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In Vitro Expression and Purification of Class I MHC Molecules

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I. Abstract

The major histocompatibility complex (MHC) is a gene family responsible for many critical functions of the immune system in most vertebrates. The MHC consists of three classes differentiated by their structure and function, and MHC class I encodes antigen binding proteins as well as chaperone and accessory proteins such as tapasin. The purpose of this project is to reconstitute several human MHC class I molecules in their peptide-filled and peptide-deficient forms, and to purify these proteins for biochemical study. The expressed proteins include wild type and mutant variants of the fusion protein human leukocyte antigen HLA-B*0801-fos, and human beta-2-microglobulin (β2m). The major experiments performed for this project include: 1) transformation of engineered plasmids into competent cells; 2) large scale protein production; 3) extraction and purification of proteins as inclusion bodies; 4) reconstitution of proteins into their native structure; 5) purification of refolded proteins. The purified MHC class I molecules can be used to study their interactions with transmembrane chaperone and accessory proteins that reside in the endoplasmic reticulum for the purpose of better understanding the class I antigen presentation pathway.

II. Table of Contents

III. Introduction

The major histocompatibility complex (MHC) is a gene family responsible for many critical functions of the immune system in most vertebrates. The MHC consists of three classes differentiated by their structure and function, and MHC class I encodes antigen binding proteins as well as chaperone and accessory proteins such as tapasin. The purpose of this project is to reconstitute several human MHC class I molecules in their peptide-filled and peptide-deficient forms, and to purify these proteins for biochemical study. Purified MHC class I molecules can be used to study their interactions with transmembrane chaperone and accessory proteins that reside in the endoplasmic reticulum for the purpose of better understanding the class I antigen presentation pathway.

The expressed proteins include wild type and mutant variants of the fusion protein human leukocyte antigen HLA-B*0801-fos, and human beta-2-microglobulin (β2m). The protein and nucleotide sequences of these genes are listed in the appendix, section IX.1. HLA-B*0801-fos was specifically designed to enhance in vitro interactions with tapasin-jun. Fos and jun interactions between these proteins allow the B*0801 and tapasin portions of the proteins to be within vicinity of one another, mimicking the environment in the Endoplasmic reticulum. β2m is the small subunit of MHC class I molecule, including HLA-B*0801. Association with β2m is generally required for the transport of class I heavy chains from the endoplasmic reticulum to the cell surface.

Essential interaction regions between HLA-B*0801-fos and tapasin-jun can be studied through experiments involving mutant variants of these proteins. The location of these regions can be pinpointed by studying changes in interactions between HLA-B*0801-fos and tapasin-jun mutated at specific residues. Residues 222, 227 and 229 of the HLA-B*0801 protein are hypothesized to be key regions of interaction with tapasin-jun. The series of experiments performed in this project attempted to reconstitute wild type HLA-B*0801fos proteins and HLA-B*0801fos proteins mutated at the specific residues 222, 227 and 229. In addition, the experiments attempted to reconstitute β2m proteins of both wild type and mutant variety.

The major experiments performed for this project include: 1) transformation of engineered plasmids into competent cells; 2) large scale protein production; 3) extraction and purification of proteins as inclusion bodies; 4) reconstitution of proteins into their native structure; 5) purification of refolded proteins.

IV. Experiment

IV.1 Plasmid preparation of PGMT7 with HLA-B*0801fos gene and PLM1 with β2m gene

Pure plasmid samples of PGMT7 containing the HLA-B*0801fos and PLM1 containing the β2m gene were extracted from a stock culture of previously transformed DH5α cells. First, the cells were streaked on Luria-Bertani (LB) agar plate with ampicillin and incubated overnight at 37℃. After incubation, cells that contained the mutant plasmid formed colonies on the plate. An isolated colony was selected and inoculated into 25ml of 2% LB nutrient broth and grown overnight at 30℃.

Pelleted cells were collected from the 25ml culture by centrifugation. The pellets were distributed into five 1.5ml microcentrifuge tubes. Plasmids were extracted from these pelleted cells using the QIAspin miniprep kit from Qiagen. This process yielded about a 500µl solution of pure plasmid. The plasmid solution was concentrated to a final volume of 100µl through vacuum evaporation. The plasmid was stored in a 1.5ml microcentrifuge tube at -20℃.

The final concentration of the plasmid solution was determined through mass spectroscopy. 5µl of the solution was mixed with 495µl of sterilized water and the optical density at 260nm wavelength (OD_{260}) of the mixture was measured. The concentration of the plasmid solution was determined by using the following formulas IV.1.1 and IV.2.2.

$$C\ (\mu g/ml) = 50*A*D \qquad (IV.1.1)$$

$$C\ (mM) = 0.15*A*D \qquad (IV.2.2)$$

C = Concentration
A = Absorbance = OD_{260} of the mixture
D = Dilution factor = 100

A total of six plasmid preparations were made: two for wild type HLA-B*0801fos and β2m genes, one for a mutant β2m gene, and three for each of the mutant HLA-B*0801fos genes “222”, “227”, “229”.

IV.2 Induced substitution mutation of PGMT7 plasmid containing HLA-B*0801fos and PLM1 plasmid containing β2m gene

Primers were designed to induce mutations in PGMT7 plasmids containing the HLA-B*0801fos gene and PLM1 plasmids containing the β2m gene through a polymerase chain reaction (PCR). A total of 4 sets of primers were designed according to the *QuikChange™ XL Site Directed Mutagenesis Kit*, as shown in table IV.2.1. Three of these sets were used to induce a codon change in the

HLA-B*0801fos gene and one was used to induce a codon change in the β2m gene. The primers were synthesized by Tufts University Core Facility.

A set of forward and reverse primers, 125ng each, were combined with 10ng of circular plasmid containing the targeted gene into a reaction buffer with 0.0002 M Deoxyribonucleotide triphosphate (dNTP) and 1µl of Pfu DNA polymerase, with a final volume of 50µl. Additionally, a control was made, which contained all the contents of the experimental mixture except the circular plasmid. PCR was performed by placing the solutions through a heating cycle outlined in Table IV.2.2.

Gel electrophoresis was used to check the result of the PCR, using 0.8% agarose gel and 5µl of the PCR product. The remainder of the product was stored at -20℃. Four induced mutation experiments were performed, one for each set of primers in table IV.2.1.

Table IV.2.1. Primers for Substitution Mutation

***B8fos222*: HLA-B*0801fos Residue 222 Glutamic Acid to Alanine mutation**
B8F222 forward primer : 5' GGC AGC GGG ATG GCG CGG ACC AAA CTC AGG 3'
B8R222 reverse primer: 5' CCT GAG TTT GGT CCG CGC CAT CCC GCT GCC 3'
***B8fos227*: HLA-B*0801fos Residue 227 Aspartic Acid to Alanine mutation**
B8F227 forward primer: 5' GGA CCA AAC TCA GGC CAC TGA GCT TGT GGA 3'
B8R227 reverse primer: 5' GTC TCC ACA AGC TCA GTG GCC TGA GTT TGG 3'
***B8fos229*: HLA-B*0801fos Residue 229 Glutamic Acid to Alanine mutation**
B8F229 forward primer: 5' CCA AAC TCA GGA CAC TGC GCT TGT GGA GAC 3'
B8R229 reverse primer: 5' CTG GTC TCC ACA AGC GCA GTG TCC TGA GTT 3'
***β2m58*: β2m Residue 58 Lysine to Alanine mutation**
β2mF58 forward primer: 5' GTG GAG CAT TCA GAT TTG TCC TTT AGC GCG GAT TGG TCG TTT TAT CTC TTG TAC TAC ACT 3'
β2mR58 reverse primer: 5' CAG TGT AGT ACA AGA GAT AAA ACG ACC AAT CCG CGC TAA AGG ACA AAT CTG AAT GCT CCA C 3'

Table IV.2.2. Heating Cycle for Polymerase Chain Reaction

Temp (℃)	time (s)	cycles
95	60	1
95	50	18
60	50	
68	540	
68	60	1

IV.3 Pure plasmid preparation of PGMT7 with mutant HLA-B*0801fos gene and PLM1 with mutant β2m gene

Products from the PCR reaction were treated with 1µl Dpn1 enzyme for two hours to break down the circular plasmids containing the wild type gene and to join the ends of the linear plasmids containing the specified mutated gene.

After Dpn1 digestion, 10µl of the mutant and control PCR products were added to separate microcentrifuge tubes each containing 50ml of thawed DH5α competent cells. In addition, 2.5µl of control puc19 plasmid was added to another 50ml of competent cells. The mixtures were incubated in ice for 30 minutes, followed by a 20 second heat shock at 37℃. After additional 2 minute incubation on ice, 950µl of LB medium is added to each mixture, followed by additional 1 hour incubation at 37℃. Each of the mixtures were spread on Luria-Bertani plus ampicillin (LBA) agar plates with the amount in the table IV.3.1

An isolated colony was selected from either plate 1 or 2 and inoculated into 25ml LB nutrient broth and grown overnight at 30℃. 500µl of the culture was combined with 15% glycerol and stored at -80℃ for future use. Plasmids were extracted from the remaining overnight culture by following the plasmid preparation procedures described in section IV.1, and stored at -20℃. A sample of the extracted plasmid was used for DNA sequencing by Tufts University Core Facility to check if the mutated gene is present. Four sets of plasmids were created that contained the specified gene and mutation according to table IV.2.1.

Table IV.3.1. LBA Agar Plate Contents for DH5α Transformation

plate	content	amount
1	Mutant	200µl
2	Mutant	400µl
3	Control	200µl
4	Control	400µl
5	Control puc19	100µl

IV.4 Transformation of PGMT7 plasmids with mutant HLA-B*0801fos gene and PLM1 plasmids with mutant β2m gene into protein producing competent cells

PGMT7 plasmids with mutant HLA-B*0801fos gene were transformed into BL21 (DE3) competent cells, while PLM1 plasmids with mutant β2m gene were transformed into BL21 (DE3) PLysS competent cells.

Two microcentrifuge tubes containing 100µl of thawed competent cells were each treated with 1.7µl of β-mercaptoethanol and incubated in ice for 10 minutes. Cells from the first tube were transformed with 1µl of plasmid solution containing the target mutant gene. Also, 1µl of puc18 plasmid solution was added to the second tube for use as a control. After additional 30 minute incubation in ice, contents in each tube were heat shocked at 42℃ for 45 seconds, followed by 2 minute ice incubation. The two tubes are then placed in a 37℃ incubator for 1 hour. Each of the mixtures were spread on LB agar plates containing the appropriate antibiotic, and in the amount shown in table IV.4.1. These plates were incubated overnight at 37℃ and used for expression test as described in section IV.5

Table IV.4.1. LBA Agar Plate Contents for BL21 Transformation

host strain	**antibiotics used**
BL21 (DE3)	ampicillin
BL21 (DE3) PLysS	ampicillin, chloramphenicol

plate	**content**	**amount**
1	Mutant	100µl
2	Mutant	200µl
3	Control puc18	100µl

IV.5 Protein Expression Test of transformed BL21 (DE3) and BL21 (DE3) PLysS cells

5 isolated colonies were selected from either plate 1 or 2 from experiment IV.4 and inoculated into separate sterile tubes each containing 2ml LBA nutrient broth and grown overnight at 30℃. Afterwards, 50µl was taken from each of these cultures and combined with 4.95ml of LB nutrient agar to make 1/100 dilutions. The diluted cultures were placed in a shaker set at 37℃ for about 3 hours, until the contents reached an optical density at 600nm wavelength of OD_{600} = 0.600. 300µl were collected from each culture and pelleted cells were separated from the supernatant and placed aside in separate 1.5 ml microcentrifuge tubes, and stored at -20℃. Also, 500µl from each tube was collected to create 15% glycerol stock cultures for future use. The remaining cultures were induced with 1mM IPTG and returned to the shaker for additional 3 hour incubation. Another 300ml were collected from each tube and the pelleted cells were stored in separate

microcentrifuge tubes for SDS gel electrophoresis testing. The remaining cultures were disposed.

The collected cells were each combined with 40µl of loading buffer and boiled at 100℃ for 5 minutes, then centrifuged. 20µl of the supernatant from each tube were applied to 12% SDS gel for HLA-B*0801fos, and 15% SDS gel for B2m. For each gel, electrophoresis was conducted for 100 minutes at 130V and 2A. The finished gels were stained and used to confirm protein expression of the targeted mutant genes.

IV.6 Inclusion body Preparation of mutant HLA-B*0801fos β2m proteins

Cells from a stock culture containing the target gene, made from section IV.5, were streaked on an LB agar plate with ampicillin (and chloramphenicol for BL21 (DE3) PLysS) and incubated overnight. An isolated colony from the plate was selected and inoculated into 50ml LB nutrient broth and grown overnight at 30℃. 10µl was taken from the culture and added to a flask containing 990ml LB nutrient broth and 0.4% glucose to make a 1/100 dilution. Four of these flasks were prepared for a total of 4 liters of culture. The diluted cultures were placed in a shaker set at 37℃ for about 3 hours, until the contents reached OD_{600} = 0.600. 1ml were collected from each culture and the pelleted cells placed aside in separate 1.5ml microcentrifuge tubes and stored at -20℃. The remaining cultures were induced with 1mM IPTG and returned to the shaker for additional 3 hour incubation. Another 1000ml were collected from each tube and the pelleted cells were stored in separate microcentrifuge tubes for SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Pelleted cells were collected from the remaining culture by centrifugation. The cells were then homogenized in 20ml of sucrose solution (table IV.6.1) and then lysed by adding 75ml of deoxycholate/triton solution (table IV.6.2)with 30mg of lysozyme. The lysing reaction lasted 20 minutes, at which the solution became very viscous. 5mM of magnesium chloride and 5-10mg deoxyribonuclease I was added to the solution to break the viscosity.

The solution was frozen using liquid nitrogen and then thawed in warm water, at which 1ml of 1M MgCl2 and 1ml 0.5 M EDTA was then added. All the contents were placed into 50ml centrifuge tubes and spun at 8000 rpm and at 4℃ for 20 minutes.

The supernatants were discarded and the pellets were suspended in 40 ml of Triton solution (table IV.6.3) and homogenized. The homogenized mixture was once again placed into 50ml centrifuge tubes and spun at 8000 rpm and 4℃ for 20 minutes. This washing procedure was done three times. Pellets collected after three washes underwent a forth wash following the same procedures except that Triton solution was substituted with Tris solution (table IV.6.4).

Pellets collected after the forth wash were dissolved and homogenized in 10ml Urea solution (table IV.6.5), and then placed into 10 1.5ml microcentrifuge tubes and spun at 12000 rpm and at room temperature for 10 minutes. The supernatants were aliquoted into 10 1.5 ml microcentrifuge tubes and frozen at -80℃.

Bradford assay was used to determine the concentration of the collected inclusion bodies. A standard absorbance versus concentration curve was created using stock 2 mg/ml bovine serum albumin (BSA) with the dilutions outlined in table IV.6.6. 100ul of each dilution was added to 5ml of 20% Bradford dye and the OD at 595nm wavelength was measured. This curve was used to calculate the unknown concentration of the inclusion bodies based on its measurable OD and known dilution factor.

Table IV.6.1. Sucrose Solution

reagent	**Concentration**
Tris pH 8.0	50mM
sucrose	25%
EDTA	1mM
Sodium Azide	0.10%

Table IV.6.2. Deoxycholate/triton Solution

reagent	**Concentration**
Deoxycholate	1%
Triton x100	1%
Tris pH7.5	20mM
Sodium Chloride	0.1M
Sodium Azide	0.10%

Table IV.6.3. Triton Solution

reagent	**Concentration**
Triton x100	0.50%
Tris	50mM
Sodium Chloride	100mM
EDTA	1mM
Sodium Azide	0.10%

Table IV.6.4. Tris Solution

reagent	**Concentration**
Tris	50mM
EDTA	1mM
Sodium Azide	0.10%

Table IV.6.5. 8M Urea Solution

reagent	**Concentration**
Urea	8M
MES pH6	25mM
EDTA	1mM
DTT	0.1mM

Table IV.6.6. Bradford Assay Standard Dilutions

Concentration	0mg/ml	0.2mg/ml	0.4mg/ml	0.6mg/ml	0.8mg/ml
BSA (2mg/ml)	0µl	50µl	100µl	150µl	200µl
8M Urea	500µl	450µl	400µl	350µl	300µl

IV.7 Refolding HLA-B*0801fos Inclusion Bodies

Table IV.7.1 lists the components in the refolding buffer that was used to refold the inclusion bodies into their native structure. First, 2µm of B2m inclusion was added to the refolding buffer, followed by 10mg of synthetic peptide dissolved in 100ml DMSO, and finally 1µm of the HLA-B*0801fos heavy chain.

The mixture was stored at 4℃ for 48 hours, and then placed in 10000 MWCO dialysis bags, which were placed in 10L 20mM Tris dialysis buffer (table IV.7.2). The dialysis procedure lasted 24 hours, at which then concentration procedures began.

Contents from the dialysis bags were concentrated to a volume of 15-30ml using the Stir Cell apparatus, using 10000 MWCO membranes. The contents from the Stir Cell were then placed in 1-2 Centripreps and concentrated to a final volume of about 1ml and placed in a 1.5ml microcentrifuge tubes and spun at 12000 rpm and at 4℃ for 30 minutes. The supernatant, which contained refolded HLA-B*0801fos and β2m, were transferred to another clean 1.5ml microcentrifuge tube.

Refolded HLA-B*0801fos and β2m were each separated from aggregates and other unwanted items according to their mass by using a fast protein liquid chromatography (FPLC) system. The collected purified HLA-B*0801fos and β2m were placed into a 10000 MWCO and a 3000 MWCO Centriprep, respectively, and each concentrated to 1ml.

The final concentrations of the samples were determined using mass spectroscopy. 5µl from a sample was combined with 600µl of 6M Guanidine Hydrochloride and stored at room temperature for one hour. The OD of this mixture was measured and used to calculate the concentration of the sample using formulas IV.7.1 and IV.7.2.

$$C(M) = A/E*D \quad (IV.7.1)$$
$$C(mg/ml) = A/E*D*MW \quad (IV.7.2)$$

C = concentration
A = absorbance = OD_{280}
D = dilution factor = 605/5
*MW HLA-B*0801fos = 50080kDa*
MW β2m = 11400kDa

Table IV.7.1. 1L Refolding Buffer

reagent	**Concentration (mM)**	**Comments**
Tris pH 8	50mM	50 ml added from 1M stock
Arginine pH8	400mM	200 ml added from 200ml stock (filtered)
EDTA pH 8	1mM	2ml added from stock 0.5M
GSSH	0.5mM	1.5g added directly
GSSG	0.5mM	0.38g added direclty
PMSF	0.5mM	2.5ml of 200mM fresh stock added
Water		add up to 1L

Table IV.7.2. 20mM Tris buffer

reagent	**Concentration (mM)**
Tris pH 8	20mM
Sodium Chloride	150mM

IV.8 Creating peptide-less HLA-B*0801fos proteins

From IV.7, 500µl of refolded HLA-B*0801fos was denatured in 1500µl of 8M Guanidine Hydrochloride (table IV.8.1) and stored at room temperature for 4 hours. The HLA-B*0801fos heavy chain was separated from B2m using the FPLC with 6M Guanidine Hydrochloride gel filtration buffer. The collected HLA-B*0801fos was combined with the collected β2m and additional β2m from other sources, if necessary, such that the molar ratio is 1:2 Heavy chain to β2m.

The mixture was diluted with 6M Guanidine Hydrochloride (table IV.8.2) to a concentration of 1mg HLA-B*0801fos per 1ml, and placed in 3000 MWCO dialysis bags and dialyzed against 1L of 8M Urea buffer (table IV.8.3) for 24 hours at 15°C. The dialysis buffer was then changed to 1L of 20mM Tris (table

IV.7.2) plus 10% Glycerol for 16 hours, at which then the buffer was replaced with another 1L of 20mM Tris plus 10% Glycerol. This process was done for 48 hours and 3 1L 20mM Tris plus 10% Glycerol buffers were used.

The dialyzed contents were concentrated to 30 ml using a Stir Cell and then to 1ml using a Centriprep. Pure peptide-less folded HLA-B*0801fos was collected from the concentrated sample using the FPLC with 20mM Tris gel filtration buffer, followed by a final Centriprep concentration to 1ml. The final concentration of the sample was determined using mass spectroscopy as described in IV.7

Table IV.8.1. 8M Guanidine Hydrochloride

reagent	**Concentration**
Guanidine Hydrochloride	8M
Sodium Chloride	150mM
Tris pH 7.5	20mM

Table IV.8.2. 6M Guanidine Hydrochloride

reagent	**Concentration**
Guanidine Hydrochloride	6M
Sodium Chloride	150mM
Tris pH 7.5	20mM

Table IV.8.3. 8M Urea Buffer

reagent	**Concentration**
Urea	8M
MES pH6	25mM
EDTA	1mM
DTT	0.1mM

V. Results and Discussion

V.1 Plasmid preparation of PGMT7 with HLA-B*0801fos gene and PLM1 with β2m gene

The final concentration of the PGTM7 plasmid solution with HLA-B*0801fos gene was 380 µg/ml and was sufficient for further PCR experiments. Absorbance for the solution was low at OD_{260} = 0.076 and a lower dilution factor can be used to increase this value to be in the range of 0.1 – 0.9. An increase in plasmid yield can be obtained by increasing the amount of cell culture used.

V.2 Induced substitution mutation of PGMT7 plasmid containing HLA-B*0801fos and PLM1 plasmid containing β2m gene

Gel electrophoresis results of several PCR mutation reaction products are shown in figures V.2.1 – V.2.3. PCR results with *B8fos222* primers (figure V.2.1) were somewhat inconclusive as the bands for control (lane 4) and mutant (lane 5) were quite faint and can only be seen under careful inspection of the gel itself. The photograph of the gel renders these bands invisible. These results indicated that 5µl of sample may not be enough. For PCR reactions using *B8fos227, B8fos229* and *β2m58* primers, 10µl of the products were used for gel electrophoresis, which produced much better clarity, as shown by figures V.2.2 – V.2.4.. Despite the lack of clarity of the *222* gel, the results confirm that the PCR reactions were successful.

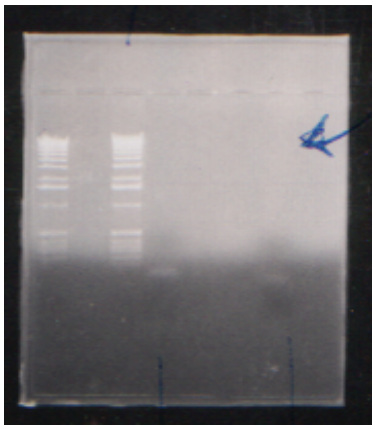

Figure V.2.1. PCR results with *B8fos222* primers. Starting from the left, lanes 1 and 3 are makers, lane 4 is control and lane 5 is the mutant. Lane 4 contains no bands near 30kb, which shows that PCR did not occur for the control. Although not visible in this photo, a faint band is present in lane 5 at the location indicated by the arrow near 30kb, showing that the PCR reaction was successful and that the plasmids were produced.

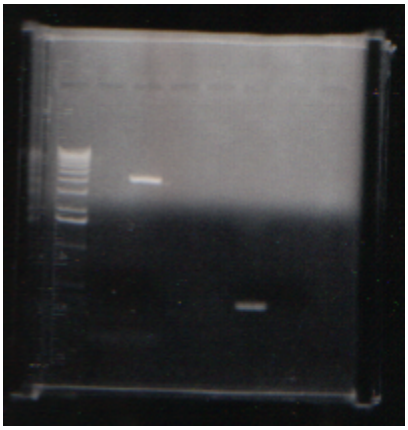

Figure V.2.2. PCR results with *B8fos227* primers. Starting from the left, lane 1 is a maker, lane 3 is the mutant and lane 6 is the control. Lane 3 contains only one band at around 30kb, which suggests that all the primers were used up to create mutant plasmids. Lane 6 contains a strong band near 50b, which represents unused *227* primers.

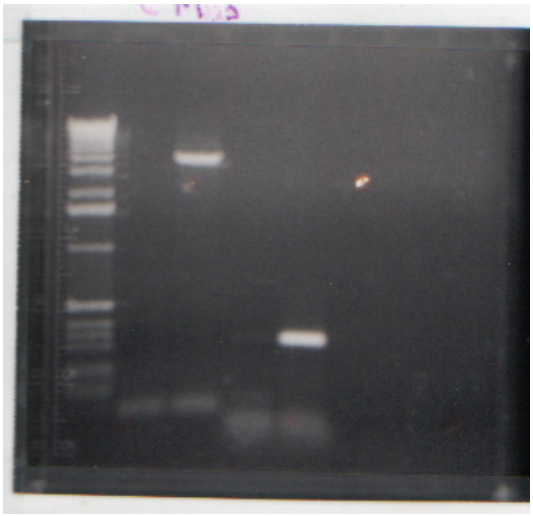

Figure V.2.3. PCR results with *B8fos229* primers. Starting from the left, lane 1 is a maker, lane 3 is the mutant and lane 5 is the control. A band at around 30kb, suggests that the PCR reaction was successful and plasmids were replicated. Lane 5 contains a strong band near 50b, which represents unused *229* primers.

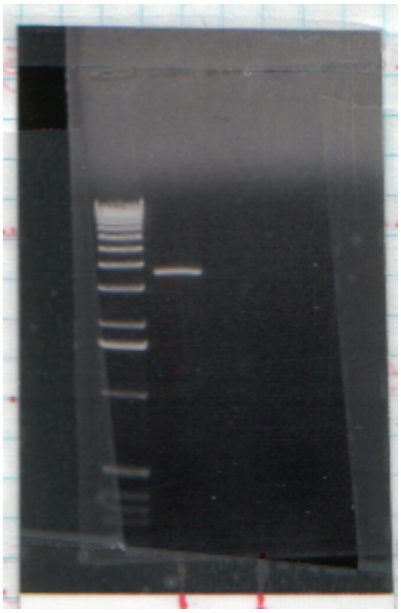

Figure V.2.4. PCR results with *β2m58* primers. Starting from the left, lane 1 is a maker, lane 2 is the mutant and lane 3 is the control. A band at around 30kb for lane 1 suggests that the PCR reaction was successful and plasmids were replicated.

V.3 Pure plasmid preparation of PGMT7 with mutant HLA-B*0801fos gene and PLM1 with mutant β2m gene

The transformation results for each plasmid were expected. Colony growth was observed in LBA agar plates with competent cells strains transformed with a mutant sample from PCR or the control puc19 plasmid. Cells transformed with a control sample from PCR did not form colonies on LBA agar plates.

The sequencing results each plasmid are in Appendix – IX.3. The results show that induced mutation using primers *B8fos222*, *B8fos227*, and *B8fos229* were successful and produced the specified point mutation. However, the first β2m mutation was unsuccessful as the sequencing results show a combination of insertion and frameshift mutations in the β2m gene, as opposed to the expected point substitution mutation. The original set of primers (see Appendix – IX.2) had a melting temperature slightly lower than the recommended values according to the *QuikChange™ XL Site Directed Mutagenesis Kit* and was also relatively short compared to the primers *B8fos222*, *B8fos227* and *B8fos229*. A second set of primers were designed to carry out the β2m mutation and is listed in table IV.2.1. This primer set was significantly longer than the first set and induces additional point mutations, but the codons remain unchanged. Sequencing results indicates that PCR using this primer yielded the expected point mutation in the β2m gene contained in the PLM1 plasmid.

V.4 Transformation of PGMT7 plasmids with mutant HLA-B*0801fos gene and PLM1 plasmids with mutant β2m gene into protein producing competent cells

The transformation results for each plasmid were expected. Colony growth was observed in LBA or LBAC agar plates with competent cells strains transformed with a mutant sample from PCR or the control puc18 plasmid. Cells transformed with a control sample from PCR did not form colonies on LBA or LBAC agar plates.

V.5 Protein Expression Test of transformed BL21 (DE3) and BL21 (DE3) PLysS cells

SDS-PAGE results of several expression tests are shown in figures V.6.1 – V.6.3. Leaked expression was observed for expression tests for the BL21 (DE3) strain transformed with PGMT7 plasmids containing the *B8fos222, B8fos227, B8fos229* mutations. This problem was not observed in the expression test done with the BL21 (DE3) PLysS strain transformed with PLM1 plasmids containing the β2m58 mutation. Despite leaked expression, the results confirmed that the transformed cells produced the specified proteins.

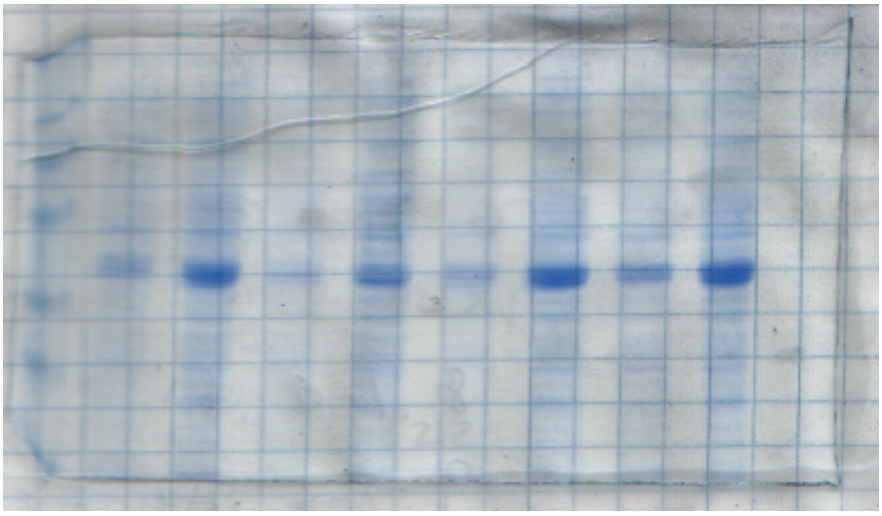

Figure V.5.1. SDS-PAGE result of *B8fos222* expression test. Starting from the left, lane 1 is a maker, lanes 2,4,6,8 are cells contents before IPTG induction. Lanes 3,5,7,9 are cells after induction. Dominant bands near 35 kDa suggested that HLA-B*0801fos proteins were produced.

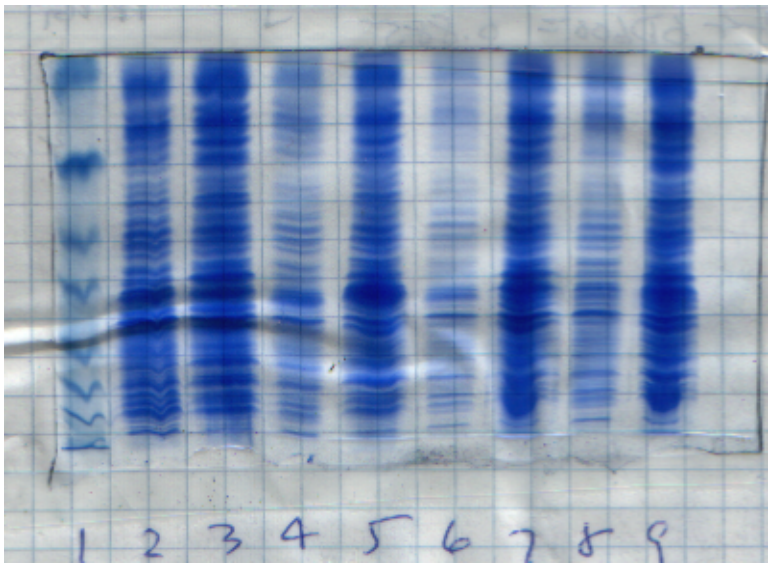

Figure V.5.2. SDS-PAGE result of *B8fos227* expression test. Starting from the left, lane 1 is a maker, lanes 2,4,6,8 are cells contents before IPTG induction. Lanes 3,5,7,9 are cells after induction. Bands near 35 kDa suggested that HLA-B*0801fos proteins were produced, although large amounts of other proteins were produced as well.

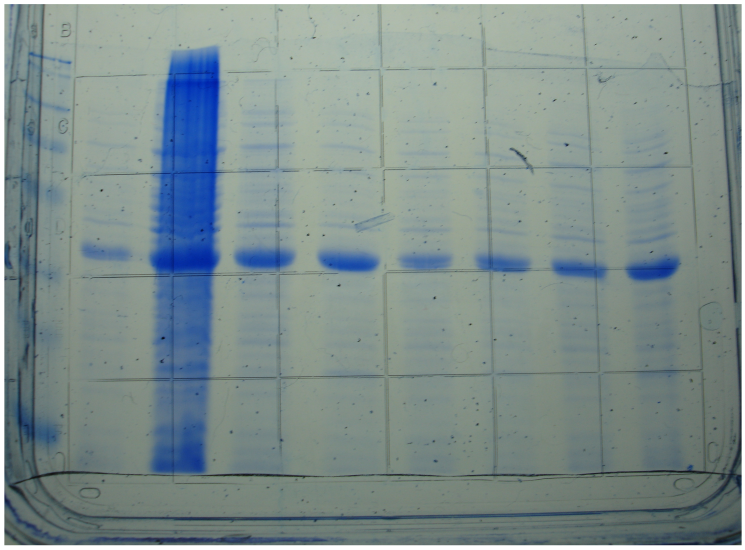

Figure V.5.3. SDS-PAGE result of *B8fos229* expression test. Starting from the left, lane 1 is a maker, lanes 2,4,6,8 are cells contents before IPTG induction. Lanes 3,5,7,9 are cells after induction. Dominant bands near 35 kDa suggested that HLA-B*0801fos proteins were produced.

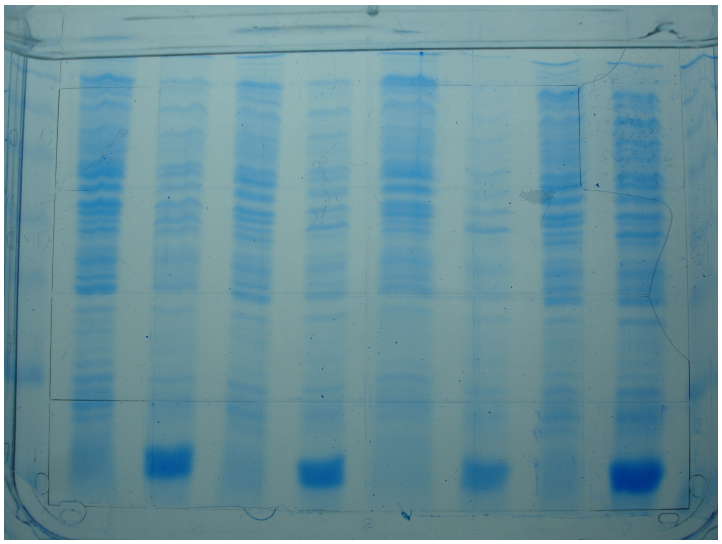

Figure V.5.4. SDS-PAGE result of *β2m58* expression test. Starting from the left, lane 1 is a maker, lanes 2,4,6,8 are cells contents before IPTG induction. Lanes 3,5,7,9 are cells after induction. Dominant bands near 16 kDa for lanes 3,5,7,9 suggested that HLA-B*0801fos proteins were produced. The use of BL21 (DE3) PLysS cell line may have prevented leaked expression.

V.6 Inclusion body Preparation of mutant HLA-B*0801fos β2m proteins

SDS-PAGE results of the inclusion bodies are shown in figures V.6.1 – V.6.3. For HLA-B*0801fos, lanes that have a large band near 36 kDa compared with other relatively weak bands shows that the inclusion body preparations contained mostly HLA-B*0801fos protein and small amounts of other proteins. A large band near 16 kDa for β2m also confirms that the inclusion body preparation contained mostly β2m proteins.

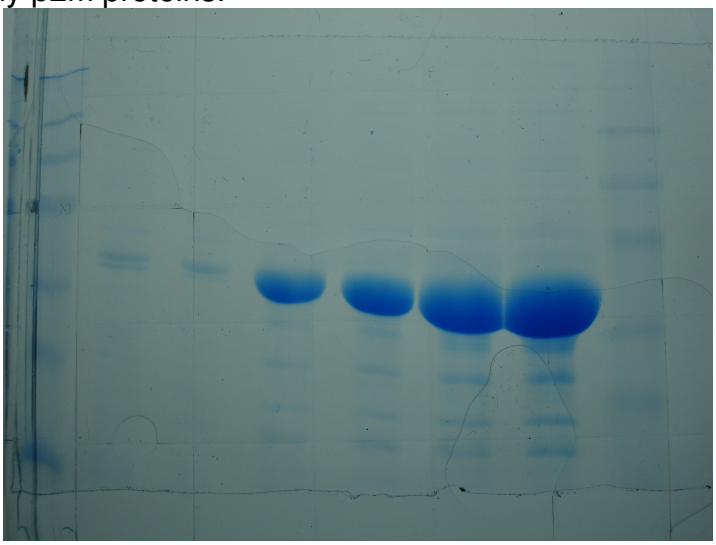

Figure V.6.1. SDS-PAGE result of *B8fos222* inclusion bodies. Lanes 4,5,6,7 are inclusion body samples after each subsequent wash, with lane 4 being after the first wash. The dominant bands observed at these lanes are near 36 kDa.

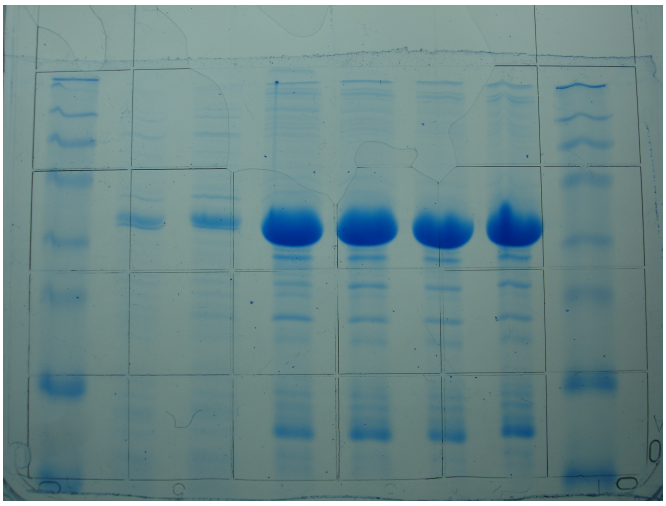

Figure V.6.2. SDS-PAGE result of *B8fos227* inclusion bodies. Lanes 4,5,6,7 are inclusion body samples after each subsequent wash, with lane 4 being after the first wash. The dominant bands observed at these lanes are near 36 kDa.

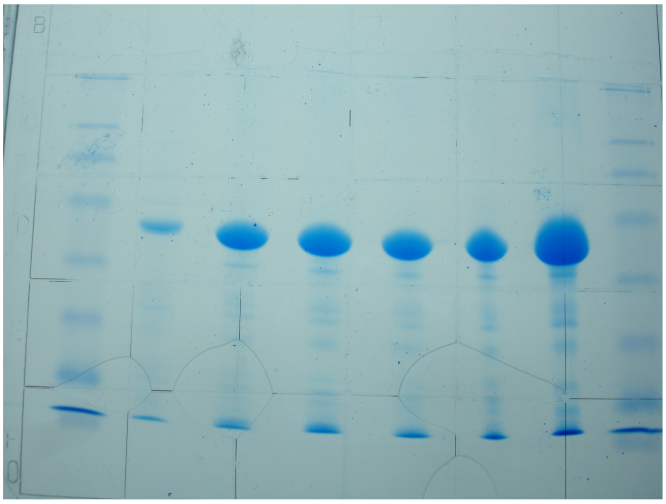

Figure V.6.3. SDS-PAGE result of *B8fos229* inclusion bodies. Lanes 4,5,6,7 are inclusion body samples after each subsequent wash, with lane 4 being after the first wash. Lane 3 contains inclusion body sample before any wash. The dominant bands observed at these lanes are near 36 kDa.

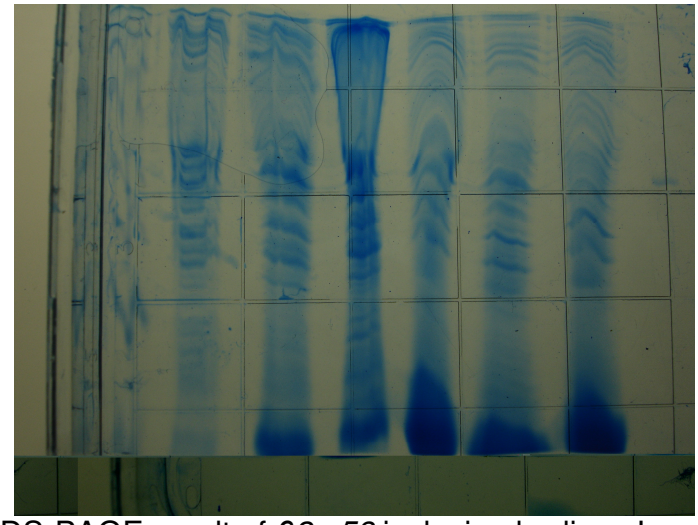

Figure V.6.4. SDS-PAGE result of *β2m58* inclusion bodies. Lanes 4,5,6,7 are inclusion body samples after each subsequent wash, with lane 4 being after the first wash. Lane 3 contains inclusion body sample before any wash. The dominant bands observed towards the bottom of these lanes are near 16 kDa.

V.7 Refolding HLA-B*0801fos Inclusion Bodies

Chromatograph results from FPLC purification of HLA-B*0801fos proteins are shown in Figure V.7.1. Generally, the HLA-B*0801fos peak is at 29 minutes, followed by excess β2m proteins at 38 minutes. The aggregate peak at 18 minutes varies in strength depending on the concentration of the sample. A high concentration usually resulted in a high aggregate peak (figure V.7.1) whereas a low concentration yielded a low peak (figure V.7.2).

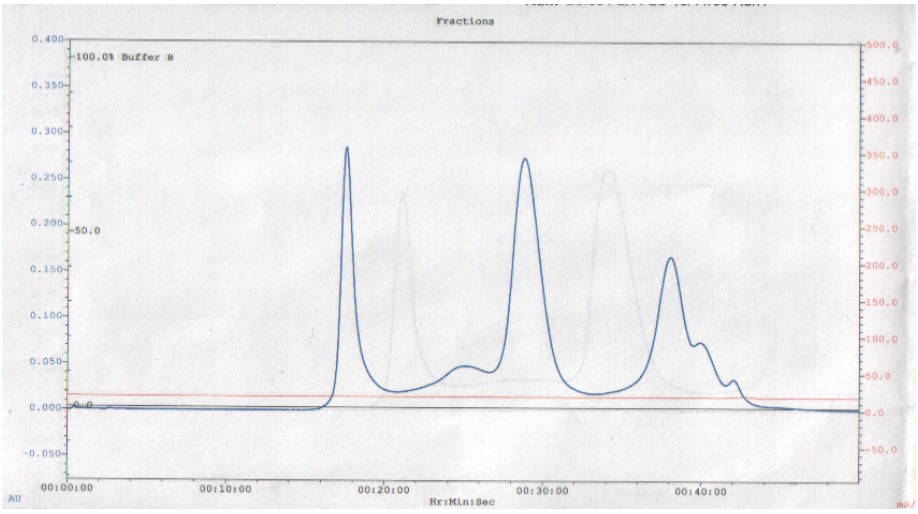

Figure V.7.1. Chromatograph of FPLC purification of HLA-B*0801fos proteins. A 1L refold was concentrated to 500ml before purification. The high concentration lead to a large aggregate peak.

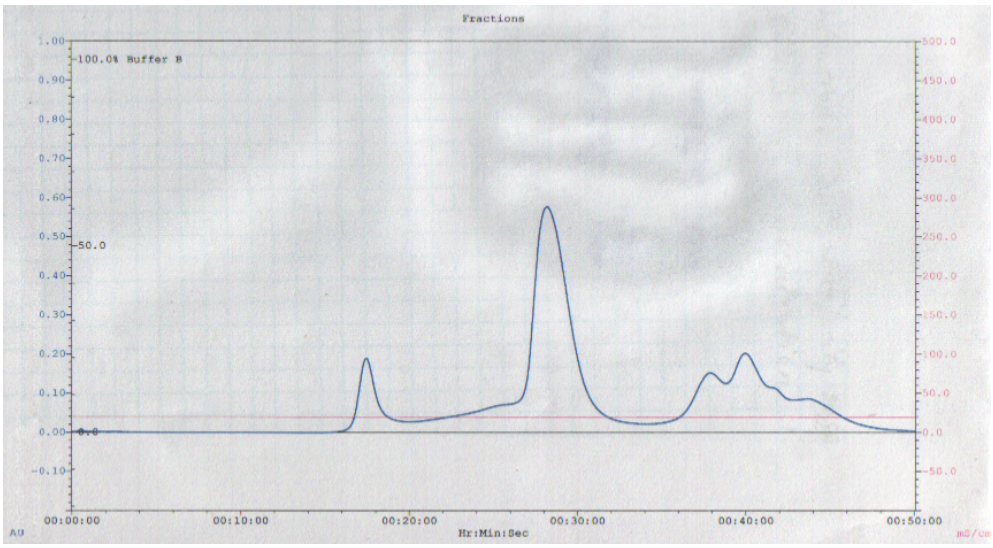

Figure V.7.2. Chromatograph of FPLC purification of HLA-B*0801fos proteins. A 1L refold was concentrated to 5ml before purification. The low concentration significantly reduced the aggregate peak and lead to a larger HLA-B*0801fos peak.

V.8 Creating peptide-less HLA-B*0801fos Proteins

Chromatograph results from FPLC purification of denatured HLA-B*0801fos proteins are shown in Figure V.8.1, and results from FPLC purification of refolded peptide-less HLA-B*0801fos proteins are shown in Figure V.8.2. Aggregates are almost undetectable by these chromatographs as they have been removed from previous purifications.

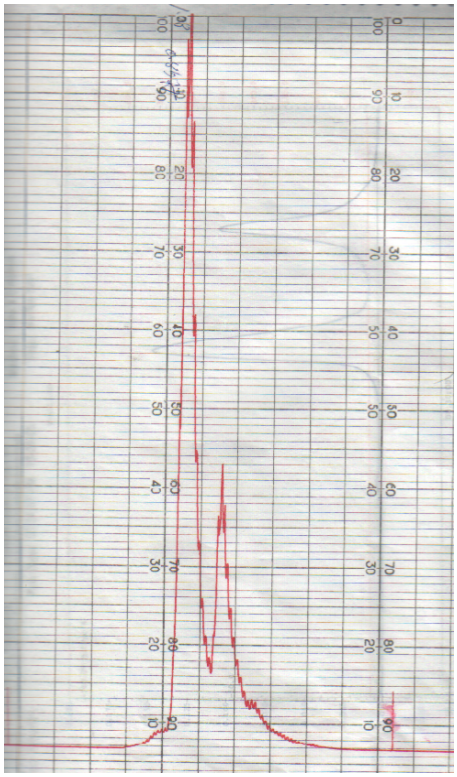

Figure V.8.1. Chromatograph of FPLC purification of denatured HLA-B*0801fos proteins. The first large peak represents denatured HLA-B*0801fos and the second peak represents denatured β2m

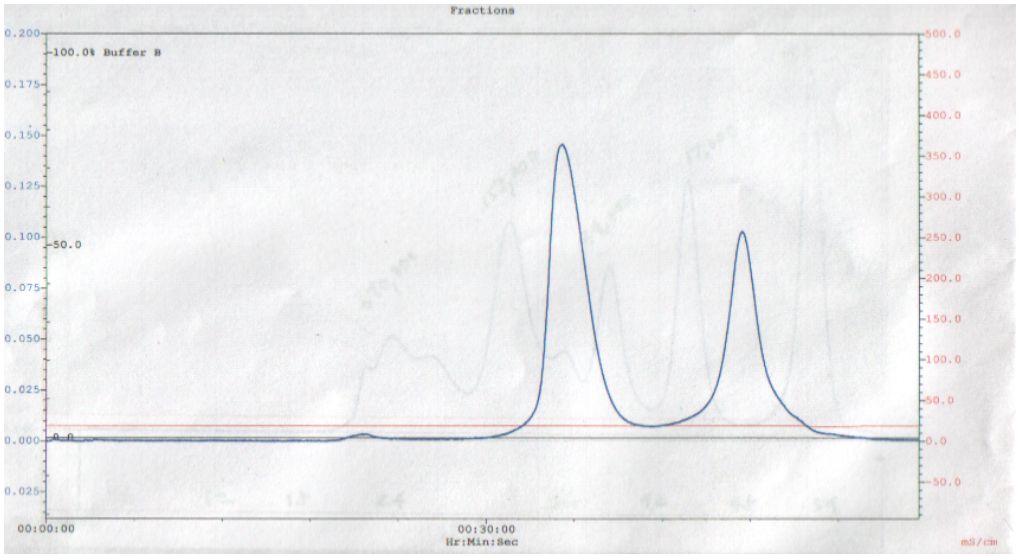

Figure V.8.2. Chromatograph of FPLC purification of peptide-less HLA-B*0801fos proteins. The first large peak represents peptide-less HLA-B*0801fos proteins and the second peak represents excess refolded β2m.

VI. Conclusion

Inclusion body preparation of mutant HLA-B*0801-fos and β2m was successful, as well as the reconstitution of wild type HLA-B*0801-fos protein in both peptide-filled and peptide deficient forms. Several unexpected problems occurred during the series of experiments. First, the first set of mutations that involved the β2m primers in IX.1.2 did not produce the desired codon change. As a result, the primers were redesigned and the second mutation attempt produced the expected results. Second, an unusual amount of aggregates were formed in the refolding process for HLA-B*0801-fos. This problem was overcome by decreasing the concentration at which the sample was purified. Other than these minor problems, the experiments proceeded as expected and yielded acceptable results.

VII. Acknowledgements

I thank Dr. Marlene Bouvier for providing me with the opportunity to work and learn at her lab. I also thank Mingnan Chen, Hong Liu and Jie Fu for continually answering my questions and teaching me new procedures.

VIII. References

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5. Cheng, Loi. **HLA-B8fos mutants** (Lab Manual). Dr. Marlene Bouvier Laboratory. University of Connecticut School of Pharmacy.

IX. Appendix

IX.1 DNA sequences of HLA-B*0801fos gene and β2m gene

IX.1.1 Human Leukocyte Antigen HLA-B*0801fos

```
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Soluble domain of B0801 Heavy chain
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IX.1.2 Human beta-2-microglobulin (**β2m)**

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(Ribosome binding site) (atg)
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121 aatttcctga attgctatgt gtctgggttt catccatccg acattgaagt tgacttactg
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Ribosome binding site = aag gag gat att aaa

IX.2 Original B2m primers

***β2m58*: β2m Residue 58 Lysine to Alanine mutation**
β2mF58 forward primer: 5' CAG ACT TGT CTT TCA GCG CGG ACT GGT CTT TCT ATC TC 3'
β2mR58 reverse primer: 5' GAG ATA GAA AGA CCA GTC CGC GCT GAA AGA CAA GTC TG 3'

IX.3 Plasmid sequencing results of mutant PGMT7 and β2m from TUFTS University Core Facility in FASTA format

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>B82271-T7
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