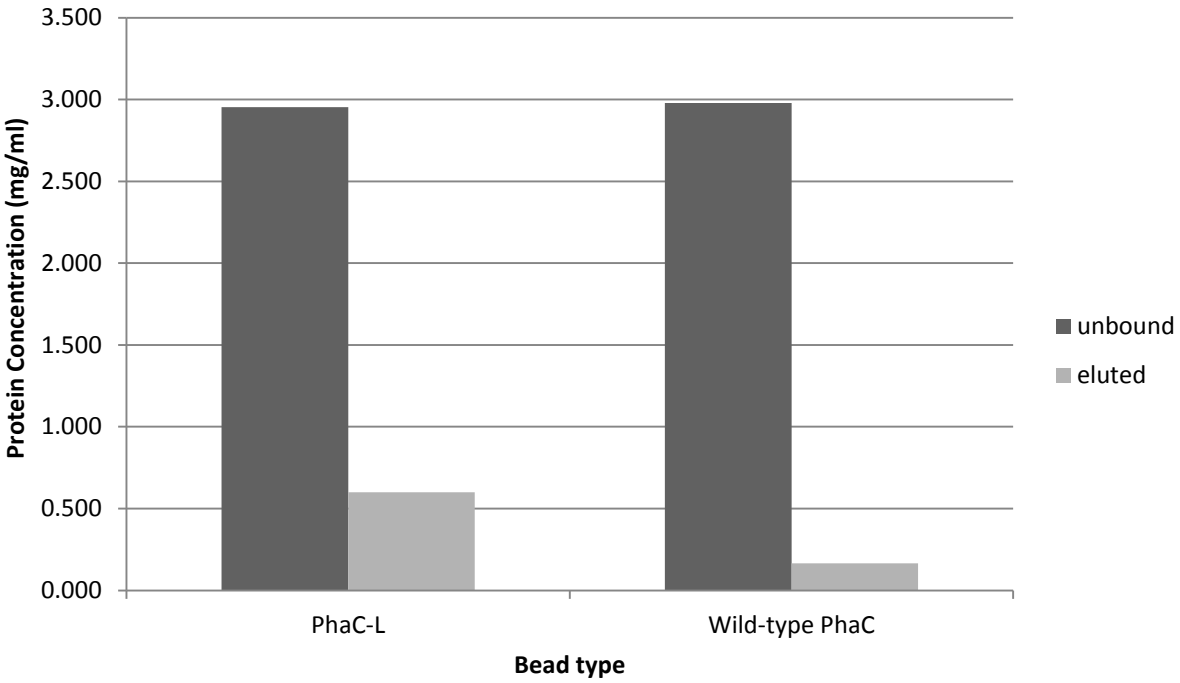


Figure 11: Binding experiment of 100 mg beads with 1ml crude hybridoma supernatant. Measured protein concentrations of the unbound and elution fractions obtained from the binding assay of 1ml crude hybridoma supernatant (3.2 mg/ml) with 100 mg of PhaC-L PHA beads and Wild-type PhaC PHA beads.

Based on the binding experiment results (Figure 11), we determined that the PhaC-L PHA beads (displaying the L-domain) were capable of purifying 17% more protein when the elution fraction was compared to that obtained from binding with Wild-type PhaC beads. We then proceeded to confirm the identity of the additional purified protein using SDS-PAGE analysis (Figure 12).

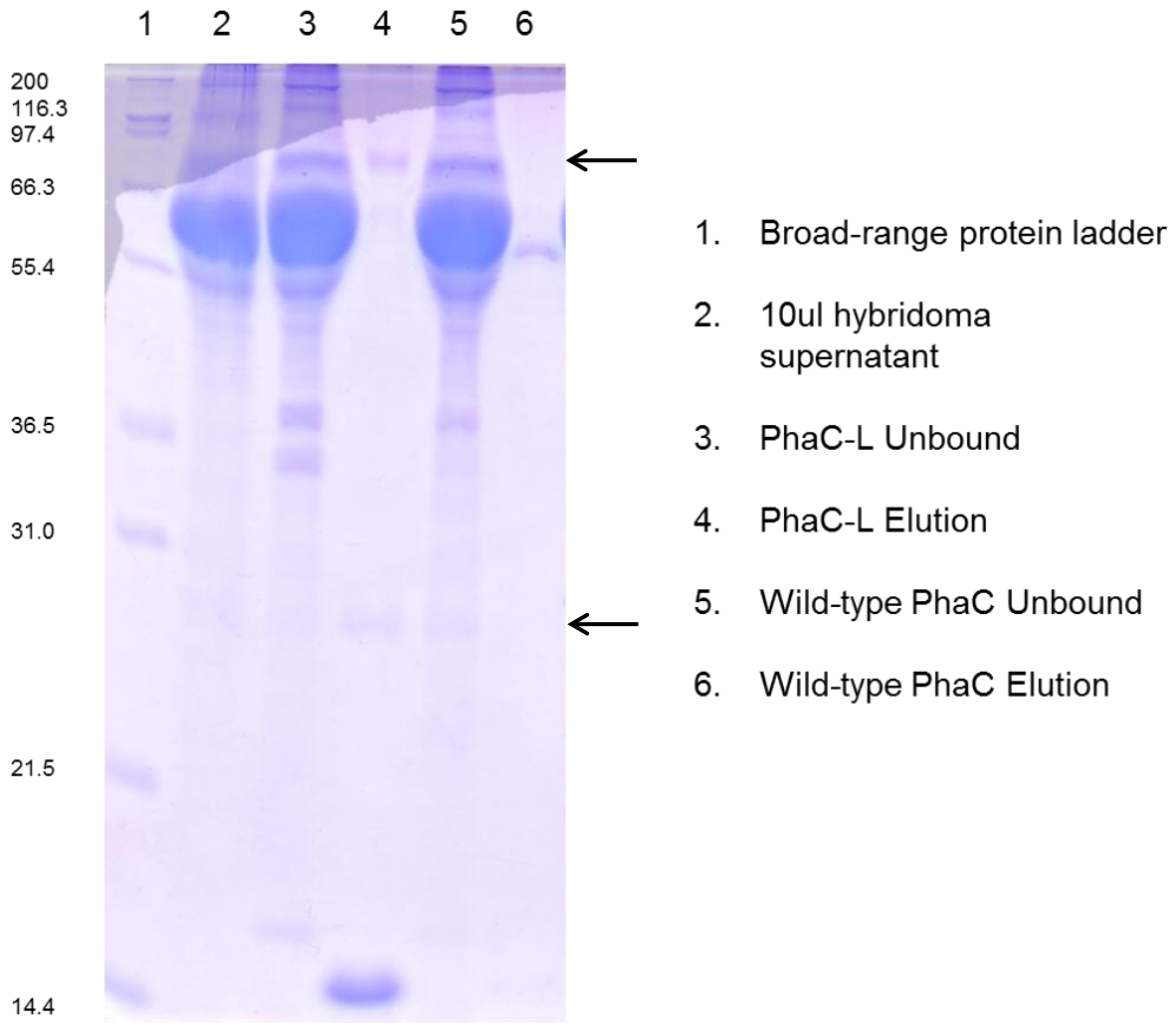
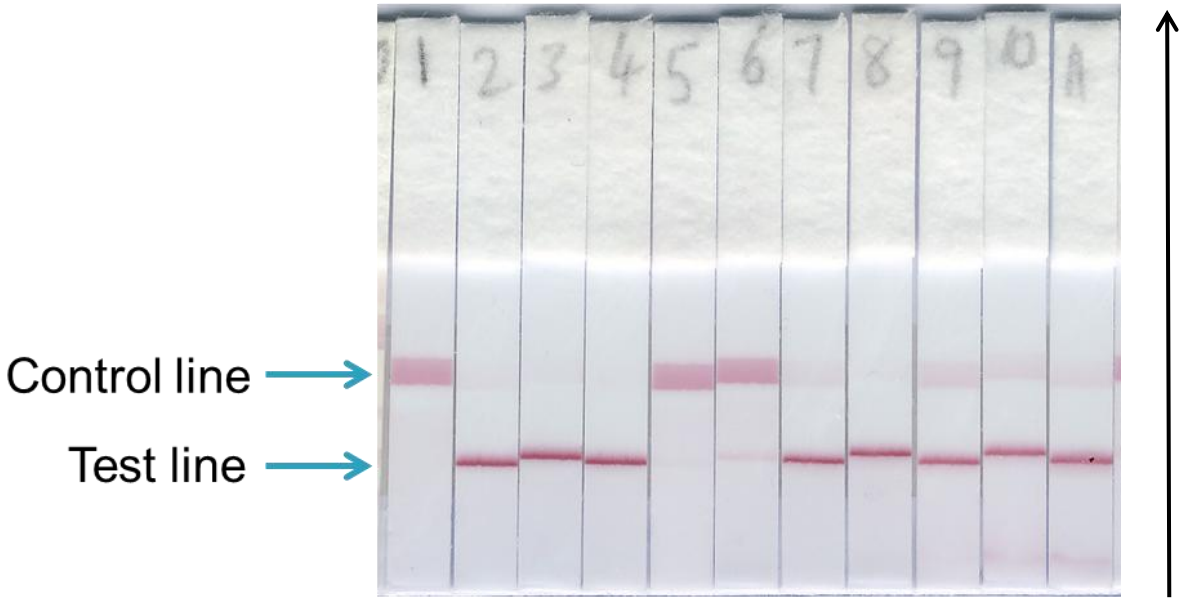


Figure 12: SDS-PAGE analysis of unbound and elution fractions of the binding experiment with crude hybridoma supernatant. 10 ul of each sample was used for SDS-PAGE analysis. Lanes were loaded as follows: 1. Broad range protein ladder, 2. Crude hybridoma supernatant (as provided by Science Haven Ltd), 3. Unbound fraction from binding with Phac-L PHA beads (L-domain), 4. Eluted fraction from binding with Phac-L PHA beads (L-domain), 5. Unbound fraction from binding with Wild-type PhaC PHA beads, 6. Eluted fraction from binding with Wild-type PhaC PHA beads. Arrows indicate heavy chains (76-92 kDa) and light chains (25-30 kDa) of IgM.

SDS-PAGE analysis of the unbound and eluted fractions from the binding experiment (Figure 12) indicated that the PhaC-L bead had successfully bound IgM as indicated by the protein bands at 76-92 kDa corresponding to the estimated size of the IgM heavy chain; and 25-30 kDa corresponding to the estimated size of the IgM light chain. These bands are not visible in the elution fraction obtained from binding with Wild-type PhaC beads.

The unbound and elution fractions obtained from the binding assay were then analysed by half strip lateral flow assay (2.1.4). Resulting immunostrips were analysed for band formation on the control and test lines. The apparent absorption was then measured and plotted against bead type.

The results obtained from the half strip lateral flow assay (Figure 13) confirmed that there was IgM present in the elution fraction obtained from the binding assay between 100 mg of PhaC-L PHA beads and 1ml crude hybridoma supernatant. This was indicated by the formation of the intense test band on the immunostrip. This is in contrast with the elution fractions obtained from binding with Wild-type PhaC beads which do not display the L-domain. The intense control band and lack of an intense test band indicate the absence of IgM in the fraction. The intensity, or apparent absorption of the bands formed on the immunostrips were subsequently measured using the Stripscan (USA) program for further analysis.



Strip	Tube
1	Negative Control (16µl PBS)
2	Positive control (Crude supernatant)
3	Positive control (Crude supernatant)
4	Positive control (Crude supernatant)
5	1ml elution fraction from Wild-type PhaC
6	1ml elution fraction from Wild-type PhaC
7	1ml unbound fraction from Wild-type PhaC
8	1ml unbound fraction from Wild-type PhaC
9	1ml elution fraction from PhaC-L
10	1ml elution fraction from PhaC-L
11	1ml elution fraction from PhaC-L

Figure 13: Immunostrips from the half strip lateral flow assay. Strip numbers correspond to a specific sample indicated in the table above. Lateral flow assay performed by Jenness Guthrie (Institute of Fundamental Sciences, Massey University, New Zealand) using the methodology explained in 2.14. Flow direction of the running buffers is indicated by the black arrow. Formation of a band at the test line indicates the presence of IgM in a given sample. Formation of a band at the control line indicates that the assay is complete.

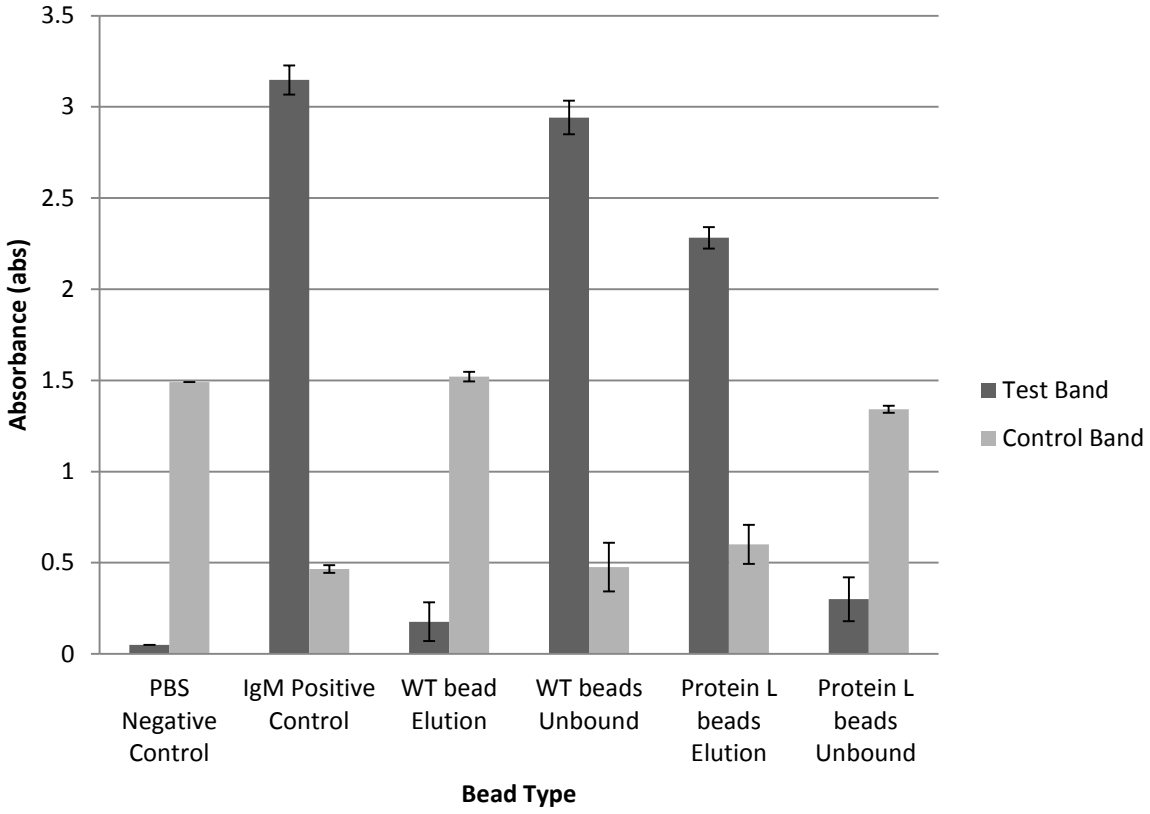


Figure 14: Apparent absorption (Aapp) or band intensity of the bands formed on immunostrips from lateral flow assay. Half strip lateral flow assay was performed on unbound and eluted fractions generated from the hybridoma binding assay with PHA beads. 1x PBS buffer was run as a negative control, and 1 ml IgM from crude hybridoma supernatant (3.2mg/ml) was run as a positive control. Intensity of bands formed on the immunostrips (Figure 13) were measured using Stripscan (USA). Increased intensity in the test band is indicative of high IgM concentrations in the sample. Intensity in the control band indicates increased binding of the detection reagent, and therefore suggests the absence of IgM in a given sample.

Comparing the sample results (Figure 14) to that of the controls, we observed low test band intensity in the elution fraction of the Wild-type PhaC beads, and a high intensity in the unbound material collected after the purification procedure. This is consistent with our

expectations since Wild-type PhaC does not have the L domain displayed on the bead surface. In contrast, there is a much greater intensity in the test band of the elution fraction from the purification experiment with PhaC-L beads as well as a low intensity test band in the unbound material. This confirmed that the functional L-domain displayed on the surface of the PhaC-L beads successfully bound IgM from the crude supernatant. Further analysis suggested that 73% of the total IgM was purified from the original hybridoma supernatant, with a loss of 18% of the IgM during the purification procedure, wash steps and subsequent low-pH elution. Using the PhaC-L beads as a purification tool had therefore also allowed for subsequent purification of IgM from crude hybridoma supernatant without adversely affecting the antibody concentrations or functional epitopes.

CHAPTER 4: DISCUSSION

4.1 ZZ-PhaC to ZZ-Linker-ZZ-PhaC

The ZZ domain of protein A is a well-established example of a successful and functional covalent attachment to the PHA synthase and thus the PHA granule surface. At the onset of this study, we proposed that the ideal target for attachment and subsequent display of bacterial immunoglobulin binding proteins such as protein G and L would be to the C-terminus of PhaC, thus creating a fusion protein displaying dual functionality on a single platform without any additional cost. A functional construct from a previous study, pET-14b-ZZ-phaC-GFP, was an ideal starting point. The GFP fragment could be excised via restriction endonuclease digest and the respective gene encoding for a desired protein designed with identical restriction endonuclease cut-sites to the excised fragment could be digested and subsequently ligated with the construct, thus replacing GFP with the gene of interest.

However, immunoglobulin binding experiments with the resulting pET-14b-ZZ-phaC-$GB1_3$ indicated poor binding efficiency. Most of the IgG was found in the unbound fraction, with less than 50% of added IgG obtained in the elution fraction, indicating poor binding both with IgG from human serum and IgG from goat serum. This changed in 2011 when a new construct was generated, linking 2 separate ZZ domains with a linker region to produce ZZ-Linker-ZZ-PhaC. The importance of this linker region was described in a previous study (Jahns & Rehm, 2009). The effect of the described linker on the AG-bead construct was then tested. A human IgG binding assay with 3mg IgG from human serum was added to 50mg of each type of bead. The protein concentration of the elution fractions obtained is indicated below.

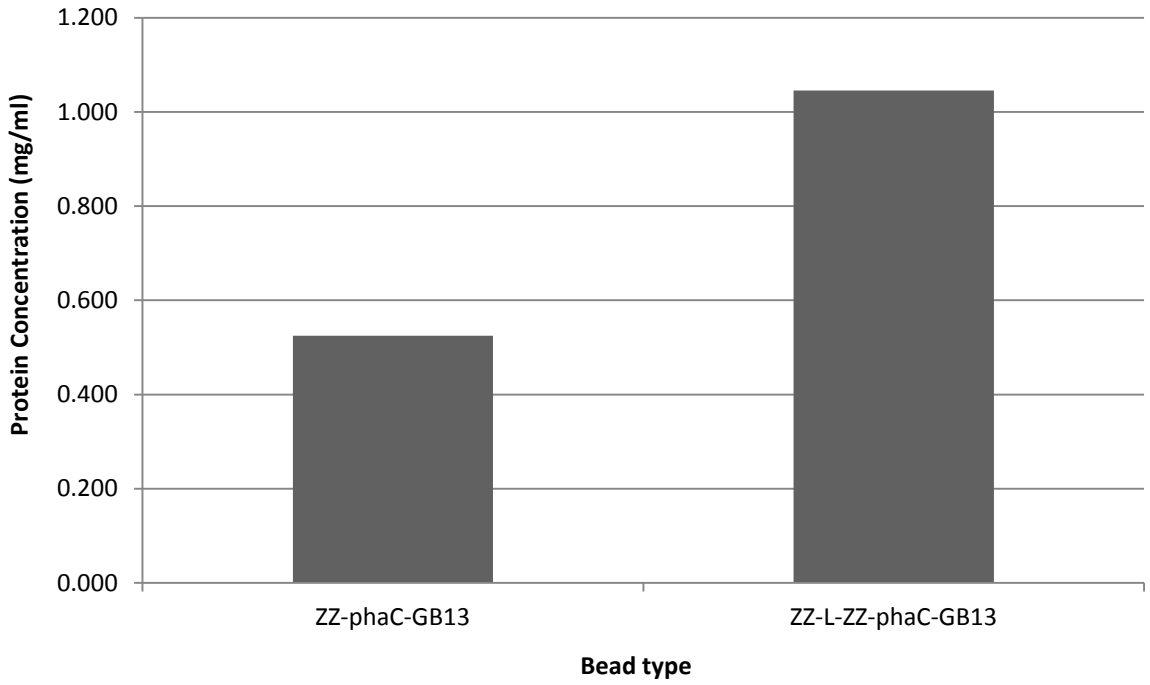


Figure 15: Protein concentration of elution fractions obtained from binding experiment of 50mg PHA beads with 3mg IgG from human serum.

The increase in binding efficiency (Figure 15) seen in the new ZZ-linker-ZZ-PhaC-$GB1_3$ construct (49.7% increase in eluted protein concentration) further confirms that the addition of the linker region facilitates the correct display of proteins on the surface of the PHA granule while maintaining the hydrophobic environment close to the synthase (Jahns &Rehm, 2009). The linker is not necessary for the C terminal fusion of $GB1_3$ to PhaC. As in the case of GFP, the length of the fusion protein and the confirmed functionality of the $GB1_3$ domain suggest that it inherently maintains the hydrophobic region required for the synthase to remain active. The ZZ-Linker-ZZ-PhaC fragment was therefore used for the generation of AG- and AL-fusion beads.

4.2 ZZ-Linker-ZZ-PhaC-L

As mentioned in Chapter 3 (3.2), the analysis of the AL-fusion bead by SDS-PAGE yielded unexpected results. The fusion protein obtained was not of the expected size.

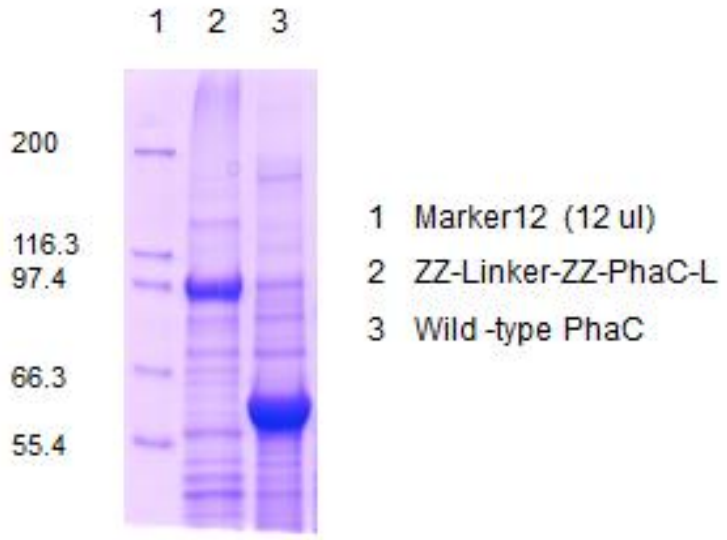


Figure 16: SDS-PAGE analysis of isolated and purified AL-beads. The expected fusion protein size of ZZ-Linker-ZZ-PhaC-L is 142kDa. Wild-type PhaC expected size is 64KdA.

As seen in the 8% SDS-PAGE above, there is no intense band signifying overexpression at 142kDa. Instead, a band suggesting overexpression is seen at 97kDa. The beads were subsequently harvested and purified once more; however, the same result was obtained.

The DNA sequence of the construct used to transform the production strain (pET14b-ZZ-linker-ZZ-phaC-L) was re-sequenced and checked for possible errors such as previously unnoticed stop codons. No such errors were found. Protease inhibitor tablets were then used during downstream processing to rule out the possibility that the fusion protein was being cleaved during the purification process.

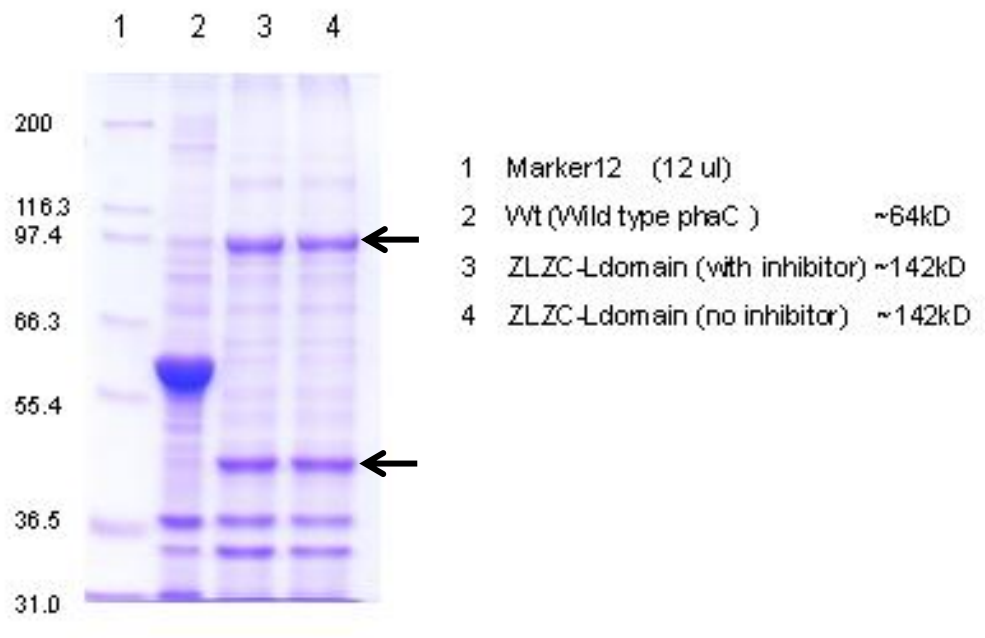


Figure 17: SDS-PAGE of isolated and purified AL-beads with and without the addition or protease inhibitor. Expected molecular weights are indicated. PHA beads run in lane 3 were processed in 50 mM potassium phosphate buffer (pH 7.5) and 1 tablet of Complete Mini EDTA-free protease inhibitor cocktail (Roche, Switzerland). PHA beads run in lane 4 were processed without the protease inhibitor being added. Arrows indicate fragments resulting from the cleavage of the fusion protein.

The addition of protease inhibitor did not alter the unexpected result, and the fusion protein of the expected size was still not expressed. The cleavage of the 142 kDA fusion protein is clearly seen in the SDS-PAGE above. We hypothesize that the 142 kDa fusion protein was cleaved into 2 separate fragments of 97 kDa and 45 kDa as indicated by the overexpressed band at those molecular weight marks. This initially seemed to correspond with 2 separate parts of the fusion protein, the 97 kDa fragment matching the estimated molecular weight of ZZ-linker-ZZ-PhaC and the 45 kDa fragment matching the L-domain protein. To assess if

either of the proteins were still functional, we performed an IgG binding assay using the purified ZZ-linker-ZZ-PhaC-L beads.

Results from immunoglobulin binding experiments with both IgG from rat serum and human serum suggested that neither the L domain nor the ZZ domains of the fusion protein were functional, suggesting that the proteins were inactive and most likely misfolded.

In order to display both domains on a single construct, we then proposed and partially executed a method involving the use of Polymerase Chain Reaction (PCR) to produce a fusion protein with the L-domain displayed on the N-terminus of PhaC instead of the C-terminus. This is achieved by introducing new restriction endonuclease cutsites (*Nde*I) flanking the L-domain synthetic gene thus allowing for subsequent ligation to the 5' end of the *phaC* gene. Similarly, protein A's ZZ domain would be displayed on the C-terminus of PhaC connected to the synthase by a linker region (Jahns & Rehm, 2009). However, due to time constraints, this experiment was not included in this particular study and will be pursued as future work on the topic.

4.3 Summary

The performance of the novel, engineered protein G, protein L and protein AG-fusion PHA bead met the expectations we set in the hypothesis of this study. Through the methodology above we have demonstrated the ability to display these proteins on the surface of a bio polyester bead produced in recombinant *E.coli* using PHA synthase immobilization technology. The ability to display 2 different immunoglobulin binding proteins derived from different bacteria on a single platform greatly increases the value of the novel PHA bead by adding functionality and binding efficiency without increasing the cost it would require to produce a single-function bead. The tests of functionality conducted on the novel protein L

PHA beads also suggests that these beads could serve as an critical tool for purification of immunoglobulin from serums and hybridoma supernatants.

In this study, we have confirmed that it is possible to express multiple binding domains on the surface of the PHA granule while maintaining functionality (and even improving on binding performance set by commercial standards). This in turn opens up many other possibilities, such as the creation of beads displaying combinations of other binding domains. The convenient, one-step cultivation and subsequent purification of PHA granules would mean that these highly-specific affinity purification tools could be produced on-demand on a large scale. The process of eluting the bound immunoglobulin from the beads does not have an adverse effect on the epitopes of the immunoglobulin molecule as demonstrated in the results of the lateral flow assay. There also exists the possibility of adding more functionality to the beads by expressing more domains via PHA synthase attachment and utilizing the linker sequence to maintain correct orientation and display of the binding domains. A "super-bead" with the binding domains of protein A, G, L and mannan-binding protein (MBP) displayed on a single platform could be produced.

APPENDIX

GC/MS results (analysis performed by Plant & Food Research, NZ)

Data File Name	PHB Std - 2mg		
Peak#	Ret.Time	Area	Name
1	6.297	721002	Cyclotrisiloxane, hexamethyl-
2	7.513	66429775	methyl 3-hydroxybutanoate
3	7.706	2699686	Sulfuric acid, dimethyl ester
4	8.728	4261062	2-Propanol, 1- 1-methyl-2-(2-propenyloxy)ethoxy -
5	31.334	1096806	
6	34.778	559909	
7	36.125	2249921	Hexadecanoic acid, methyl ester
8	38.14	826300	
9	39.957	525580	
10	40.443	962605	

Data File Name	PHB Std - 4mg		
Peak#	Ret.Time	Area	Name
1	7.513	141794326	methyl 3-hydroxybutanoate
2	7.667	2877365	Sulfuric acid, dimethyl ester
3	36.142	2391490	methyl hexadecanoate, C16:0 Me ester

Data File Name	PHB Std - 10mg		
Peak#	Ret.Time	Area	Name
1	7.623	378137860	methyl 3-hydroxybutanoate
2	36.112	7626342	Hexadecanoic acid, methyl ester
3	38.095	696828	Octadecanoic acid, methyl ester

Data File Name	PHB Std - 15mg		
Peak#	Ret.Time	Area	Name
1	7.675	504407324	methyl 3-hydroxybutanoate
2	36.103	11316068	methyl hexadecanoate, C16:0 Me ester
3	37.883	621469	
4	38.087	1073081	methyl octadecanoate, C18:0 Me ester

Data File Name	ZZ-Linker-ZZ-PhaC-GB1$_3$		
Peak#	Ret.Time	Area	Name
1	7.57	202016426	methyl 3-hydroxybutanoate
2	7.694	1750301	Sulfuric acid, dimethyl ester
3	8.688	9213489	2-Propanol, 1- 1-methyl-2-(2-propenyloxy)ethoxy -
4	32.648	4142081	Tetradecanoic acid, methyl ester
5	35.833	9968489	9-Hexadecenoic acid, methyl ester, (Z)-
6	35.958	544148	
7	36.099	32147925	methyl hexadecanoate, C16:0 Me ester
8	37.544	508030	
9	37.907	18815567	C18:1n9c FAME - oleic
10	38.079	1012360	methyl octadecanoate, C18:0 Me ester
11	39.115	1066691	

Data File Name ZZ-Linker-ZZ-PhaC-L

Peak#	Ret.Time	Area	Name
1	7.582	255823905	methyl 3-hydroxybutanoate
2	7.687	1705396	Sulfuric acid, dimethyl ester
3	32.649	3669269	C14:0 Me ester
4	35.829	10409365	9-Hexadecenoic acid, methyl ester, (Z)-
5	35.95	896471	
6	36.096	36265493	methyl hexadecanoate, C16:0 Me ester
7	37.541	597728	
8	37.906	10287996	C18:1n9t FAME - elaidic
9	38.078	1228365	Octadecanoic acid, methyl ester
10	39.119	563140	
11	39.376	559563	

Data File Name PHB Std - 2mg

Peak#	Ret.Time	Area	Name
1	7.468	47903345	methyl 3-hydroxybutanoate
2	7.673	1761061	Sulfuric acid, dimethyl ester
3	8.702	2793341	Propanoic acid, 2-hydroxy-2-methyl-, ethyl ester
4	36.159	1569662	Hexadecanoic acid, methyl ester

Data File Name PHB Std - 4mg

Peak#	Ret.Time	Area	Name
1	7.535	112084628	methyl 3-hydroxybutanoate
2	7.694	2409628	Sulfuric acid, dimethyl ester
3	8.705	6199633	2-Propanol, 1- 1-methyl-2-(2-propenyloxy)ethoxy -
4	36.16	2028951	methyl hexadecanoate, C16:0 Me ester

Data File Name PHB Std - 10mg

Peak#	Ret.Time	Area	Name
1	7.632	305942071	methyl 3-hydroxybutanoate
2	7.717	736938	Sulfuric acid, dimethyl ester
3	8.695	16234249	2-Propanol, 1- 1-methyl-2-(2-propenyloxy)ethoxy -
4	36.136	5131962	methyl hexadecanoate, C16:0 Me ester
5	38.107	525558	Octadecanoic acid, methyl ester

Data File Name PHB Std - 15mg

Peak#	Ret.Time	Area	Name
1	7.693	430232870	methyl 3-hydroxybutanoate
2	8.702	22375108	2-Propanol, 1- 1-methyl-2-(2-propenyloxy)ethoxy -
3	36.127	8077232	Hexadecanoic acid, methyl ester
4	38.108	762105	Octadecanoic acid, methyl ester

Sample	**Area**	**Weight (mg)**	**PHB**	**%PHB/mg**
ZZ-Linker-ZZ-PhaC-GB1$_3$	202016426	15	6.72	44.82
ZZ-Linker-ZZ-PhaC-L	255823905	15	8.52	56.78

MALDI-TOF (analysis performed by The Centre for Protein Research, Otago University)

ZZ-linker-ZZ-PhaC-GB1$_3$

Protein View

Match to: **Seq2_10081** Score: **2692**
(10081_Seq2) K2
Found in search of 10081_K2_combined.txt

Nominal mass (M_r): **103690**; Calculated pI value: **5.29**
NCBI BLAST search of Seq2_10081 against nr
Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Deamidation (NQ),Oxidation (M),Pyro-glu (N-term E),Pyro-glu (N-term Q)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **53%**

Matched peptides shown in **Bold Red**

```
   1 MAQHDEAVDN KFNKEQQNAF YEILHLPNLN EEQRNAFIQS LKDDPSQSAN
  51 LLAEAKKLND AQAPKVDNKF NKEQQNAFYE ILHLPNLNEE QRNAFIQSLK
 101 DDPSQSANLL AEAKKLNDAQ APKVDANSSS VPHMATGKGA AASTQEGKSQ
 151 PFKVTPGPFD PATWLEWSRQ WQGTEGNGHA AASGIPGLDA LAGVKIAPAQ
 201 LGDIQQRYMK DFSALWQAMA EGKAEATGPL HDRRFAGDAW RTNLPYRFAA
 251 AFYLLNARAL TELADAVEAD AKTRQRIRFA ISQWVDAMSP ANFLATNPEA
 301 QRLLIESGGE SLRAGVRNMM EDLTRGKISQ TDESAFEVGR NVAVTEGAVV
 351 FENEYFQLLQ YKPLTDKVHA RPLLMVPPCI NKYYILDLQP ESSLVRHVVE
 401 QGHTVFLVSW RNPDASMAGS TWDDYIEHAA IRAIEVARDI SGQDKINVLG
 451 FCVGGTIVST ALAVLAARGE HPAASVTLLT TLLDFADTGI LDVFVDEGHV
 501 QLREATLGGG AGAPCALLRG LELANTFSFL RPNDLVWNYV VDNYLKGNTP
 551 VPFDLLFWNG DATNLPGPWY CWYLRHTYLQ NELKVPGKLT VCGVPVDLAS
 601 IDVPTYIYGS REDHIVPWTA AYASTALLAN KLRFVLGASG HIAGVINPPA
 651 KNKRSHWTND ALPESPQQWL AGAIEHHGSW WPDWTAWLAG QAGAKRAAPA
 701 NYGNARYRAI EPAPGRYVKA KALEVLAVID KRGGGGGSGG GSGGGSGGGG
 751 SKTDTYKLIL NGKTLKGETT TEAVDAATAE KVFKQYANDN GVDGEWTYDD
 801 ATKTFTVTEK PESGGGSGGG SGGGGSKTDT YKLILNGKTL KGETTTEAVD
 851 AATAEKVFKQ YANDNGVDGE WTYDDATKTF TVTEKPESGG GSGGGSGGGG
 901 SKTDTYKLIL NGKTLKGETT TEAVDAATAE KVFKQYANDN GVDGEWTYDD
 951 ATKTFTVTEK PE
```

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
15 - 34	2485.2549	2484.2476	2484.2033	0.0443	0	K.EQQNAFYEILHLPNLNEEQR.N (Ions score 143)
35 - 56	2360.2366	2359.2293	2359.2019	0.0274	1	R.NAFIQSLKDDPSQSANLLAEAK.K (Ions score 84)
154 - 169	1858.9371	1857.9298	1857.9050	0.0249	0	K.VTPGPFDPATWLEWSR.Q (Ions score 116)
196 - 207	1309.7419	1308.7346	1308.7150	0.0197	0	K.IAPAQLGDIQQR.Y (Ions score 95)
211 - 223	1453.6975	1452.6902	1452.6707	0.0195	0	K.DFSALWQAMAEGK.A (Ions score 74)

211 - 223	1469.6899	1468.6826	1468.6656	0.0170	0	
K.DFSALWQAMAEGK.A	Oxidation (M)	(Ions score 24)				
224 - 233	1066.5544	1065.5471	1065.5203	0.0268	0	
K.AEATGPLHDR.R	(Ions score 50)					
248 - 258	1256.6993	1255.6920	1255.6713	0.0207	0	
R.FAAAFYLLNAR.A	(Ions score 84)					
259 - 272	1416.7524	1415.7451	1415.7143	0.0308	0	
R.ALTELADAVEADAK.T	(Ions score 60)					
279 - 302	2664.3423	2663.3350	2663.2802	0.0549	0	
R.FAISQWVDAMSPANFLATNPEAQR.L	(Ions score 168)					
279 - 302	2680.3162	2679.3089	2679.2751	0.0339	0	
R.FAISQWVDAMSPANFLATNPEAQR.L	Oxidation (M)	(Ions score 71)				
303 - 313	1173.6736	1172.6663	1172.6400	0.0263	0	
R.LLIESGGESLR.A	(Ions score 84)					
328 - 340	1438.7090	1437.7017	1437.6735	0.0282	0	
K.ISQTDESAFEVGR.N	(Ions score 107)					
341 - 367	3115.6604	3114.6531	3114.5912	0.0619	0	
R.NVAVTEGAVVFENEYFQLLQYKPLTDK.V	(Ions score 100)					
383 - 396	1695.9159	1694.9086	1694.8879	0.0207	0	
K.YYILDLQPESSLVR.H	(Ions score 102)					
397 - 411	1793.9689	1792.9616	1792.9372	0.0244	0	
R.HVVEQGHTVFLVSWR.N	(Ions score 129)					
412 - 432	2320.0588	2319.0515	2319.0226	0.0290	0	
R.NPDASMAGSTWDDYIEHAAIR.A	(Ions score 89)					
412 - 432	2336.0583	2335.0510	2335.0175	0.0336	0	
R.NPDASMAGSTWDDYIEHAAIR.A	Oxidation (M)	(Ions score 74)				
439 - 468	3045.6870	3044.6797	3044.6327	0.0470	1	
R.DISGQDKINVLGFCVGGTIVSTALAVLAAR.G	Carbamidomethyl (C)	(Ions score 112)				
469 - 503	3750.0137	3749.0064	3748.9311	0.0753	0	
R.GEHPAASVTLLTTLLDFADTGILDVFVDEGHVQLR.E	(Ions score 131)					
504 - 519	1513.8076	1512.8003	1512.7718	0.0285	0	
R.EATLGGGAGAPCALLR.G	Carbamidomethyl (C)	(Ions score 128)				
520 - 546	3200.7183	3199.7110	3199.6341	0.0769	0	
R.GLELANTFSFLRPNDLVWNYVVDNYLK.G	(Ions score 47)					
547 - 575	3469.7136	3468.7063	3468.6389	0.0674	0	
K.GNTPVPFDLLFWNGDATNLPGPWYCWYLR.H	Carbamidomethyl (C)	(Ions score 79)				
576 - 584	1145.6191	1144.6118	1144.5876	0.0242	0	
R.HTYLQNELK.V	(Ions score 52)					
576 - 584	1145.6198	1144.6125	1144.5876	0.0249	0	
R.HTYLQNELK.V	(Ions score 53)					
612 - 631	2171.1375	2170.1302	2170.1058	0.0244	0	
R.EDHIVPWTAAYASTALLANK.L	(Ions score 108)					
634 - 651	1748.0022	1746.9949	1746.9780	0.0169	0	
R.FVLGASGHIAGVINPPAK.N	(Ions score 103)					
697 - 706	1004.5153	1003.5080	1003.4835	0.0245	0	
R.AAPANYGNAR.Y	(Ions score 53)					
722 - 732	1226.7783	1225.7710	1225.7394	0.0317	1	
K.ALEVLAVIDKR.G	(Ions score 62)					
767 - 781	1493.6984	1492.6911	1492.6893	0.0019	0	
K.GETTTEAVDAATAEK.V	(Ions score 43)					
785 - 803	2144.9084	2143.9011	2143.8606	0.0405	0	
K.QYANDNGVDGEWTYDDATK.T	Pyro-glu (N-term Q)	(Ions score 58)				
785 - 803	2161.8940	2160.8867	2160.8872	-0.0005	0	
K.QYANDNGVDGEWTYDDATK.T	(Ions score 123)					
804 - 827	2097.9668	2096.9595	2096.9609	-0.0014	0	
K.TFTVTEKPESGGGSGGGSGGGGSK.T	(Ions score 146)					
954 - 962	1051.5243	1050.5170	1050.5233	-0.0063	0	
K.TFTVTEKPE.-	(Ions score 35)					

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