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Biotechnological production of a stereoselective scFv antibody fragment

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Biotechnological Production of a Stereoselective scFv Antibody Fragment

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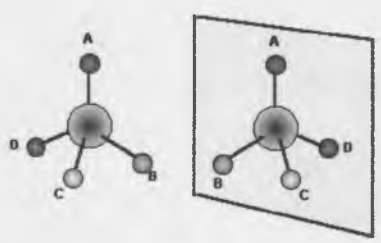
ABSTRACT

Antibodies are proteins of the immune system that exhibit exquisite specificity to their binding partners, called antigens. Binding occurs at the hypervariable region (Fv), which is capable of discriminating even enantiomeric molecules. By targeting on the DNA sequences that code for Fv and expressing it as a protein, the binding properties of an antibody can be assessed. In this project, the heavy and light chains of the Fv region (V_H and V_L) of an anti-amino acid antibody (anti-phenylalanine 67.36) were linked using a short nucleotide linker and four primers designed from previously determined sequences of V_H and V_L. The resulting V_H-linker-V_L(HIL) construct - a single chain variable fragment (67.36 HIL scFv) - was inserted into a TOPO cloning vector and transformed into *E. coli* BL 21 expression cells. 1 L cultures were grown at 37°, 30° and 27 °C. Cells were collected, resuspended and lysed to release the protein of interest. Isolation and purification was performed using a metal (cobalt) affinity chromatography resin. Characterization by SDS-PAGE indicated a correlation between incubation temperature and extent of expression. Noncompetitive ELISA tests revealed a functional 67.36 HIL scFv. However, further tests e.g., competitive ELISA, must be carried out to confirm an enantiomeric preference.

INTRODUCTION

I. Chirality



Chirality is the structural characteristic that makes it impossible to superimpose a molecule with its mirror image. The most common form of chirality occurs in molecules that possess an asymmetric center. The two mirror images, called *enantiomers*, are identical in atomic constitution and bonding, but differ in the three-dimensional arrangement of its substituents. Such molecules have essentially the same physical properties, but may show different behavior in a chiral environment (e.g., the human body). Most biological molecules are chiral and all organisms on Earth and their numerous physiological processes are *stereoselective*, utilizing, e.g., amino acids and sugars in only one of the two possible mirror-image forms. Important biological macromolecules that are stereoselective are enzymes, receptors, and antibodies.

II. The Antibody

Antibodies are proteins produced by B-cells to combat the numerous foreign invaders our immune system encounters on a daily basis. The basic four-chain structure of antibodies is shown in figure 1. Generally Y-shaped, it is comprised of two identical light and heavy chains. Antigen recognition and interaction occurs in the antigen-binding site located at the ends of the "arms" of the molecule.

There are generally five different classes of antibodies in existence: IgA, IgD, IgE, IgG, and IgM. The general four-chain structure is common for all monomeric immunoglobulin molecules. Polymeric immunoglobulins of higher molecular weights are formed by 2-6 four-chain subunits. Therefore they differ not only in their physiological roles but also in their overall structures. For example, IgA is a dimer comprised of two Y-shaped antibody subunits. In this research project, 67.36, a type of IgG antibody, was studied and observed.

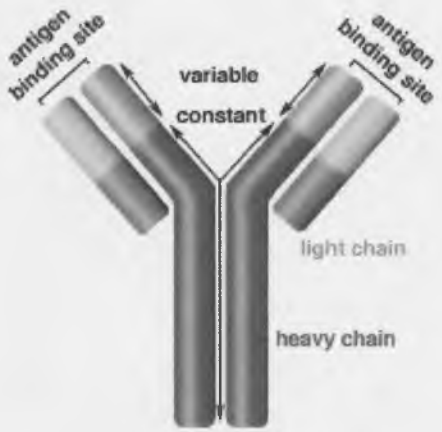


Figure 1. General Structure of Antibodies

II. A Closer Look – Structural Basis for Specificity

Form a structural point of view (figure 2), IgG antibodies are a particular class of immunoglobulins that have been extensively studied because of the dominant role they play in a mature immune response.

The IgG molecule can be broken down into two regions: Fc and Fab. Each immunoglobulin light (L) or heavy (H) chain is structurally segmented into domains that are either of the variable type (V_L and V_H) or the constant type (CH_1, CH_2, or CH_3). The Fab region contains the antigen-binding site, which is comprised of the hypervariable Fv region (V_L + V_H). It is important to note that this area of highly variable amino acids are confined to 6 protein loops

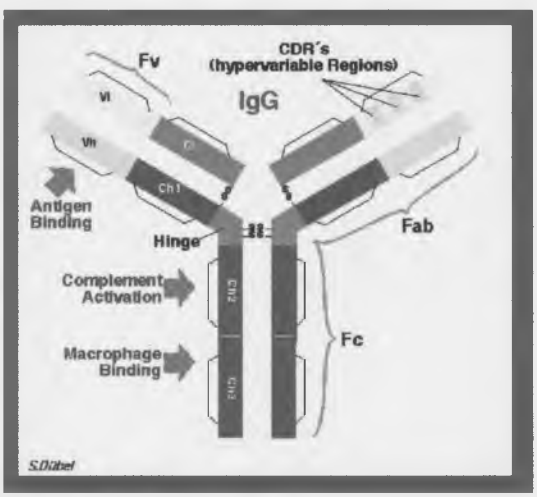


Figure 2. IgG Structure

that cluster together at the end of the Fab fragments to form a continuous hypervariable surface. It is this region that is responsible for antibody specificity and is the area of focus for this research project.

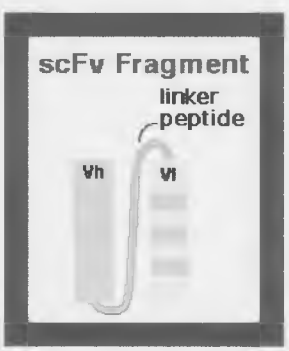
The specificity of antibodies is incredible, able to differentiate between enantiomers. As mentioned earlier, enantiomers are molecules that are optical isomers, or mirror images, of one another. These non-superimposable molecules can be distinguished by the direction in which they rotate the plane of polarization of polarized light and are referred to, therefore, as being dextrorotatory (D-type or right handed) or laevorotatory (L-type or left handed).

Since antibodies can be raised against virtually any compound, practical applications of antibody stereoselectivity is currently being researched; this can range from the detection of enantiomeric impurity in drugs to the chromatographic separation of enantiomeric mixtures.

III. Importance and Goals of this Project

All organisms on Earth use amino acids and sugars in only one of the two possible mirror-image forms. Since enantiomers are essentially the same molecule, possessing the same molecular weight and physical properties, separating the two forms is not only difficult but also time-consuming. This makes it challenging to separate racemic samples, which contain both forms. This is especially relevant in drugs manufactured for human consumption because minor enantiomeric impurities may cause sever pharmacologic and toxicologic side effects. Thus antibody practical applications for antibody stereoselectivity can range from the detection of enantiomeric impurity in drugs to the chromatographic separation of enantiomeric mixtures

Because of the effectiveness of antibody stereoselectivity, applications are being developed to attain analytical techniques for the precise determination of biorelevant chrial molecules. Currently there are several analytical techniques used for separating



My research project focuses on the production of a scFv fragment from the antigen-binding site (Fab) of anti-AA 67.36, an antibody to phenylalanine, and the investigation of its binding properties

BACKGROUND INFORMATION

I. Monoclonal Antibodies

In order to perform the experiments and tests necessary to investigate antibody specificity, one needs a constant source of a single type of antibody that are all built alike and manufactured by a single clone of plasma cells, or *monoclonal antibodies*. Possible sources for monoclonal antibodies are spleen cells. Since they are responsible for B-cell production, spleen cells contain the genetic information that give rise to antibody production. B-cells are significant because each carry a different membrane-bound antibody molecule on its surface. Therefore if an antigen is introduced and binds to a particular B-cell, it stimulates this cell to divide. The result is the secretion of large amounts of the same antibody in soluble form.

However, there are drawbacks if one intends to harvest monoclonal antibodies through this procedure. First of all, the response of the immune system to any antigen, even the simplest, is polyclonal. That is, the system manufactures antibodies of a great range of structures both in their binding regions as well as in their effector regions. In addition, even if the isolation of a single antibody-secreting cell were successful and the cell is placed in culture, it would die out after a few generations because of the limited growth potential of all normal somatic cells.

The solution to this problem is Hybridoma Technology where single antibody forming cells are fused to tumor cells grown in culture, producing hybridoma cells. In this process, antibodies are "raised" by injecting an animal (usually mice or rabbits) with an antigen, X for example. Repeated injections of the same antigen at intervals of several weeks stimulates specific B-cells to secrete large amounts of anti-X antibodies in the bloodstream. The cells that produce these antibodies are fused with tumor cells and grown in a selective medium, HAT (hypoxanthine/aminoptherin/ thymine), which only allows hybridoma cells to grow. The supernatant was extracted from microcultures and screened for secretion of the desired antibody. Lastly, the cells with positive results were subculture by limiting dilution such that each culture contains hybridomas with single antibody specificity. Since there is an excess of these anti-X antibody-producing cells, the likelihood of attaining the antibodies of interest is much greater. The result are cells monoclonal antibodies can be raised by fusing single antibody forming cells to tumor cells grown in culture. The resulting cell is called hybridoma.

II. Degenerate Primers

Once the specific hybridoma cell has been attained, it is now possible to gain access to the genes of the cell that code for the antibody molecule. Within the cell is the mRNA for both light chain and heavy chain production. The next step is now to purify the mRNA using QuickPrep mRNA purification kit. Once the mRNA has been isolated, we can have the cDNA for the chains since mRNA is not very stable and cDNA is. To focus on these hypervariable regions, degenerate primers were used. Degenerate primers are a library of primers that allow wobbles at specific locations.

III. Sequences for Variable Fragments V_H and V_L

Chain	Sequence
Heavy Chain	GTT CCG GAATTC GAG GTG CAG CTG GAG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGC GCA GCC TCT GGA TTC ACT TTC AGT AAC TAT GAC ATG TCT TGG GTT CGC CAG ACT CCA GAG AAG AGA CTG GAG TGG GTC ACA TCT ATT AGT AGT GGT GGT TAC ACC TAC TAT CCA GAC AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAT AAT GTC AGG AAC ATC CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAA GAC ACG GCC ATG TAT TAC TGT GCA AGA GCC CTT TAT GAC TAC ACT ACT AGC CCC TGG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GCC AAA ACG ACA CCC CCA TCT GTC TAT AGATCT TCC
Light Chain	TGG GAGCTC GAT ATT GTG CTG ACC CAA ACT ACA GCA ATC ATG TCT GCA TCT CCA TGG GAA AAG GTC ACC ATG ACC TGC AGG GCC AGC TCA AGT GTA AGT TCC AGT TAC TTG CAC TGG TAC CAG CAG AAG TCA GGT GCC TCC CCC AAA CTC TGG ATT TAT AGC ACA TCC AAC TTG GCC TCT GGA GTC CCT GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC AGT GTG GAG GCT GAA GAT GCC GCC ACT TAT TAC TGC CAG CAG TAC AGT GGT ACC CCG CTC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAG CGG GCT GAT GCT GCA CCA ACT GTA TCA GTTAAC GGC

Methods

I. Amplifying V_H and V_L via PCR

Polymerase chain reaction (PCR) is a common method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many billions of molecules. It creates copies by mimicking how DNA is replicated in a cell, however using temperature as a means of manipulating the structure of the double stranded DNA (dsDNA):

TABLE 1. Steps in PCR

Step	Temp (°C)	Time (min)	# of Cycles	Purpose
Initial denaturation	94	1	1	Separates dsDNA into two template strands
Denaturation	94	1	35	Same as above
Annealing	55	1		Anneals primers to template
Elongation	72	2		Polymerase elongates primers to create new strands from template
Final Elongation	72	10	1	Finish elongation Reform dsDNA

To amplify V_H and V_L ,the following solutions were added into a small Eppendorf PCR tube:

		V_H		V_L
Buffer		2 uL		2 uL
MgCl2		2		2
*Primers	*67.36 HIL HR	0.4	*67.36 HIL LR	0.4
	*67.36 HIL HF	0.4	*67.36 HIL LF	0.4
dNTP		0.4		0.4
Sterile H2O		13.9		13.9
*Template	*1' stock	0.4	*4' stock	0.4
Taq polymerase		0.2		0.2
TOTAL VOLUME		20 ul		20 ul

After mixing well, a drop of mineral oil was added to the top of the solution to prevent evaporation during denaturation. The samples were placed in the PCR machine and allowed to run for 35 cycles according to the specifications on temperature and time as shown in table 1 (program #9 in PCR). At the end of the program, the tubes were placed in –70° C freezer and the mineral oil drop was removed from the surface; since mineral oil does not solidify, freezing is an efficient way to remove the mineral oil from the surface without disturbing or removing any of the precious DNA sample that remains in solid-form. Then, agarose-gel electrophoresis was utilized to assure that amplfication was carried out and to check final molecular weight of fragments.

Electrophoresis is a way of separating out charged particles. When a sample is placed in a semi-solid matrix or gel and exposed to an electric field, the ions or molecules migrate through the

gel in response to the field. Smaller particles travel faster and move further than larger ones. Highly charged particles are also more responsive to the electric field.

The sugar phosphate backbone of DNA has a negative charge in neutral to basic solution. Therefore when an electric charge is placed through the sample, it will move from the negative (cathode) pole to the positive (anode) pole. A buffer solution in the unit stops the solution from becoming acidic. The DNA sample was placed in a gelatinous matrix, agarose (1.2% agarose: 1.2 g/60 mL TAE buffer) and exposed to an electric field. Along with the sample, loading dye was added to increase the density of the solution causing it to sink to the bottom of the agarose gel. In addition, the dyes contain bromophenol blue and xylene cyanol, both negatively charged dyes that will migrate to the positive pole as well.

Since DNA is not visible in the gel, the dyes give an indication of how fart he nucleic acids have migrated. Once the samples have migrated to the bottom of the gel, the electric current was stopped. The separated bands were visualized by staining the gels with ethidium bromide, which become fluorescent under ultraviolet light. A photograph of the gel was taken as shown below displays two distinct bands. These bands correspond with the known molecular weights of V_H and V_L :

- Heavy Chain - 408 bp
- Light chain - 370 bp

II. Designing the Linker

The linker sequence is a short nucleotide chain that must connect the V_H and V_L domains of a given Fv without perturbing contacts or interfering with domain folding. Various factors are taken into consideration to optimize the chances of obtaining a functional fragment. The overall length and %GC holds great significance since both directly affects the melting temperature (Tm). The melting temperature is the temperature in which double stranded DNA can be separated into single strands. Since the linker will be directly attached to the heavy and light chains, its melting temperature must be relatively close to the Tm of the scFV.

The longer the length of the sequence, the more energy required to separate the strands, thus an increased Tm. Increased %GC or percent guanine-cytosine has a similar effect. Base-pairing nucleotides make up the ladder of double helix DNA. Guanine-cytosine base pairs are stabilized by 3 hydrogen bonds, compared to 2 hydrogen bonds between adenine-thymine. The more stabilized G-C pairing requires more energy to separate thus increasing Tm as more GC are placed.

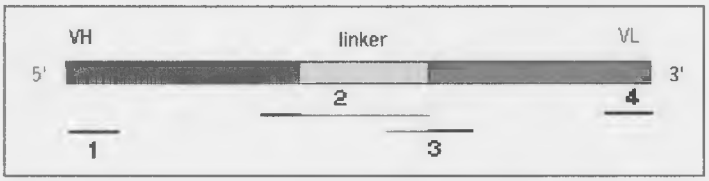
For the linker in creating 67.36 scFV, the linker must remain flexible, thus Gly and Ser residues were introduced. Solubility is also important since the resulting protein samples must be kept in solution during use and long-term storage. To enhance solubility, Lys and Glu were incorporated. Degradation from proteases is imminent for proteins that are not in its native environment. To prevent protease susceptibility, Pro residues were included. Lastly, the linker sequence must be in an optimal length to reduce multi-dimer formation.

	Sequence	Length
Linker	**5'** AAC TTT AGT GCT ADD TTC ACC ACT CCC GGG TTT GCC GCT ACC GGA AGT AGA GCC **3'**	54 bp

III. Designing the Primers

The steps in creating primers is similar to creating the linker, considering the length and %GC to remain in a safe range. However with the primers, previously determined sequences of the heavy and light chain were used to determine the exact amino acid composition. In addition:

- Primers were created to incorporate the linker sequence
- Proper length: approx 21-24 bases
- Proper alignment with sequences; must occur in 5' → 3' direction.
- Proper melting temp Tm = 60-65 C (annealing temp = 55 C)
  % GC = below 50% ideal
  acceptable if 50-55%

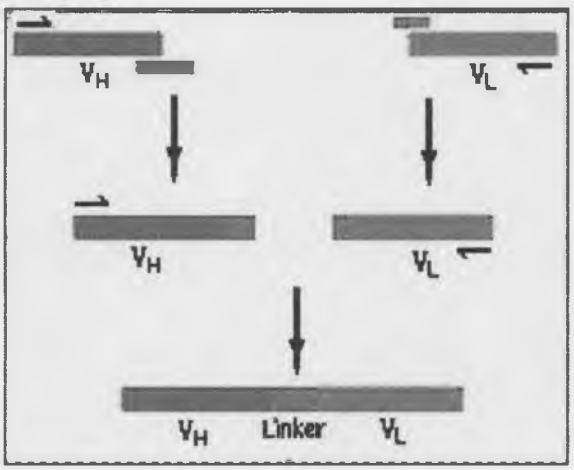


Primer	Sequence	Length	Tm (°C)	%GC
1. Heavy Forward	**5'** GTT CCG GAA TTC GAG GTG CAG **3'**	21	59.4	57.1%
2. Heavy Reverse	**5'** GGA AGA TCT ATA GAC AGA TGG GGG TGT **3'**	27	60.8	48.1%
3. Light Forward	**5'** TGG GAG CTC GAT ATT GTG CTG ACC **3'**	24	62.3	54.2%
4. Light Reverse	**5'** GCC CTT AAG TGA TAC AGT TGG TGC **3'**	24	59.5	45.8%

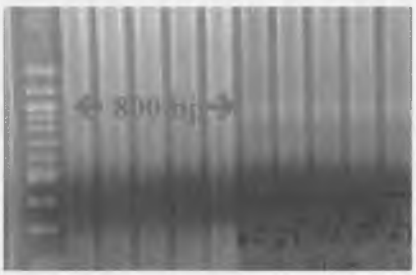
IV. Creating scFV

Using polymerase chain reaction (PCR), the primers were elongated to form scFv HIL. Since the length of the linker, V_H and V_L were known (54, 370 and 408 bp, respectively), the molecular weight of 67.36 scFv HIL could be calculated. The primers are strategically placed, accompanied by portions of the linkers sequence in order for elongation to occur in the 5' → 3' direction (see figure 5). This resulted in a single chain fragment.

To begin ligation, the following solutions were added to an eppendorf tube:



ScFV resolved by agarose gel



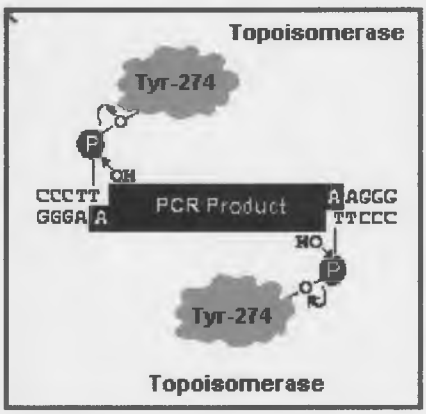
Electrophoretic separation in an agarose gel revealed a band at about 800 bp, which corresponds to 67.36 scFv HIL. The band was then isolated from the gel via Qiagen® Gel Extraction Kit. This kit is designed to extract and purify DNA fragments from standard or low-melt agarose gels in TAE or TBE buffer.

- The DNA fragment was excised from the agarose gel with a clean, sharp scalpel.
- The gel slice was weighed in a colorless tube. 3 volumes of Buffer QG was added to 1 volume of gel.
- To dissolve the gel, it was incubated at 50 C for 10 min. After the gel slice has been dissolved completely, the color of the mixture was checked to assure it remained yellow. This indicates it is in the optimal pH.
- 1 gel volume of isopropanol was added to the sample and mixed thoroughly
- A QIAquick spin column is placed into a 2 mL collection tube.
- The sample was applied onto the QIAquick column to bind the DNA. The tube was centrifuged for 1 min
- The flow-through was discarded and 0.5 mL of Buffer QG was added to the column and centrifuged for 1 min. This step removed all traced of agarose
- The column was then washed by adding 0.75 mL of Buffer PE and centrifuged for 1 min. The flow through was discarded and the tube was centrifuged for an additional 1 min
- The QIAquick column was placed into a clean, 1.5 mL Eppendorf tube.
- The DNA was eluted by adding 50 uL of H2O at the center of the column. The tube was centrifuged at high speed for 1 min.

IV. Inserting scFV into TOPO Cloning Vector

At this point in the experiment, 67.36 is nothing more than a short fragment of DNA. In order for 67.36 HIL to be expressed as a protein, it must first be inserted into a cloning vector. A cloning vector is a plasmid DNA that allows the introduction of foreign DNA fragments. In this experiment, we used a TOPO cloning vector.

TOPO TA Cloning provides a highly efficient, 5-minute, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector has a single, overhanging 3' deoxythymidine (T) residues. This allows PCR insert to ligate efficiently with the vector.



67.36 scFV HIL was directly inserted into a TOPO cloning vector and transformed into TOP 10 cloning cells. First, 400 ng of the DNA fragment was added with 1 uL Dilute Salt Solution and 1 uL TOPO vector. Water was added to bring up the total volume to 6 uL. The solution was incubated for 5 minutes at room temperature. Then it was transformed into TOP 10 cells using electroporation. 250 uL of SOC medium was added and the solution was allowed to incubate for 1 hr at 37 °C in order for cells to recover. 250 uL of the solution was plated on agar medium and incubated overnight at 37 °C. Each resulting colony was analyzed by PCR in order to determine if the insertion was successful. The agarose gel photograph displays colonies that contained the insert. Only plasmids of positive colonies were isolated and transformed into *E. coli* BL 21 expression cells.

Buffer		2 uL
MgCl2		2
*Primers	*67.36 HIL LR	0.4
	T7	0.4
dNTP		0.4
Sterile H2O		14.6
*Template	*Cells	Varies
Taq polymerase		0.2
TOTAL VOLUME		20 uL per colony

VI. Expression of Protein

Once positive colonies were identified, a DNA purification kit (Promega Wizard® *Plus* Midipreps) was used to remove and isolate plasmids with the 67.36 HIL scFv insertions from cells. Plasmids were then transformed into ampicillin resistant *E. coli* BL 21 expression cells by electroporation. Transformed expression cells were placed in TB broth containing 1mL/mL

ampicillin, 0.17 M KH_2PO_4, and 0.72 M K_2HPO_4. 1L cultures were incubated at 37°, 30° and 27° C. After a 24-hour incubation period, cells were induced with IPTG. 1.5 hours after induction, cells were collected by centrifugation at 5,000 x g. The supernatant was discarded and the cell pellet was frozen overnight at –20° C.

VII. Isolation and Purification of protein

Prior to cell lysis, cells were thawed on ice. 3 mL of extraction/wash buffer (50 mM NaH_2PO_4, 300 mM NaCl) per 1 g of cells were added to resuspend the pellet. DNAse was then added (100 – 300 mL) to decrease the viscosity of the solution and allow more efficient cell lysis. Cells were lysed in a French Press at 2000 psi. The resulting solution was collected and centrifuged at 18,000 x g. The supernatant, containing the protein of interest, was saved while the cell pellet was discarded. The protein of interest contained a histidine(His)-tag, which was captured on a metal affinity chromatography resin. The TALON® cobalt resin contains sites with a high affinity for His-tags and, therefore, allows purification of the protein from impurities. After consecutive washings, the protein was eluted with elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 150 mM Imidazole). 1 mL fractions were collected.

SDS-polyacrylamide gel electrophoresis was used to characterize the first five elution fractions as well as the supernatant, pellet, and flow-throughs obtained in the washing steps. Results demonstrate that much of the protein was retained on the cobalt column even after repeated washings. The protein was not found in the supernatant, pellet, or flow-throughs. At lower incubation temperatures, the extent of expression was more pronounced.

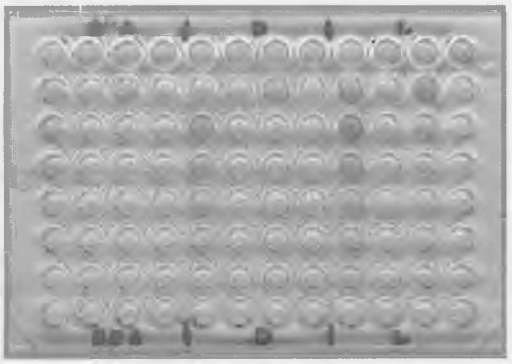
Elution Fractions 37 °C

Elution Fractions 30 °C

Elution Fractions 27 °C

VIII. Determining Functionality

Antibody binding was monitored in a noncompetitive enzyme-linked immunosorbent assay (ELISA) using bovine serum albumin (BSA), *p*-azo-D-Phe-BSA, and *p*-azo-L-Phe-BSA as solid phase coatings (100 mL/well; 1 mg/mL in 50 mM carbonate buffer, pH 9.6; 14 h at 4 °C). Unoccupied adsorption sites were blocked with 1% gelatin in PBS/Tween (250 mL/well; 2 h at 37 °C). Fraction #3 (of 27 and 30 °C incubations) was diluted as follows: 1:1, 1:2, 1:5, 1:10, 1:20, 1:40. 50 mL- samples were added to each well and incubated for 2 h at 37 °C. Bound antibody was detected with anti-His- or anti-mouse-horseradish peroxidase-conjugated antibodies. (1:5,000 in PBS; 100 mL/well; 2 h at 37 °C).



Antibody binding to the immobilized antigen was determined through a visible color change resulting from the enzymatic conversion of o-phenylendiamine (100 mL/well).

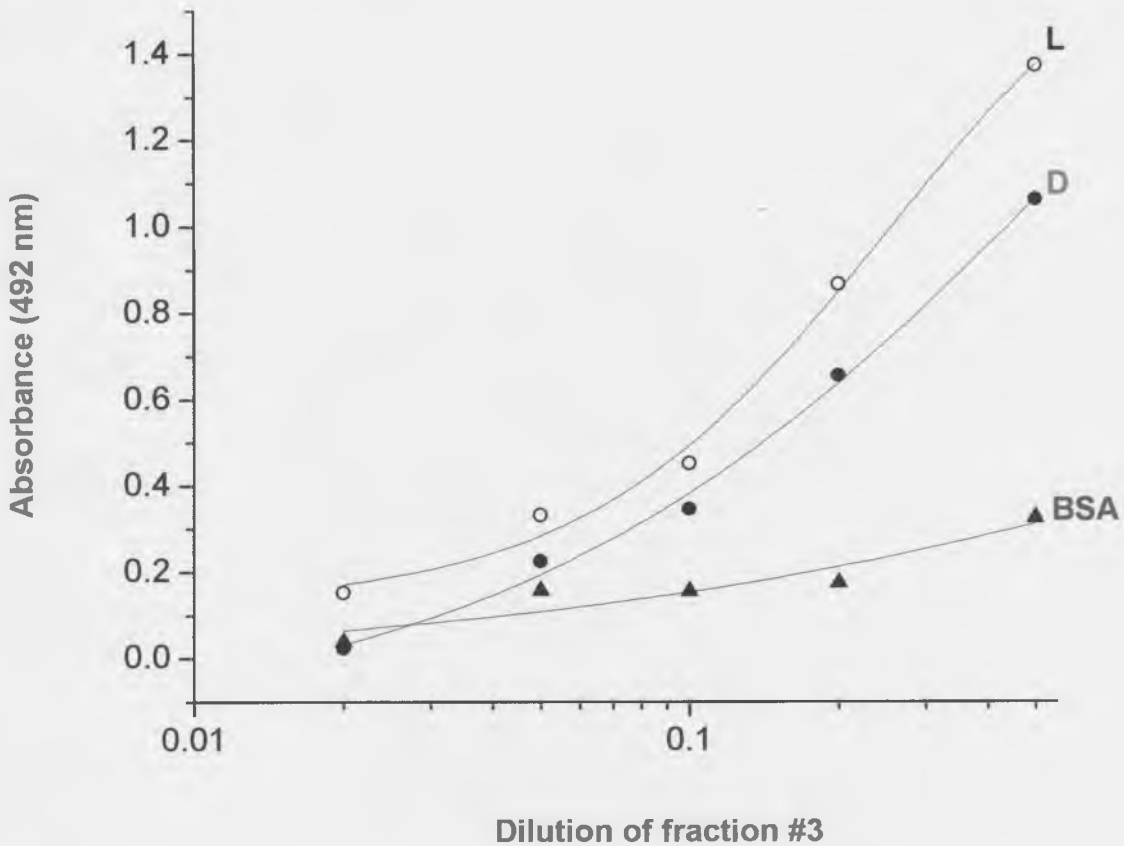
Using a spectrophotometer, the absorbance of each sample was taken. Then the absorbance was plotted against the concentration (see graphs in end pages).

ELISA RESULTS & DISCUSSION

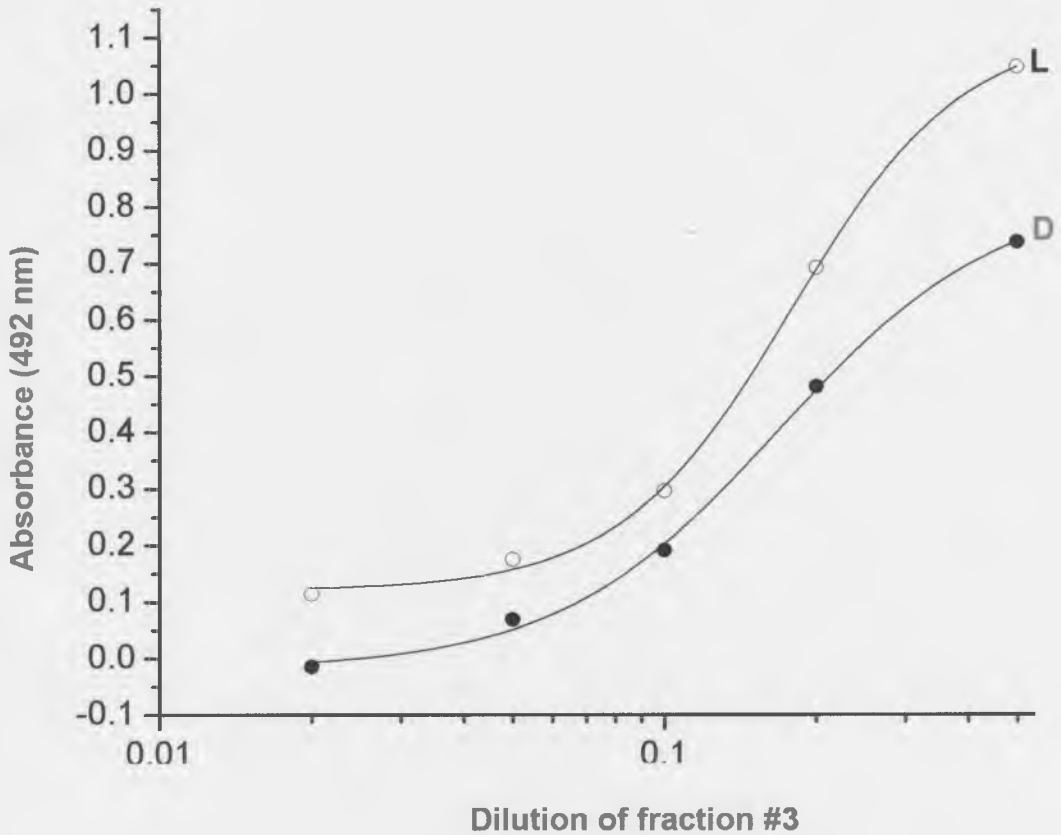
Result noncompetitive ELISA results show that cells grown at 27 °C and 30 °C expressed a functional 67.36 HIL scFv. At 27 °C, the protein binds to both *p*-azo-D-Phe-BSA and *p*-azo-L-Phe-BSA, with a slight preference to the latter. At 30 °C a more pronounced preference is observed. While these results suggest that the antibody 67.36 HIL scFv is stereoselective, it is imperative that further tests, e.g., competitive ELISAs, are carried out to verify its enantiomeric preference. A competitive ELISA, in which antibodies are allowed to bind to free antigen in solution, is much more suited to unambiguously determine antibody specificity and stereoselectivity

In conclusion, we successfully ligated the light and heavy variable regions to create 67.36 HIL scFv. We were able to express 67.36 HIL scFV as a protein and found a temperature dependence on the extent of expression. We determined that the expressed protein was functional. Noncompetitive ELISA tests revealed that cells grown at 30 °C expressed protein with a preference towards *p*-azo-L-Phe-BSA. In the future, competitive ELISA tests will be performed to confirm the observed enantiomeric preference

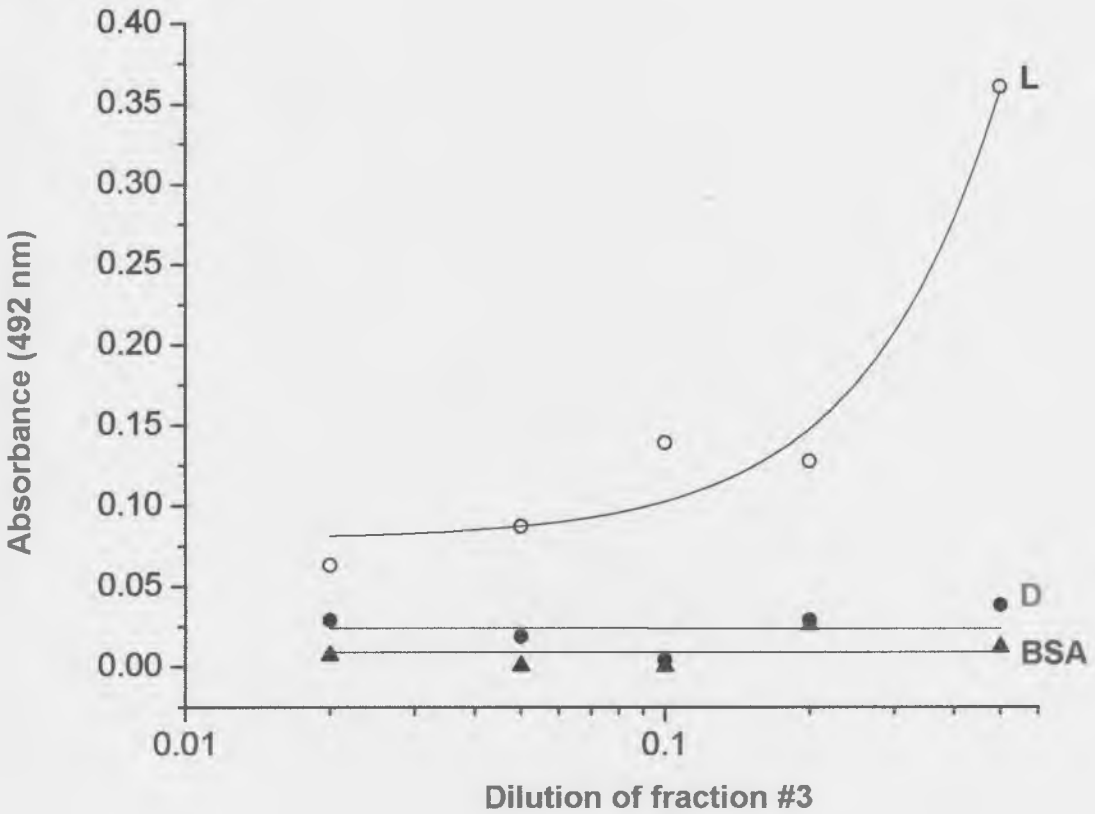
GRAPH 1
67.36 HIL Binding Curve 27 °C



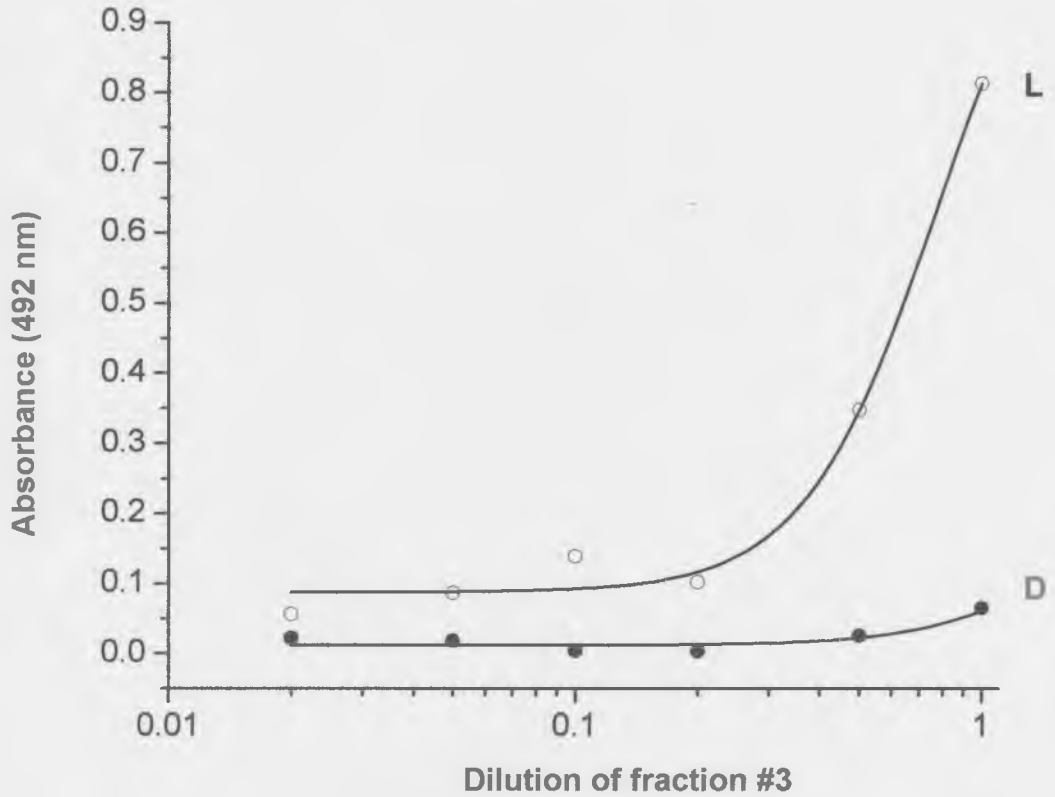
GRAPH 2
67.36 HIL Binding Curve 27 °C
BSA values subtracted



GRAPH 3
67.36 HIL Binding Curve 30 °C



GRAPH 4
67.36 HIL Binding Curve 30 °C
BSA values subtracted



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