Extraction and detection antibiotic

Result

Extraction and detection of Virginiamycin

Collect the supernatant from culture broth after cultivation of *Streptomyces virginiae* for 24 hours and perform bioassay against *Bacillus subtilis.* The result of bioassay was shown in figure 1.

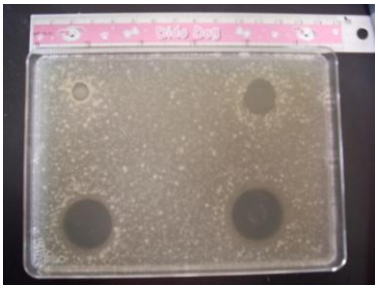


Fig.1. Bioassay result against *Bacillus subtilis*
1 : 0 µl of culture supernatant (diameter of clear zone = 0 cm.)
2 : 10 µl of culture supernatant (diameter of clear zone = 1.4 cm.)
3 : 100 µl of culture supernatant (diameter of clear zone = 2.2 cm.)
4 : 200 µl of culture supernatant (diameter of clear zone = 2.2 cm.)

Virginiamycin was analyzed by HPLC. The result was shown in figure 2 and the concentration shown in table 1.

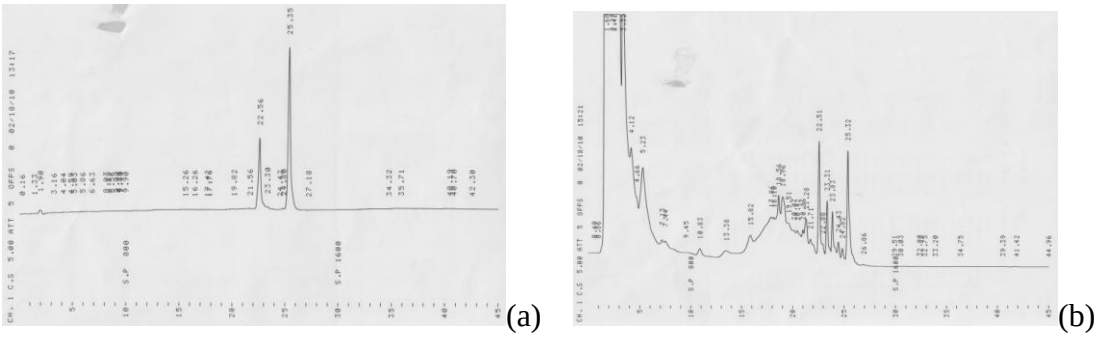


Fig.2. (a) Standard curves for virginiamycin m and s
(b) HPLC result of culture supernatant

	Standard (100 ng/µl, 20 µl)		Culture supernatant		
	RT	area	RT	area	concentration (ng/µl)
Virginiamycin m	22.56	124210	22.51	185527	14.95
Virginiamycin s	25.35	246814	25.32	225843	9.15

Table.1. The result of HPLC, virginiamycin m and s

Extraction and detection of Thaxtomin

Collect the supernatant from culture broth after cultivation of *Streptomyces scabies* for 48 hours and extract with n-butanol, evaporate the n-butanol layer, dissolve with methanol. The color of solution becomes yellow. Perform TLC analysis compared with authentic thaxtomin. The result of TLC was shown in figure 3.

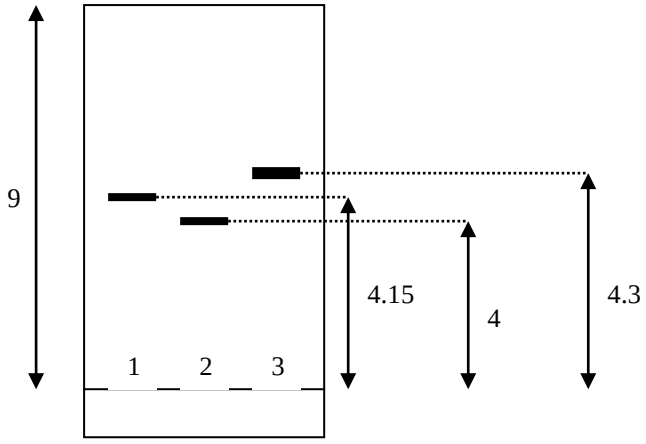


Fig.3. The result of TLC

1 : Authentic thaxtomin 5 µl (R_f = 0.461)
2 : Culture supernatant 5 µl (R_f = 0.44)
3 : Culture supernatant 10 µl (R_f = 0.478)

Discussion

Virginiamycin is a cyclic polypeptide antibiotic from *Streptomyces virginiae*. It consist of 2 major components, virginiamycin m and s. It is used to treat infection with gram-positive bacteria such as *Bacillus subtilis*. It inhibit bacterial cell growth by inhibiting protein synthesis. Both virginiamycin m and s binds to 50s ribosomal subunit. Virginiamycin m causes a conformational change and virginiamycin s causes the release of incomplete peptides.

From the culture supernatant found that the retention time of the first expected peak is 22.51 and the second expected peak is 25.32. Compared with the retention time of standard virginiamycin m (22.56) and s (25.35) suggested that the first peak is virginiamycin m and the second peak is virginiamycin s. In addition, we can calculate the amount of virginiamycin by using the area of peak compared with the area of standard virginiamycin.

TLC analysis, the mobility of spot was measured, compared with authentic thaxtomin and calculated the retention factor or R_f by using the equation.

"R_f = distance traveled by the compound / distance traveled by the solvent"

Found that the R_f of compound is similar to the R_f of authentic thaxtomin. This result suggested that the compound is thaxtomin.

Principle of TLC

Thin layer chromatography (TLC) is a chromatography technique used to separate the mixture compound. TLC consist of two phase, stationary phase and mobile phase. When apply sample to the TLC by spot on the glass or aluminium which is coated by absorbent material such as cellulose or siliga gel called stationary phase, then put the plate on the solvent called mobile phase. The solvent is drawn up on the plate by capillary force.

The separation of mixture compound is based on the property of compound in the dissolution in solvent and the movement on the stationary phase (interaction with the stationary phase). If that compound has strong interaction with the stationary phase, that compound cannot move on the plate in long distance. On the other hand, if another compound is less stronger interaction with stationary phase than other compounds, that compound can move in long distance on the plate (resulting in a higher R_f value).

Production and crystalization of *Pseudomonas* sp MIS38 lipase (PML)

Project –based Training Course Report

Dian Anggraini Suroto(28A09085)

Introduction

Bacterial produce different classes of lipolytic enzyme, including carboxylesterases which hydrolyse small containing molecules at least partly soluble in water, true lipases which display maximal activity towards water insoluble long-chain triglycerides, and various types of phospholipases (1). Lipolytic enzyme are attracting a lot of attention due to their biotechnological potential, include their addition to detergents, the manufacture of food ingredients, pitch control in pulp and paper industry, and biocatalysis of stereoselective transformation (2). *Pseudomonas* sp MIS38 lipase (PML) belongs to sub family 1.3 lipase, which are true lipases and consist of an N-domain (residues 1-370) and C-domain (residues 371-617) (3). PML accumulates in *Escherichia coli* cells in an insoluble form upon overproduction using a pET system (3). Therefore a system for extracellular production and / or overproduction of PML by *Escherichia coli* harbouring LipBCD genes from *Serratia marcescens* SM800 have been performed (4,5) and crystal structure of PML also had been determined (5,6).

Material and methods

Protein production and purification

Escherichia coli DH5α containing two plasmid, pUC-PML containing PML gene and pYBCD20 harbouring *lip*BCD gene were grown in 1 litre LB medium with addition 50 mg/L ampicillin and 30 mg/L chloramphenicol at 37 ^{o}C for 24 hour, 120 rpm. The culture than centrifugated at 10000g for 30 min at 4^{o}C. The supernatant was collected and its pH was adjusted to 8,0 by adding 1/20 (v/v) 2 M Tris HCl pH 8,0. PML was collected by 80% ammonium sulfate precipitation. The protein precipitate was redisolved in 50 ml 5 mM Tris-HCl pH 8,0 containing 5% glycerol and 50 mM NaCl and dialyzed overnight against the same buffer in 4^{o}C. The dialysate was centrifugated at 25000 g for 30 min , and then applied to HiTrap Q HP anion exchange column (GE Health Care) equilibrated with the same buffer. The flowtrough was collected and applied onto HiLoad 16/60 Superdex gel-filtration column (Amersham Biosciences) equilibrated with 5 mM Tris –HCl pH 8,0 containing 50 mM NaCl. The protein fractions containing PML were collected , concentrated to final concentration 8 mg/ml using Centricon YM-50 (Milipore). Protein concentration was determined by UV spectrophotometer, and the purity was analyzed by SDS-PAGE followed by staining with Coomassie Briliant Blue.

Crystallization

Crystallization was performed using hanging-drop vapour-diffusion method. Drops were prepared by mixing 2 µl protein solution with 1 ul crystallant solution (0,1 M MES buffer pH 6,5, 10% Polyethylene Glycol (PEG) 20K, 0,2 M Calcium acetate, 5mM Zinc acetate). The drops were vapour equilibrated against 300 µl of a modified crystallant solution that contains 5% PEG 20K instead of 10%. After 10 days incubation at 4^{o}C, crystals were observed using light microscope.

Result and discussion

The amount of protein purified from 1 L culture approximately 2 mg, this amount was lower than previously reported 5 mg (5) this lower amount of purified protein may be due to some loss during purification especially leakage during dialysis. The molecular weight of PML was around 66.4 kDa and it is monomer confirmed by SDS –PAGE, this result is along with previously reported (5).

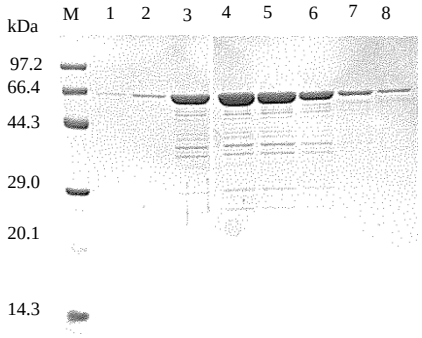


Figure 1. Analysis of PML after gel filtration, lane 1-8 are correspond to fraction no.78 – 85.

PML then was crystallized using hanging-drop vapor diffusion method as descripted above, The strategy for induced crystallization here was to bring the system very slowly toward state of minimum solubility and thus achieve a limited degree of super saturation using PEG 20K as precipitant / crystallant. PML crystals were appear after 10 days incubation.

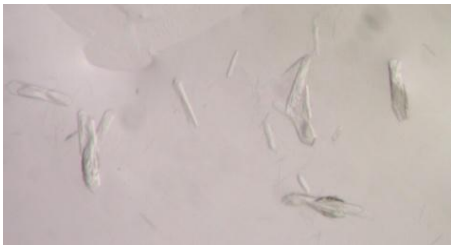


Figure 2. PML crystals obtained by hanging-drop vapor diffusion method

In this experiment, the large single crystal seem not to be formed, this means that saturation has proceed to rapidly (7), may me due to the lower concentration of protein and some protein already degraded. For making large single crystal the protein / macromolecules concentration should be from 10 to 100 mg/ml (7), as previously reported for crystallization of PML concentration was 10 mg/ml (5). Large single crystal is important, because only in that form

protein can be subjected to X –ray diffraction for further analysis of its structure. To increase the chances of growing large single crystal there are many requirement should be fulfilled, purity and amount of protein should be first priority (7).

References

1. Arpigny, J.L. and Jaeger, K.E.: **Bacterial lipolytic enzymes : classification and properties**. *Biochem. J*. 1999, **343**: 177-183.
2. Jaeger, K.E. and Reetz, M.T : **Microbial lipases from versatile tools for biotechnology.** *Trends in Biotechnology* 1998, **16**: 396-403.
3. Amada, K., Haruki, M., Imanaka, T., Morinaka, M, and Kanaya, S: **Overproduction in *Escherichia coli*, purification and characterization of a family I.3 lipase from *Pseudomonas* sp MIS38**. *Biochim. Biophys. Acta* 2000, **1478** : 201-210.
4. Kwon, H.J., Haruki, M., Morikawa, M., Omori, K. and Kanaya, S.: **Role of repetitive nine-residu sequence motifs in secretion, enzymatic activity and protein conformation of a family I.3 lipase**. *J. Biosci. Bioeng*. 2002, **93**: 157-164.
5. Angkawidjaja, C., You, D.J., Matsumura, H., Koga, Y., Takano, K., and Kanaya, S. **Extracellular overproduction and preliminary crystallographic analysis of a family I.3 lipase.** *Acta Crystallographica* 2007, **F63**: 187-189.
6. Angkawidjadja, C., You, D.J., Matsumura, H., Kuwahara, K., Koga, Y., Takano, L., and Kanaya, S. **Crystal structure of a family I.3 lipase from Pseudomonas sp. MI38 in a closed conformation**. *FEBS Letters* 2007, **581**: 5060-5064.
7. McPherson, A. **Preparation and analysis of protein crystal.** John Willey and Sons 1982, 82-135.

.

PRODUCTION AND PURIFICATION OF HISTONE DEMETHYLASE JMJD2A

Abstract

JMJD2A is an enzyme that converts specific trimethylated histone residues to the dimethylated form, and also acts as a transcriptional repressor. JMJD2A is widely expressed in human tissues and cell lines. In this experiment, JMJD2A has successfully been produced by E. coli BL21 (DE) after transformation using plasmid PGeX4T-2. SDS-PAGE confirmed this enzyme which has 67 KDa in size. The concentration of enzyme that has been produced is 16.3 μM. Further purification using the more effective method is needed to purify this enzyme.

1. Introduction

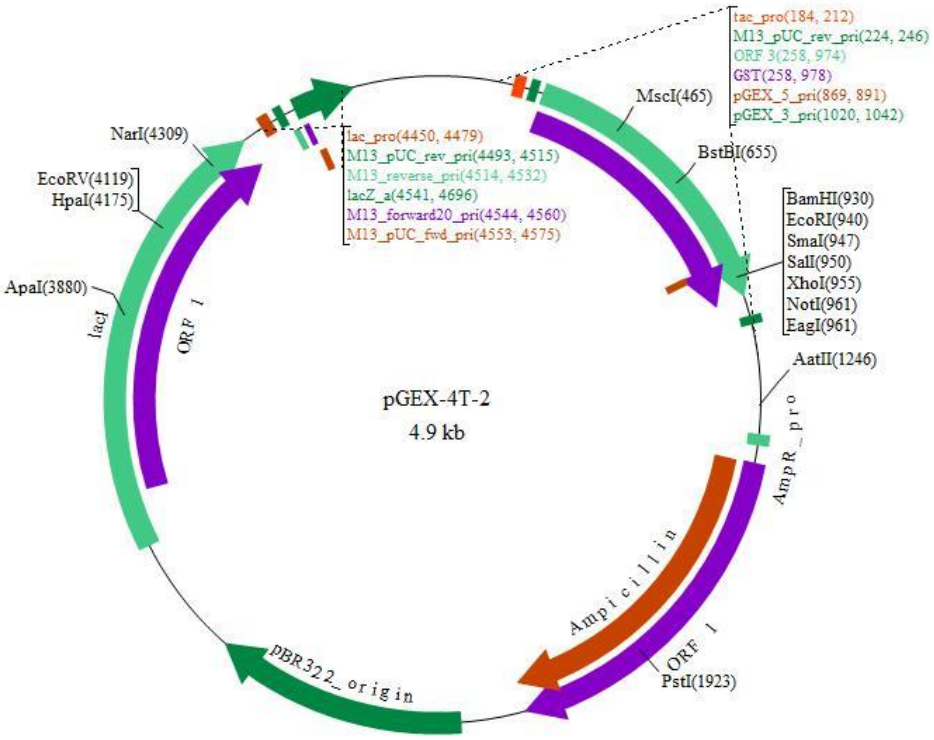
The posttranslational modification of histones plays an important role in chromatin regulation. Histone methylation influences constitutive heterochromatin, genomic imprinting, X-chromosome inactivation and gene transcription. Histone demethylase catalyzes the removal of methyl groups on lysine or arginine residues of histones. Two kinds of histone lysine demethylases have been identified, including lysine specific demethylase 1 and Jumonji C (JmjC) domain family proteins. These histone demethylases are involved in the regulation of gene expression. Histone modification is a dynamic process, and the imbalance of histone methylation has been linked to cancers. Therefore, histone demethylases may represent a new target for anti-cancer therapy.

JMJD2A is an enzyme that in humans is encoded by the *KDM4A* gene. This gene is a member of the Jumonji domain 2 (JMJD2) families and encodes a protein with a JmjN domain, a JmjC domain, a JD2H domain, two TUDOR domains, and two PHD-type zinc fingers. This nuclear protein functions as a trimethylation-specific demethylase, converting specific trimethylated histone residues to the dimethylated form, and as a transcriptional repressor. JMJD2A is widely expressed in human tissues and cell lines, and high endogenous expression of JMJD2A mRNA was found in several cell types,

JMJD2A can be expressed by bacteria such as *E. coli*. BL21 (DE) is *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$. This is one of the commonly used strains of *E. coli* in research, because this strain and its derivatives have a vigorous growth rate. Transformed plasmids containing T7 promoter driven expression are

repressed until IPTG induction of T7 RNA polymerase from a lac promoter. It is derived from B834 by transducing to Met$^+$. To transform *KDM4A* gene that encode JMJD2A enzyme to *E. coli*, a plasmid is needed.

Plasmid PGeX4T-2



Transformation is the genetic alteration of a cell resulting from the uptake, genomic incorporation, and expression of foreign genetic material (DNA). Because transformation usually produces a mixture of rare transformed cells and abundant non-transformed cells, a method is needed to identify the cells that have acquired the plasmid. Plasmids used in transformation experiments will usually also contain a gene giving resistance to an antibiotic that the intended recipient strain of bacteria is sensitive to. Cells able to grow on media containing this antibiotic will have been transformed by the plasmid, as cells lacking the plasmid will be unable to grow.

To separate protein that has been expressed, SDS-Page is one of the promising techniques. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS gel electrophoresis of samples having identical charge per unit mass due to binding of SDS results in fractionation by size

Purification of protein can be done using affinity chromatography method. Affinity chromatography is a method of separating biochemical mixtures, based on a highly specific biological interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Proteins with a known affinity are protein tagged in order to aid their purification. Tags include glutathione-S-transferase tags, have an affinity for nickel or cobalt ions which are Coordinate covalent bond coordinated with a chelator for the purposes of solid medium entrapment. For elution, an excess amount of a compound able to act as a metal ion ligand, such as imidazole, is used. GST has an affinity for glutathione-commercially available immobilized as glutathione agarose. For elution, excess glutathione is used to displace the tagged protein.

Another purification method of protein is using fluorescence spectroscopy. Fluorescence spectroscopy is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light of a lower energy, typically, but not necessarily, visible light. At low concentrations the fluorescence intensity will generally be proportional to the concentration of the fluorophore.

2. Methods

	Procedures	Observation/explanation
	Transformation	
Day 1	Competent cells ↓ • Thaw the competent cells on ice • Add 1 µL plasmid PGEX4T-2 • Gently mix the competent cells with plasmid • Heat shock for 30 second • Chill on ice for 2 minutes • Add 180 µL of room temperature media • Incubate at 37°C for 1 hour • Spread onto the appropriate solid medium • Incubate overnight at 37°C Cell culture	After incubation for about 17 hours, there was no colony on medium. Perhaps, it's caused by JMJD2A gene is more difficult to be transformed. So, for the next step, I used another colony which has successfully been transformed with JMJD2A gene.

	LB medium and Buffer Preparation	
Day 2	LB Medium (1L) 10 g tryptone, 5 g yeast extract, and 20 g NaCl ↓ • Add 950 ml MilliQ H_2O, mix the solution • Adjust pH to be about 7 • Add MilliQ H_2O up to 1 L • Sterilize the medium LB Medium	LB Medium was sterilized by autoclave. Then was kept in the refrigerator.
	Bind/Wash Buffer (200 mL) 10xPBS 20 mL ↓ • Add 180 mL MilliQ H_2O • Gently mix the solution Wash buffer	Compositions of 1xPBS: 8.1 mM Sodium Phosphate 1.47 mM Potassium Phosphate 137 mM NaCl 2.68 mM KCl
	Elution Buffer (40 mL) 50 mM Tris-HCl, 10 mM Glutathione (pH 8.0) 1.333 mL Tris-HCl 1.5 M, 123 mg Glutathione ↓ • add 34 mL MilliQ H_2O • Gently mix the solution • Adjust pH to be 8.0 • Add MilliQ H_2O up to 40 mL Elution buffer	
Day 2	Assay Buffer (100 mL) 50 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, 10% (w/v) glycerol (pH 7,5) 3.333 mL Tris-HCl ↓ • add 2.5 mL NaCl 4M and 10 g glycerol • add 65 mL MilliQ H_2O • Gently mix the solution, adjust pH to be 7.5 • Add MilliQ H_2O up to 100 mL Assay buffer	All buffers (Wash buffer, elution buffer, and assay buffer were sterilized by filtration, then were kept in the cold temperature)

	Up-scale cell culture LB medium (5 mL) ↓ • add antibiotic • inoculate a single colony from solid medium • Incubate overnight at 37°C Cell culture	In liquid medium (LB), the growth of the cells could be observed by its turbidity
Day 3	**IPTG Induction**	
	1 L LB Medium ↓ • add 1 mL ampicillin to the LB medium • Dilute 1 mL overnight culture • Grow with vigorous shaking at 37^{o}C • Leave for hours • Check the OD at 600 nm • Add IPTG until final concentration = 1 mM • Shake vigorously overnight Cell Culture	• Checking turbidity Blank : LB medium OD culture : 0.95 (0.1-1.0) • Sampling before and after IPTG induction
	Gel Preparation (For SDS Page)	
	Preparation of SDS-PAGE running gel ↓ • Wash glass plates, wipe down with ethanol • Set up templates, long plate goes in back • Add running buffer compositions • Let polymerize • Make stacking gel in tube • Add stacking gel • Place comb between glass plates Gel's ready for SDS page	Compositions of Running buffer: • 45 mL MilliQ H_2O • 5.3 acrylamide • 4.0 mL Tris-HCl 1.5M, pH 8.8 • 160 µL SDS 10% • 53 µL APS 20% • 8 µL TEMED Compositions of Stacking buffer: • 45 mL MilliQ H_2O • 5.3 acrylamide • 4.0 mL Tris-HCl 1.5M, pH 8.8 • 160 µL SDS 10% • 53 µL APS 20% • 8 µL TEMED

Day 4

Glutathione Chromatography Column

1L Cell culture ↓ • Centrifuge at 4000xg, 20 menit, 4^{o}C • Decant supernatant and allow cell pellet to drain completely • Resuspend cell in bind/wash buffer • Sonicate the sample in a tube on ice, 5 times, 1 minute each • Centrifuge the sample • Filter the sample after centrifugation • Purify using glutathione column crhomatography Protein fraction	Sampling the supernatant after centrifugation, sample fraction within chromatography and also washing buffer fraction Before sonication, protease inhibitor was added to inhibit the protease activity in the cell (Protect the protein). Protease inhibitor that was used can inhibit a broad spectrum of serine and cystein proteases

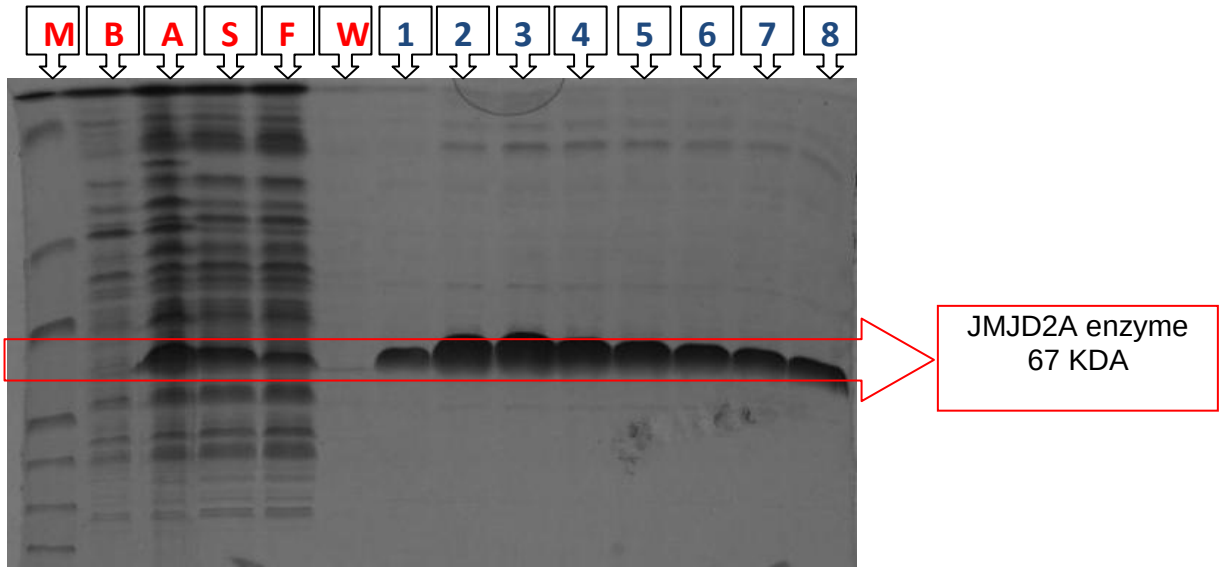
SDS-PAGE

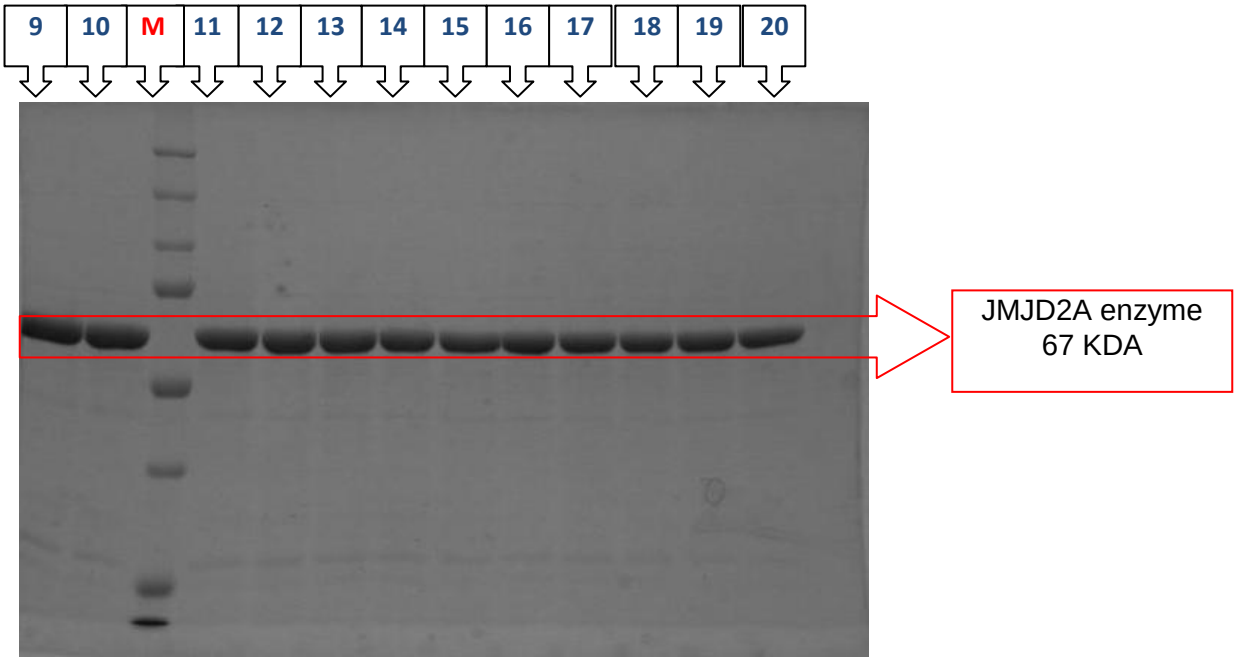
<u>Preparation of Sample</u> Protein samples ↓ • add loading buffer • add dye • boil 3 minutes before loading gel Samples for SDS-PAGE <u>SDS-PAGE running</u> SDS-PAGE instrument ↓ • set the instrument, fiil up with SDS solution • fill up the wells with samples and marker • run gel at 140V until dye is in the same line • increase voltage (250V) until dye is just off • decant SDS solution • transfer gel on to dye solution • shake and wait for 1 hour • change the dye, shake again overnight Protein's sizes are known	

	<u>Protein concentration determination</u> Sample solution ↓ • check the OD • determine proteins concentration protein concentration	avoid bubble in the cuvet because it can interfere with the readings ε_{280} JMJD2A = 116660 $Absorbance_{280}$ = 0.475 (4 fold) $Concentration = \frac{Absorbance_{280}}{\varepsilon_{280}}$
Day 5	**Fluorescence Spectroscopy**	
	Check enzyme's fluorescence activity using probe by fluorescence spectroscopy. a. Buffer only b. Enzyme only (HDAC) c. Probe only d. Probe and enzyme	Composition: Protein : 2.3 μ L Probe : 10 μ L Mg^{2+} : 1 μ L Buffer : 86.7

3. Result and Discussion

a. SDS-PAGE





M : Marker	F : Sample Fraction
B : Before IPTG Induction	W : Washing Fraction
A : After IPTG Induction	1 – 20 : Protein Sample
S : Supernatant	

SDS-Page result showed that the JMJD2A protein has been detected. The size of this enzyme is 67 KDa (red arrow). In the samples before IPTG induction, after IPTG induction, and supernatant, there are so many bands. It determined that before purification using chromatography column, there were so many proteins, not only JMJD2A.

Well 1-20 showed relatively similar band which have size around 67 KDa. But, there were also several bands besides JMJD2A protein. There are some factors that can cause protein impurities. The less effectiveness of protease inhibitor activity that added before sonication might be one of the possibilities. After extraction process soluble proteins will be in the solvent, and can be separated from cell membranes, DNA etc. by centrifugation. The extraction process also extracts proteases, which will start digesting the proteins in the solution. If the protein is sensitive to proteolysis, it is usually desirable to proceed quickly,

Keeping the extract cooled during the experiment can slow down proteolysis process. Might be, during experiment, the sample was accidentally heated (for example by improper holding of the tube), so that the protease that's still remained degraded this protein.

Another possibility is some protease might be still remained in the samples, or the protein has been degraded naturally inside the cell.

b. Protein Concentration

Protein concentration can be determined by using this equation:

$$[\text{Protein}] = \frac{\text{Absorbance}_{280}}{\varepsilon_{280}}$$

The absorbance of JMJD2A protein at 280 nm is 0.475 (four fold), so, the concentration of this protein that have successfully been extracted is:

$$[\text{JMJD2A}] = \frac{0.475 \times 4}{116660} = \mathbf{16.3\ \mu M}$$

So, based on the calculation, the concentration of JMJD2A is 16.3 μ M

The relationship of absorbance to protein concentration is linear. Because different proteins and nucleic acids have widely varying absorption characteristics there may be considerable error. Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm

c. Fluorescence Intensity

The protein sample is banded to the probes and then checked for its fluorescence intensity. But, there was no probe for JMJD2A. So, the data below show the fluorescence intensity of another protein (HDAC8).

Buffer only	843
Protein (HDAC8) only	764
Probe only	56036
HDAC8 and probe	71426

The data above showed that the fluorescence intensity of probe and HDAC8 (71426) is higher than probe only (56036). There was an increment, but it's not so high. Perhaps the probe was not so reactive with the protein.

Fluorescence intensity is the measure of fluorescence from a sample. A beam of light excites the molecules of certain compounds and causes emission at a lower energy. The light is typically from the visible portion of the UV-Visible spectrum.

4. Conclusion

Histone demethylase JMJD2A protein has successfully been produced by *E. coli* BL21 (DE) after transformation of *KDM4A* gene using plasmid PGeX4T-2. Sonication method was used to extract the proteins and then the proteins were purified by glutathione column affinity chromatography. SDS-PAGE confirmed this enzyme which has 67 KDa in size. The concentration of enzyme that has been produced is 16.3 μ M. Further purification using the more effective method is needed to purify this enzyme.

Purification can be done by fluorescence spectroscopy. The observation of HDAC8 protein purification using fluorescence spectroscopy has been done. There was increment of fluorescence intensity of probe after banded to HDAC8 protein (71426).

5. References

Alberts, Bruce; *et al.* 2002. *Molecular Biology of the Cell*. New York: Garland Science. p. G:35

Chen, Z. et al. 2007. *Structural basis of the recognition of a methylated histone tail by JMJD2A.* Proc Natl Acad Sci *USA* 104, 10818-23.

Couture, J.F, *et al*. 2007. *Specificity and mechanism of JMJD2A, a trimethyllysine-specific histone demethylase*. Nature Structural & Molecular Biology 14, 689 – 695.

Lee, J. et al. 2008. *Distinct binding modes specify the recognition of methylated histones H3K4 and H4K20 by JMJD2A-tudor.* Nat Struct Mol Biol 15, 109-11.

Shapiro AL, Viñuela E, Maizel JV Jr. 1967. *Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels*. *Biochem Biophys Res Commun.* 28 (5): 815–820.

Shin, S. and Janknecht, R. 2007 *Biochem Biophys Res Commun* 359, 742-6.

Tian Xiaoqing and Fang Jingyuan. 2007. *Current Perspectives on Histone Demethylases.* China : Acta Biochimica et Biophysica Sinica, 39(2): 81–88 CN 31-1940/Q.

Voet and Voet. 1995. *Biochemistry*. John Wiley and Sons

Project-based training report:

Chaperone function of SIB1 FKBP22, a peptidyl prolyl *cis-trans* isomerase from the psychrotrophic bacterium.

Sintawee Sulaiman

M1 student, Noji laboratory

FKBP22 is FK506-binding protein 22 from psychrotrophic bacterium *Shewanella* sp. SIB1. This bacteria growth in the low temperature and the content of the protein at 4 ^{o}C in the cell was higher than 20 ^{o}C. This study indicates that the FKBP22 is important for the adaptation of this bacterium to survive at the low temperature (Suzuki *et al.*, 2004). This protein is a homodimeric with peptidyl prolyl *cis-trans* isomerase (PPIase) for both peptide and protein substrates (Suzuki *et al*., 2005). The enzyme catalyzes the *cis-trans* isomerization of the peptide bonds N-terminal of the proline residues (Schiene and Fischer). FKBP22 is a member of the macrophage infectivity potentiator (MIP)-like FKBP subfamily proteins which showing amino acid sequence identities of 56% to *Escherichia coli* FKBP22 (Rahfeld *et al.*, 1996) and 43% to *E.coli* FkpA (Horne and Young, 1995). Each monomer composes of 205 amino acid residues and assembles to the V-shape by interacting with each other at the N-termini. V-shape structure is important for the efficient binding to a protein substrate in order to express the chaperone function. Additionally, the monomer can exhibited PPIase activity for both peptide and protein substrates but the activity for the protein substrate was reduced five- to six-fold if compared to the homodimeric structure reaction (Budiman *et al.*, 2009).

The chaperone function of FKBP22 was observed by performing the aggregation prevention of insulin and binding to folding intermediate proteins in the α-Lactalbumin. Insulin is the hormone that functions by hexamer form. Disulfide bonds are important for holding the structure of insulin andα-Lactalbumin to function. In order to observe FKBP22 roles, dithiothreitol (DTT) was used for reducing the disulfide bonds in insulin andα-Lactalbumin. Insulin would be aggregated if added the DTT whileα-Lactalbumin would be transformed to the intermediate structure which could be observed the binding between FKBP22 and intermediate structure ofα-Lactalbumin.

Materials and methods

From the *E.coli* BL21(DE3) cells culture in 1 litre, these cells contain FKBP22 genes (with His-tag) in the pET28a for overproduction. Cells were centrifuged and harvested

into 80 ml. Sonication was required for breaking the cell and retrieved the protein solution.

Purification

Ni^{2+}-chelating column was used for separating the target protein that tagged by 6 histidine which can strongly bind to Ni^{2+}.FKBP22 carried His-tag that would bind in the Ni^{2+} column and other unspecific proteins would be eluted. For affinity chromatography, the buffer contained gradual concentration of imidazole. The high concentration of imidazole would be used to release FKBP22 from Ni^{2+} column. The concentration of imidazole would gradually increase for purifying. Therefore, the last fractions of the eluted solution were expected to contain the most purified protein (Figure 1).

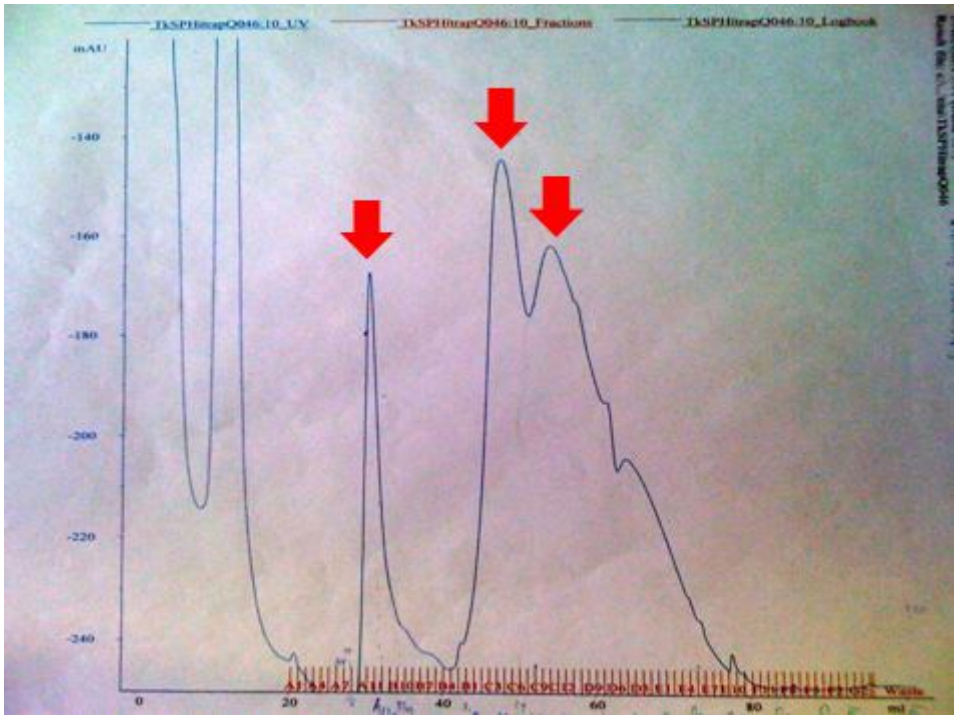


Figure 1: Graph plot from affinity chromatography (red arrow represent the selected peaks)

After selected fraction of the protein from the UV absorption intensity graph plot, SDS page was required for checking the purification of the protein (Figure 2). The molecular weight of FKBP22 could be estimated by comparing to the marker. This time, low molecular weight marker was used which indicated the size of FKBP22 is around 28 kDa.

Figure 2: SDS page from affinity chromatography

The target protein (FKBP22) would be taken to process dialysis step (eluted imidazole) for overnight.

Gel filtration method was applied to obtain more purified protein. This step would separate the protein by size. The protein solution from the affinity chromatography would be prepared for gel filtration method. The solution would be previously filtered by using centricon (Micropore). The low molecular weight molecules (less than 10 kDa) will pass through the filter of this tube and the higher molecular weight molecule will be filtered on the upper of this tube. In this case, FKBP22 solution would be stored in the upper part of the centricon. After that, the FKBP22 solution was injected to the gel column. This column will separate the molecules by size (Figure 3). The highest molecular weight molecules will be firstly eluted. In this case, the fractions of FKBP22 solution would be selected based on the graph plot (UV intensity) and then, the representative fractions will be checked by SDS page (Figure 4). Finally, the FKBP22 solution would be processed in the dialysis step for overnight.

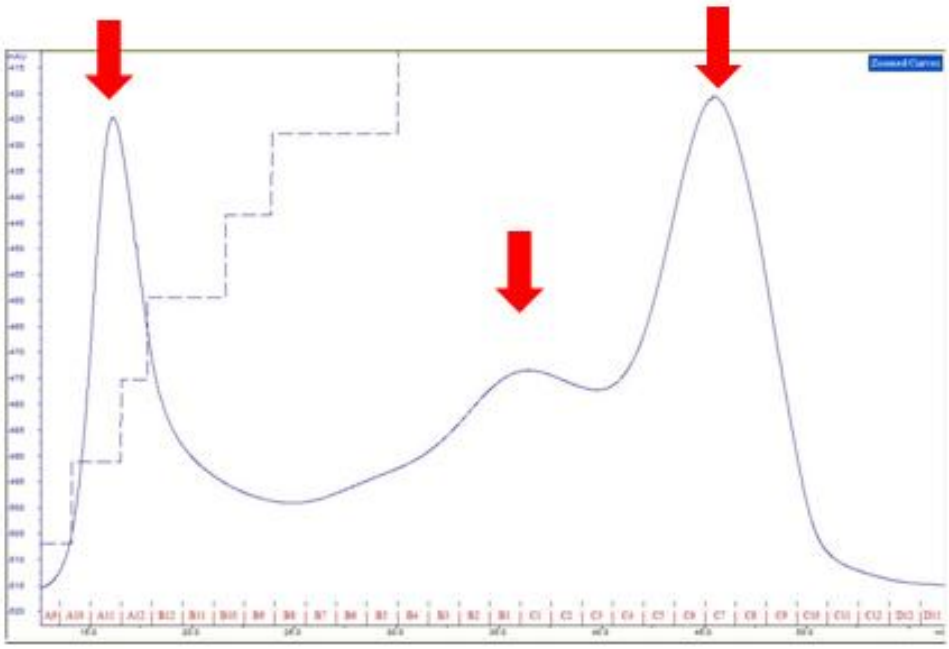


Figure 3: Graph plot from gel filtration (red arrow represents the selected peaks)

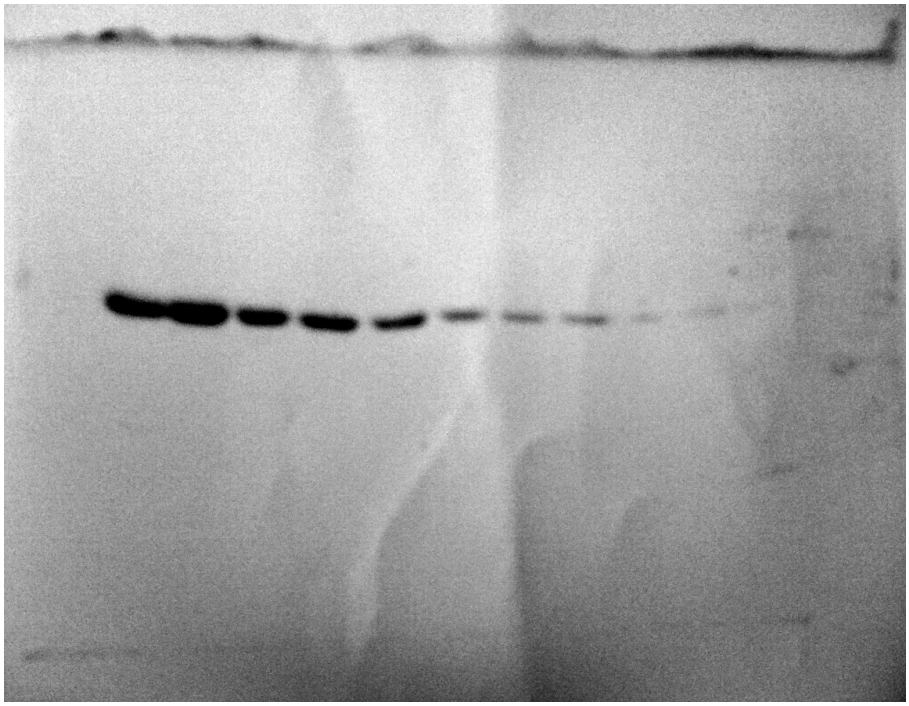


Figure 4: SDS page from gel filtration

In order to measure the concentration of the protein, spectrophotometer was applied to measure the UV absorbance at 280 nm. The absorbance value of 28 ml. protein solution

was 0.372 at Ab_{280} which revealed the concentration of the protein was about 0.5 mg/ml. Therefore, the yield of protein from 1 litre of harvested cells was 14 mg. For the next step, this protein solution would be concentrated by using centricon into 1 ml. The absorbance value was 8.78 at Ab_{280} which the protein concentration was 12.9 mg/ml.

Chaperone function would be observed via the fluorescence intensity which derived from the aggregation of insulin. Deformed insulin structure was prepared by adding DTT. Light scattering at 465 nm. was applied for measuring the fluorescence intensity which corresponding to the aggregation of insulin. The two concentration of FKBP22 (0.1 and 0.5 mg./ml.) was tested to prevent the aggregation of insulin. This step observed for 4800 seconds (Figure 5).

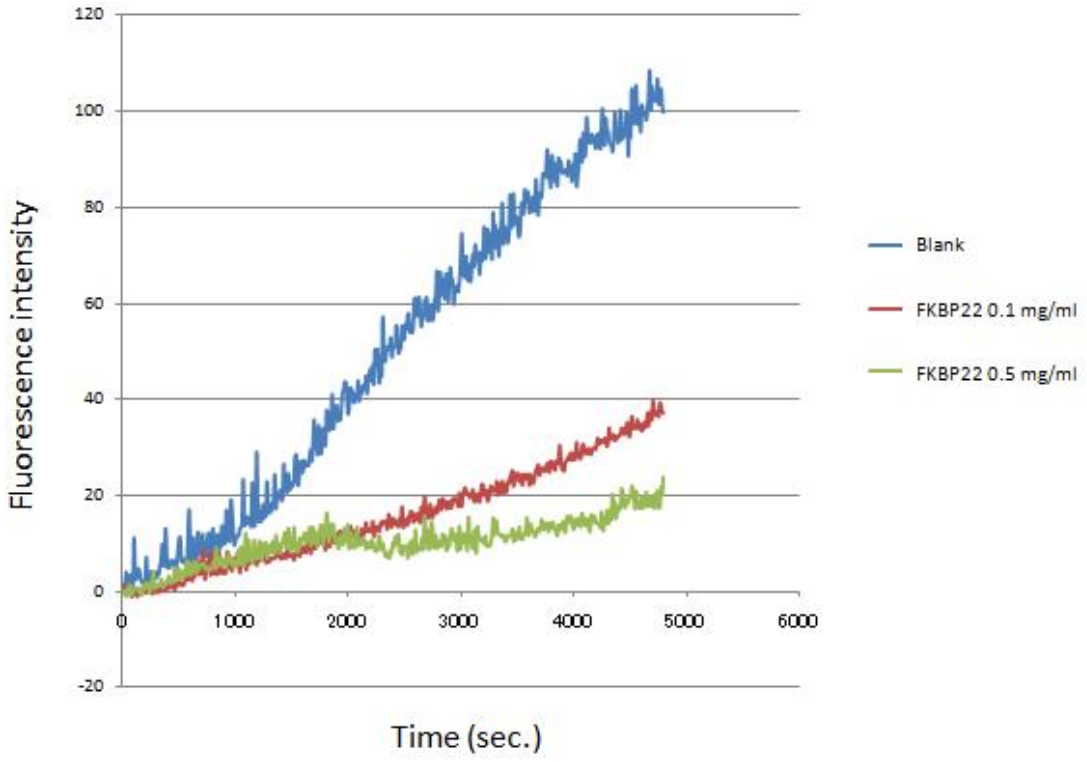


Figure 5: Fluorescence intensity from the insulin aggregation

Binding analysis by using surface plasmon resonance (Biacore)

FKBP22 was observed for the binding with the intermediate protein. This step required α-Lactalbumin as a substrate. The reduced α-Lactalbumin was prepared by using DTT which would change the conformation of α-Lactalbumin into the intermediate stage. Due to the function of FKBP22, this protein will bind to the intermediate stage of the protein folding, thus the binding step of FKBP22 could be researched via the intermediate form of α-Lactalbumin.

To examine the binding affinity of FKBP22 to the reduced α-Lactalbumin, surface plasmon resonance has been effectively used for this purpose. Upon the measurement of response unit (RU) of Biacore analysis, the monitored response unit is corresponding to the concentration of analyte and its binding affinity to the immobilized molecule.

In order to prepare this method, FKBP22 would be previously fixed on the surface of chip and the various concentration of analyte (α-Lactalbumin) would be injected into the machine to observe the binding (Figure 6). However, when higher concentrations of analyte have been injected onto the chip, the RU may not be proportionally corresponding to the concentration of analyte. This may be implied that the immobilized molecules on the chip have been more occupied and saturated with the analyte. In these points, the association or dissociation constants might be possible to be determined from the graph of RU and RU/C.

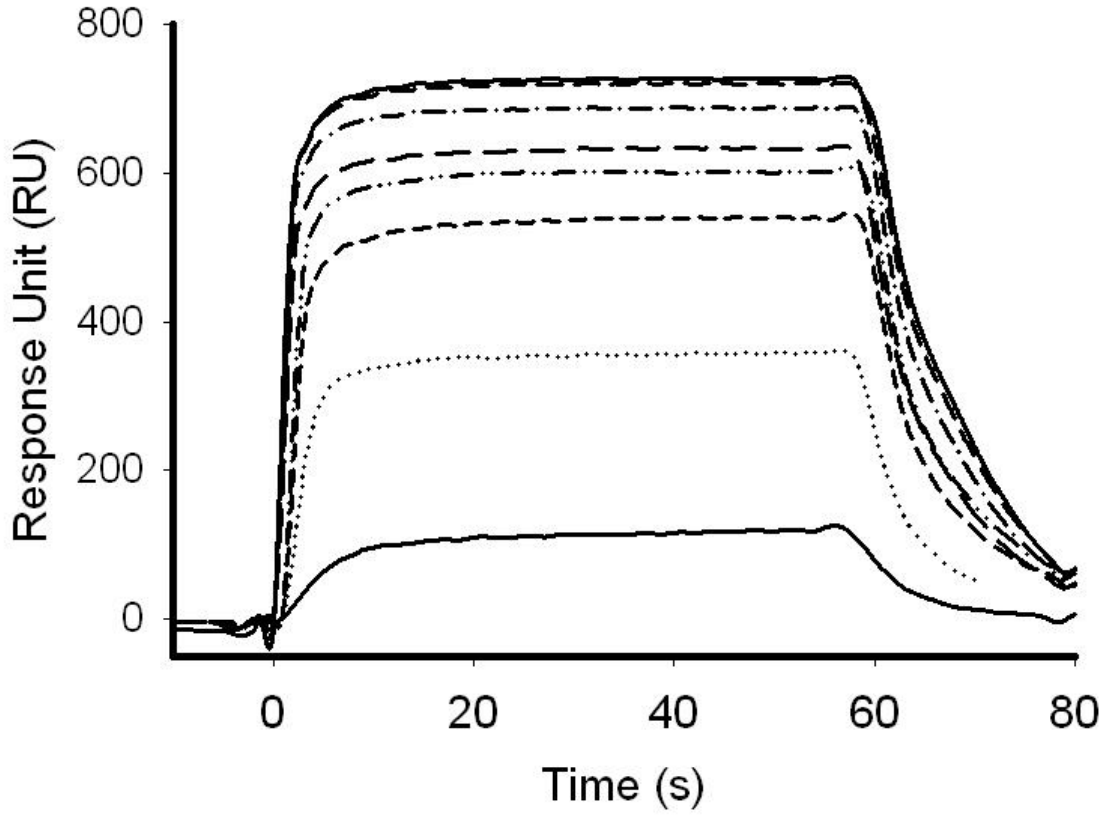


Figure 6: Binding activity between FKBP22 and α-Lactalbumin according to the response unit (RU) from Biacore machine

Result and discussion

For the chaperone function of FKBP22, this protein can significantly prevent the aggregation of deformed insulin according to Figure 5. Blank solution revealed the aggregation by fluorescence intensity by 100 while applied FKBP22 0.1 and 0.5 mg/ml, the fluorescence intensity decreased into 40 and 20 respectively. Therefore, FKBP22 can eliminate the aggregation of insulin by reducing the S-S bond forming of the insulin chains due to the chaperone protein-protein complex are formed.

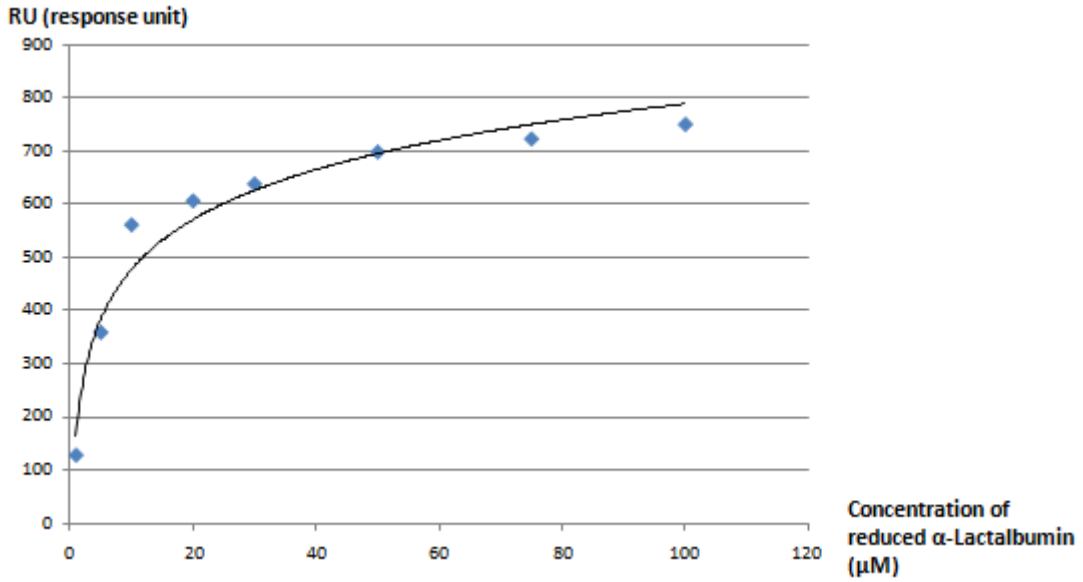


Figure 7: Saturated binding between FKBP22 and reduced α-Lactalbumin

FKBP22 can function with the intermediate form of protein. The reduced α-Lactalbumin were the intermediate form of α-Lactalbumin protein that can interact with FKBP22. When the reduced α-Lactalbumin was injected onto the chip from concentration of 1-100 μM, increase of RU has been detected from 127.5 to 749.5. This result implies that α-Lactalbumin binds to the FKBP22 and starts to be saturated for binding on the chip. The plots of the equilibrium binding responses as a function of the concentration of α-Lactalbumin gave a saturation curve as shown in Figure 7. These plots well fit a single binding site affinity model and the K_D value was determined to be 5.2 μM.

Reference

Budiman, C., Bando, K., Angkawidjaja, C., Koga, Y., Takano, K and Kanaya, S., 2009, "Engineering of monomeric FK506-binding protein 22 with peptidyl cis-trans isomerase. Importance of a V-shaped dimeric structure for binding to protein substrate", **Federation of European Biochemical Society**, Vol. 276, pp. 4091-4101.

Horne, S.M. and Young, K.D., 1995, "*Escherichia coli* and other species of the enterobacteriaceae encode a protein similar to the family of Mip-like FK506-binding proteins", **Archives of Microbiology**, Vol. 163, pp. 357-365.

Rahfeld, J.U., Rücknagel, K.P., Stoller, G, Horne, S.M., Schierhorn, A., Young, K.D. and Fischer, G., 1996, "Isolation and Amino Acid Sequence of a new 22-kda FKBP-like peptidyl-prolyl cis/trans-Isomerase of *Escherichia coli* similarity to mip-like proteins of pathogenic bacteria", **The Journal of Biological Chemistry**, Vol. 271, pp. 22130-22138.

Schiene, C. and Fischer, G., 2000, "Enzymes that catalyse the restructuring of proteins", **Current Opinion in Structural Biology**, Vol. 10, pp. 40-45.

Suzuki, Y., Haruki, M., Takano, K., Morikawa, M. and Kanaya, S., 2004, "Possible involvement of an FKBP family member protein from a psychrotrophic bacterium Shewanella sp. SIB1 in cold-adaptation", **Federation of European Biochemical Society.** Vol. 271, pp. 1372-1381.

Suzuki, Y., Win, O.Y., Koga, Y., Takano, K. and Kanaya, S., 2005, "Binding analysis of a psychrotrophic FKBP22 to a folding intermediate of protein using surface plasmon resonance", **Federation of European Biochemical Society Letters**, Vol. 579, pp. 5781-5784.