LAPORAN

Hibah Kompetitif Penelitian Untuk Publikasi Internasional Batch I
Tahun Anggaran 2009

Isolation and Identification of Java Ras Amniotic Membrane SLPI Gene

Penulis:
Dr. Elly Munadziroh,drg.MS
Dr. Ni Nyoman Tri Puspaningsih,Dra.Msi
Prof. Soetjipto,dr. MS., Ph.D
Prof.Dr. Moh. Rubianto, drg., MS., Sp.Perio

Dibiayai oleh Direktorat Jenderal Pendidikan Tinggi, Departemen Pendidikan Nasional, sesuai dengan Surat Perjanjian Pelaksanaan Hibah Kompetitif Penelitian Untuk Publikasi Internasional Nomor: 435/SP2H/PP/DP@M/VI/2009, Tanggal 30 Juni 2009

Universitas Airlangga
Desember 2009

HALAMAN PENGESAHAN

1. Judul Penelitian :

   Isolation and Identification of Java Ras Amniotic Membrane SLPI Gene

2. Ketua Peneliti :
   a. Nama Lengkap : Dr. Elly Munadziroh, drg., MS.
   b. Jenis Kelamin : Perempuan
   c. NIP : 131 653 430
   d. Pangkat/Golongan : Pembina/IVa
   e. Jabatan : Dosen
   f. Bidang Keahlian : Dental Material
   g. Fakultas/Jurusan : Kedokteran Gigi
   h. Perguruan Tinggi : Universitas Airlangga

No	NAMA PENELITI	BIDANG KEAHLIAN	FAKULTAS/JURUSAN	PERGURUAN TINGGI
1	Elly Munadziroh	Dental material	Kedokteran Gigi	Universitas Airlangga
2	Ni Nyoman Tripuspaningsih		Sains dan Teknologi	Universitas Airlangga
3	Soetjipto	Biokimia	Kedokteran	Universitas Airlangga
4	Rubianto	Periodonsia	Kedokteran Gigi	Universitas Airlangga

3. Pendanaan dan Jangka waktu Penelitian
   a. Jangka Waktu yang diusulkan : 7 bulan
   b. Biaya yang diusulkan : Rp. 200.000.000,00 (dua ratus juta rupiah)

4. Biaya yang disetujui tahun 2009 : Rp 30.000.000,00 (tiga puluh juta rupiah)

Mengetahui
Komisi Pemberdayaan Wilayah Pesisir
dan Kelautan LPPM Unair

r.Moch.Amin Alamsjah,M.Si.,PhD .
NIP. 132 129 663

Surabaya, 26 Oktober 2009
Ketua Peneliti,

Dr. Elly Munadziroh, drg., MS.
NIP.131 653 430

Mengetahui
Ketua Lembaga Penelitian dan Pengabdian Kepada Masyarakat

Prof.Dr. Bambang Sektiari L, DEA, drh.
NIP. 131 837 004

SISTEMATIKA LAPORAN EKSEKUTIF
HIBAH KOMPETITIF PENELITIAN UNTUK PUBLIKASI INTERNASIONAL BATCH I

Isolation and Identification of Java Ras Amniotic Membrane SLPI Gene

I. PERMASALAHAN DAN TUJUAN PENELITIAN

Permasalahan

Respons penyembuhan luka yang lambat pada tikus yang kekurangan SLPI akan menyebabkan kelambatan sintesis matriks dan deposisi kolagen. Kegagalan penyembuhan sering kali disebabkan karena kelebihan enzim proteolisis dan infeksi bakteri.

Tujuan penelitian ini untuk mengkloning SLPI membran amnion dan menentukan karakterisasi SLPI membran amnion dari ras jawa

II. INOVASI IPTEKS

Protein SLPI didapatkan dari membran amnion yang selama ini terbuang begitu janin telah dilahirkan

III. KONTRIBUSI TERHADAP PEMBANGUNAN

Tingkat produktifitas manusia senantiasa dituntut untuk kemajuan bangsa seiring dengan tuntutan meningkatnya tingkat harapan hidup manusia. Untuk tujuan tersebut perbaikan kualitas hidup ditingkatkan juga angka kesakitan diturunkan dan waktu kesakitan diusahakan diperpendek. Kesakitan yang dimaksudkan disini, diantaranya adalah terjadinya luka pada rongga mulut yang membutuhkan waktu untuk proses penyembuhan luka ini. Penyembuhan luka adalah usaha tubuh untuk memperbaiki kerusakan jaringan. Tubuh berusaha menormalkan kembali semua kondisi abnormal akibat luka dengan proses penyembuhan. Penyembuhan luka merupakan proses biologis yang kompleks yang melibatkan sejumlah besar sel, sitokin, faktor pertumbuhan, protease dan komponen matriks ekstraselular yang berperan dalam restorasi integritas jaringan yang rusak.

Dengan diketahui struktur dan fungsi yang meliputi mekanisme inhibisi dari SLPI dan Protease selanjutnya dapat diketahui percepatan penyembuhan luka dan dengan mengetahui struktur protein SLPI membrana amnion rekombinan ini dapat mengungkap

teori percepatan penyembuhan luka sehingga aplikasi biomaterial dari protein SLPI membrana amnion rekombinan dapat dieksplorasi

Terjadinya luka pada rongga mulut sangat menyiksa, rasa tidak nyaman dan kesulitan untuk makan dan minum, menyebabkan tingkat produktifitas akan menurun. Oleh karenanya dibutuhkan suatu bahan untuk mempercepat penyembuhan luka yang optimal.. Untuk mandapatkan hasil tersebut diperlukan langkah-langkah untuk analisis struktur dan fungsi dari bahan SLPI membrana amnion rekombinan yang telah dihasilkan. Sehingga bahan yang telah dihasilkan nantinya dapat ditingkatkan kualitasnya sehingga dapat mencapai hasil yang maksimum.

Dengan demikian, penelitian ini akan sangat berguna untuk pengembangan bahan SLPI membrana amnion rekombinan sehingga nantinya didapatkan hasil suatu bahan biomaterial yang dapat mempercepat penyembuhan luka secara optimal sehingga tingkat produktifitas manusia akan meningkat

IV. MANFAAT BAGI INSTITUSI

Penelitian ini dilakukan di Universitas Airlangga dilaboratorium Lembaga Penyakit Tropis, di laboratorium biologi molekuler FKH, laboratorium kimia Fakultas Sains dan teknologi, dengan mensinergikan dari berbagai laboratorium maka keterlibatan sumberdaya manusia dari masing-masing fakultas akan nyata.

V.PUBLIKASI

Rencana akan diterbitkan di Jurnal Dental Research

Manuscript Approved (09-0628).

To help offset the cost of the online manuscript submission system, the IADR and AADR Boards and Councils have approved a manuscript submission fee of $45, effective April, 2004. As with other publishing-related fees, the Editor has the authority to waive the submission fee if it would prevent an author from submitting.

Please click on the "Click here to make payment" button below to make a submission payment via Verisign. You will be directed to a secure Verisign web site that will gather your payment information.

Surabaya, 13 October 2009

To : Mr. Anthony J. Smith
JDR

Dear Mr. Smith,

Firstly I'd like to introduce myself; my name is Elly Munadziroh from Faculty of Dentistry, Airlangga University, Surabaya-Indonesia. As a leading author and representing my co-authors, we would like to submit a research article to be published in your Journal of Dental Research (JDR). Should there be any editing, corrections, etc. please let us know. Thank you for your attention.

Regards,
Dr. Elly Munadziroh, drg., MS.
Faculty of Dentistry, Airlangga University
Surabaya-Indonesia.

Isolation and Identification of Java Ras Amniotic Membrane SLPI Gene

E. Munadziroh*, N.N. Tripuspaningsih, Soetjipto*** M. Rubianto******
*Department of Dental Material and Technology Faculty of Dentistry, Airlangga University
** Department Biochemistry Faculty of Mathematics and Sciences, Airlangga University
***Departement of Biochemistry, Faculty of Medicine, Airlangga University
**** Departement of Periodontic, Faculty of Dentistry, Airlangga University

ABSTRACT

Secretory leukocite protease inhibitor (SLPI) is a protein found in amniotic membrane that involve in tissue repair and oral wound healing process. The oral wound healing responses are impaired in the SLPI sufficient mice and matrix synthesis and collagen deposition are delayed. Impaired healing states are characterized by excessive proteolysis and often bacterial infection. SLPI is a protein found in secretions such as whole saliva, seminal fluid, cervical mucus, as in secretions from the nose and bronchi, amniotic fluid and amniotic membrane etc.. ***The objective*** *of this research is to isolated and cloning the SLPI from human amniotic membrane and determined the expression of SLPI extracted from human amniotic membrane from Java ras.* ***Methods:*** *cDNA isolated from human amniotic membrane was amplified by reverse transcription polymerase chain reaction (RT-PCR). After the amplified, fragmen were cloned into the expression pET 101/DTOPO Vector and transformed to BL-21 Star. The insert position, size and the reading frame were right by PCR..* ***Result:*** *Through sequence analyses, SLPI cDNA was 495 nucleotide in length with a predicted molecular mass of about 12 kDa (the data was registered in genbank (htpp://www.ncbi.nib.gov) with accession number EU116331).* ***Conclusion:*** *It is demonstrated that human SLPI are highly conserved in sequence content as compared to the human SLPI from gene bank.*

Key words*: Secretory leukocyte protease inhibitor, amniotic membrane, cloning. Recombinant, wound healing*

Correspondence: Elly Munadziroh, c/o: Department of Dental Material and Technology Faculty of Dentistry Airlangga University. Jln. Mayjen Prof.Moestopo 47 Surabaya 60132, Indonesia. Email : emunadziroh@yahoo.com

INTRODUCTION

Gingival recession can occur at all age and vary from 88% (>65 years) and 50% (18-65 years). The treatment of gingival injury due to gingival traction toward coronal which might cause tension so gingival is very thin and laceration occur. Surgery is one of gingival recessive treatments. This treatment makes an injury in gingival. Sterile condition or free from bacteria is difficult to be achieved in oral cavity, therefore, various methods are developed in order to obtain more faster injury healing process

One kind of gingival recession treatment is through surgery which will cause a wound. Sterile condition or free of bacteria is hard to achieve in the oral cavity. Many methods were developed to faster wound healing. Later complication could happen if the wound won't heal caused by bacterial contamination which may lead to cellulites, abscess, or osteomyelitis. Today many wound treatment in gingival recession with surgical procedure without active material application for healing; the wound was only protected with periodontal pack.

At present, the concept of treatment and recovery has been simultaneously developed with biomaterial development. The use of natural material has been considered due to the component which supports the treatment and recovery. One of natural materials which expected to be able to cure the injury more rapidly is amniotic membrane. This material has been proved successfully in burn injury treatment as well as in ophthalmologic field. The active material of amniotic membrane which is expected to be influential on injury healing process is secretory leukocyte protease inhibitor (SLPI).

According to Ashcroft et al (2000), SLPI reduce Nuclear Factor-kB (NFkB) a transcription factor which can induce inflammatory gene activation. Cell signaling molecule would be activated by inflammation process, kinase protein would be activated and Mitogen-activated protein kinase (MAPK) would be stimulated by later process of kappa B inhibitory (IkB) would be activated and degraded into NFkB excessively.

The study was done to analyze the character of amniotic membrane SLPI protein and cloning gene coded SLPI protein of amniotic membrane to obtain great amount of recombinant SLPI protein in which is used as material to accelerate gingival injury. SLPI is a protein which has some functions in wound healing such as protease inhibitor, control leucocytes activity, reducing TGF beta, anti inflammatory, anti bacterial, control intracellular enzyme, monocytes and matrix metallo proteinase (MMP) suppressor (Zhang et al, 2002).

MATERIALS AND METHODS

Character of amniotic membrane SLPI is known by performing amplification of amniotic membrane SLPI gene with 5' ACT CCT GCC TCC ACC ATG AA3' (5' ATT CGA TCA ACT GGA CTT-3') primer. This primer produces DNA fragment in position 1530 bp. Amplification was done using two-step RT-PCR kit. For cloning preparation, PCR product which had been produced was previously purified using PCR gel kit. Cloning process with pET 101/D-TOPO was done purified PCR product

therefore resulted recombinant plasmid DNA pET-ESLPI. Further, the recombinant was transformed in *E-coli* top 10.

Characterization of recombinant plasmid PET-ESLPI was done by cutting and sequencing. Cutting using EcoRV enzyme was performed. Sequencing was done by using primer 5' CAC CAT GAA CAG CGG CCT CTT CC 3' and AGC TTT CAG AGG GGA AAC GCA GGA T 3'.

RESULTS

The PCR product of SLPI amnion membrane gene was purified by Gel Extraction Kit. The purification result was inserted into pET101/D-TOPO in order to get the DNA of plasmid recombinant pET-ESLPI as shown on Figure 1

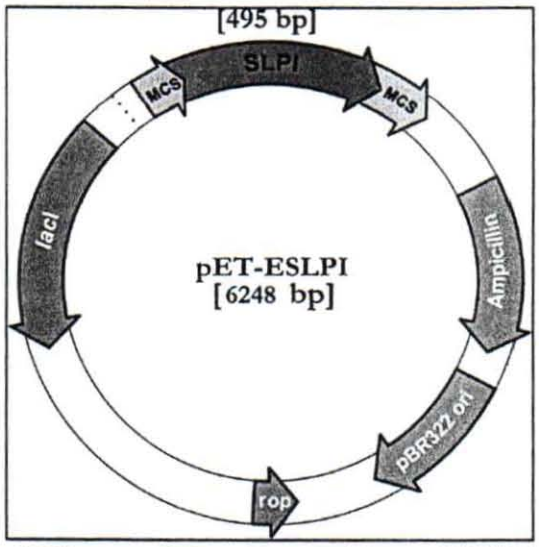

Figure 1. The DNA map of plasmid recombinant pET-ESLPI.

Transformation was done on E. coli TOP 10 and was planted into Luria Bertani (LB) solid media containing ampicillin (100 µg/ml).

The expression of SLPI amnion membrane recombinant would be seen on E. coli BL-21 star, therefore, this SLPI recombinant was transformed on E. coli BL-21 star genotype F ompT hsdSB (rBmb) gal dcm rne131 (DE3). This cell was designed to contain lambda DE3 lysogen that carry gene T7 RNA polymerase controlled by promoter lacUV5. To see the plasmid recombinant DNA before and after restriction, electrophoresis was done on gel agarose 1,5% containing ethidhium bromide.

Sequencing was done on recombinant SLPI after proven that the transformation was successful, sequencing from cloning was advantageous because whole gene could be get since the primer was not part of the gene but part of the plasmid with SLPI gene, in this case pET101/D-TOPO. Sequencing with computer

software automatically edited with PhredPhrap in the Netherlands. Sequencing recombinant plasmid on this research using primer (sense) T7 5'-TAATACGACTCACTATAGGG-3' and primer (anti sense) 5'-TAGTTATTGCTCAGCGGTGG-3'.

This research amplified signing gene SLPI using designed primer to get the whole SLPI sign gene. Primer was designed according to pET-101 D-TOPO analyzed with clone manager method.

Sequencing result of the whole DNA after analysis with clone manager software which eliminate plasmid sequence on cloning result, resulting 1 ORF and total nucleotide of 495 bp as seen on Figure 2.

```
  1 ATGAAGTCCA GCGGCCTCTT CCCCTTCCTG GTGCTGCTTG CCCTGGGAAC TCTGGCACCT
 61 TGGGCTGTGG AAGGCTCTGG AAAGTCCTTC AAAGCTGGAG TCTGTCCTCC TAAGAAATCT
121 GCCCAGTGCC TTAGATACAA GAAACCTGAG TGCCAGAGTG ACTGGCAGTG TCCAGGGAAG
181 AAGAGATGTT GTCCTGACAC TTGTGGCATC AAATGCCTGG ATCCTGTTGA CACCCCAAAC
241 CCAACAAGGA GGAAGCCTGG GAAGTGCCCA GTGACTTATG GCCAATGTTT GATGCTTAAC
301 CCCCCCAATT TCTGTGAGAT GGATGGCCAG TGCAAGCGTG ACTTGAAGTG TTGCATGGGC
361 ATGTGTGGGA AATCCTGCGT TTCCCCTGTG AAAGCTAAGG GCGAGCTCAA TTCGAAGCTT
421 GAAGGTAAGC CTATCCCTAA CCCTCTCCTC GGTCTCGATT CTACGCGTAC CGGTCATCAT
481 CACCATCACC ATTGA
```

Figure 2. Sequencing result of PCR product with forward primer against amplification product.

Sequencing result on SLPI recombinant with TOPO primer (forward) as nucleotide sequence of 495 bp, then was sequenced with gene sign SLPI in the database. The result was shown on Figure 3.

```
                    10        20        30        40        50        60        70
                     *         *         *         *         *         *         *
slpi-express ATGAAGTCCAGCGGCCTCTTCCCCTTCCTGGTGCTGCTTGCCCTGGGAACTCTGGCACCTTGGGCTGTGG
cds-stetler[ ATGAAGTCCAGCGGCCTCTTCCCCTTCCTGGTGCTGCTTGCCCTGGGAACTCTGGCACCTTGGGCTGTGG

                    80        90       100       110       120       130       140
                     *         *         *         *         *         *         *
slpi-express AAGGCTCTGGAAAGTCCTTCAAAGCTGGAGTCTGTCCTCCTAAGAAATCTGCCCAGTGCCTTAGATACAA
cds-stetler[ AAGGCTCTGGAAAGTCCTTCAAAGCTGGAGTCTGTCCTCCTAAGAAATCTGCCCAGTGCCTTAGATACAA

                   150       160       170       180       190       200       210
                     *         *         *         *         *         *         *
slpi-express GAAACCTGAGTGCCAGAGTGACTGGCAGTGTCCAGGGAAGAAGAGATGTTGTCCTGACACTTGTGGCATC
cds-stetler[ GAAACCTGAGTGCCAGAGTGACTGGCAGTGTCCAGGGAAGAAGAGATGTTGTCCTGACACTTGTGGCATC

                   220       230       240       250       260       270       280
                     *         *         *         *         *         *         *
slpi-express AAATGCCTGGATCCTGTTGACACCCCAAACCCAACAAGGAGGAAGCCTGGGAAGTGCCCAGTGACTTATG
cds-stetler[ AAATGCCTGGATCCTGTTGACACCCCAAACCCAACAAGGAGGAAGCCTGGGAAGTGCCCAGTGACTTATG

                   290       300       310       320       330       340       350
                     *         *         *         *         *         *         *
slpi-express GCCAATGTTTGATGCTTAACCCCCCCCAATTTCTGTGAGATGGATGGCCAGTGCAAGCGTGACTTGAAGTG
cds-stetler[ GCCAATGTTTGATGCTTAACCCCCCCCAATTTCTGTGAGATGGATGGCCAGTGCAAGCGTGACTTGAAGTG

                   360       370       380       390       400       410       420
                     *         *         *         *         *         *         *
slpi-express TTGCATGGGCATGTGTGGGAAATCCTGCGTTTCCCCTGTGAAAGCTAAGGGCGAGCTCAATTCGAAGCTT
cds-stetler[ TTGCATGGGCATGTGTGGGAAATCCTGCGTTTCCCCTGTGAAAGC----------------------------

                   430       440       450       460       470       480       490
```

Figure 3. Homology result on signing gene sequence of SLPI protein recombinant amnion membrane and protein gene SLPI recombinant parotid gland.

Homology analysis of amino acid on SLPI recombinant was done to find out any possibility of nucleotide sequence change that would effect amino acid formation. The result from homology product of PCR SLPI amnion membrane with SLPI membrane amnion from cloning was 100% homogeny, means that there was no change of amino acid sequence between PCR SLPI amnion membrane product and SLPI amnion membrane from cloning. The homology result could be seen on figure 4.

```
SLPI PCR  1 MKSSGLFPFLVLLALGTLAPWAVEGSGKSFKAGVCPPKKSAQCLRYKKPECQSDWQCPGK
SLPI klon 1 MKSSGLFPFLVLLALGTLAPWAVEGSGKSFKAGVCPPKKSAQCLRYKKPECQSDWQCPGK
      ************************************************************

SLPI PCR 61
KRCCPDTCGIKCLDPVDTPNPTRRKPGKCPVTYGQCLMLNPPNFCEMDGQCKRDLKCCMG
SLPI klon 61
KRCCPDTCGIKCLDPVDTPNPTRRKPGKCPVTYGQCLMLNPPNFCEMDGQCKRDLKCCMG
      ************************************************************

SLPI PCR 121 MCGKSCVSPVKA
SLPI kon 121 MCGKSCVSPVKA
           ************
```

Figure 4. Homology result of amino acid sequence of PCR SLPI amnion membrane product with SLPI amnion membrane from cloning.

SLPI amnion membrane gene were amplified using designed primer which can remove whole SLPI gene according to pET 101 D-TOPO. Primer analysis result with clone manager method was shown on table 1.

Table 1. Primer analysis with clone manager method

Primer A forward-ext		
Sequence	:	5' CACC-ATGAAGTCCAGCGGCCTCTTCC 3'
Length	:	26
Meets criteria	:	No
Pos	:	1
Composition	:	A: 5 C:11 G: 5 T: 5
Primer A forward-ext		
Sequence	:	5' AGCTTTCACAGGGGAAACGCAGGAT 3'
Length	:	25
Meets criteria	:	No
Pos	:	396 C
Composition	:	A: 8 C:5 G: 8 T: 4

Nucleotide sequence of protein sign gene SLPI amnion membrane in this research has been registered on Gene bank (http://www.ncbi.nib.gov) with accession number EU116331 for nucleotide sequence (attachment 4), on NCBI Core Nucleotide search site with keyword SLPI synthetic on first row from 28 core nucleotide and accession numberABV21606 for amino acid sequence, on the third row of four protein data SLPI synthetic (attachment 5). Nucleotide sequence and amino acid sequence were homologize, the homology result is shown on table 2.

Table 2. Homology of nucleotide SLPI recombinant amnion membrane sequence with nucleotide sequence and SLPI amino acid from database

No	Accessed number *database* NCBI	Gene size (bp)	Amino acid	DNA source	Homology identity nucleotide(%)	Homology identity amino acid (%)
1.	DQ894545	439	132	*Human*	100%	100%
2	DQ891365	439	132	*Human*	100%	100%
3	**ABV21606**	**495**	**132**	***Human***	**100%**	**100%**
4	PIGALP	600	132	*Pig*	77,835%	100%

Analysis of pET-ESLPI recombinant protein with SDS-PAGE

Recombinant protein of pET-ESLPI that were transformed on E. coli BL-21 star can be analyzed for function. The expression of recombinant protein were analyzed with SDS-PAGE.

The result from E. coli BL-21 star containing SLPI amnion membrane recombinant plasmid was processed with alkali lyses to open cell walls of E. coli therefore recombinant protein could exit E. coli and put into column nickel containing resin Ni-NTA agarose. Nickel would bind His-Tag from recombinant plasmid so the unwanted protein could be flushed away from the column. After that, the column was flushed and continued with elusion process to get the pure recombinant protein.

In order to erase the His-Tag from the recombinant protein, Taqzyme kit was used. SLPI recombinant protein partial purification could be seen on figure 5.

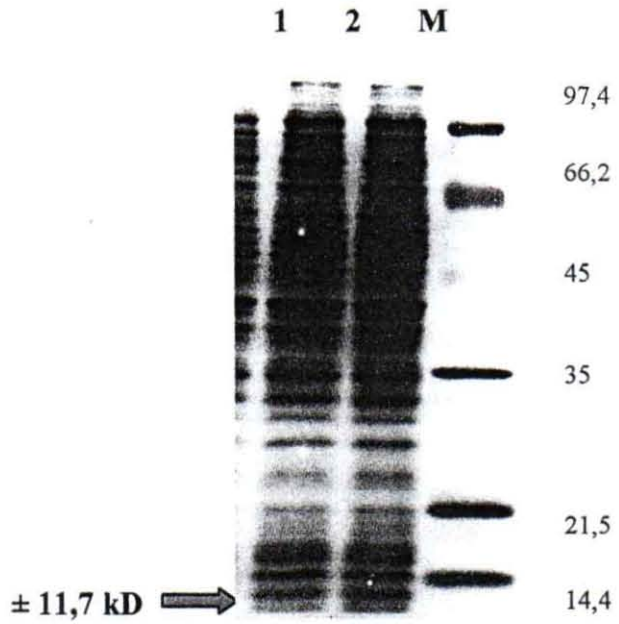

Figure 5. The result of SDS-PAGE protein SLPI membrane amnion recombinant: M: Marker protein rainbow broad range from Biorad; 1-2: protein SLPI amnion membrane.

Western blotting was done on semi dry system transfer device. Protein antigenic of SLPI could be known by western blotting technique. SDS-PAGE was done first to get the band of recombinant protein. After that, transfer was done to nitrocellulose membrane using blotter. After band of protein was moved to nitrocellulose membrane, reaction was done with antibody I (from mice serum inoculated with SLPI). After washing, the process was continued by reaction with antibody II (antimouse labeled conjugate alkaline phosphatase) that will react with substrate alkaline phosphatase (AP). Western blotting analysis result can be seen on figure 6.

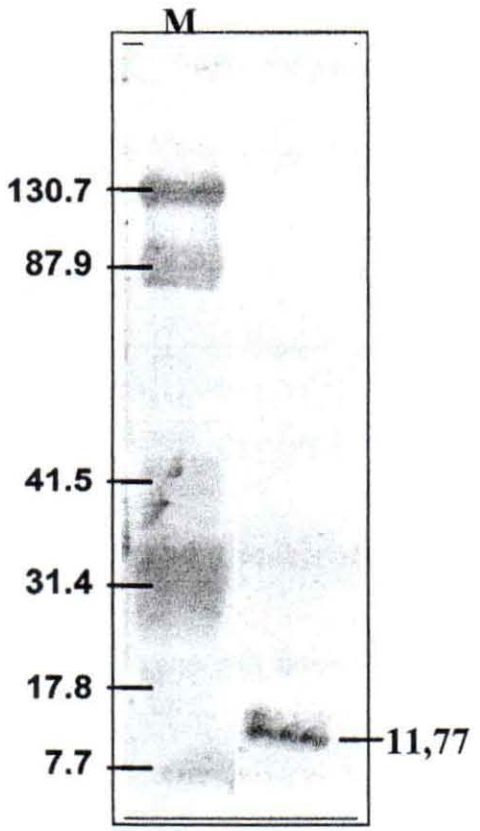

Figure 6. Western blotting of SLPI recombinant protein against SLPI monoclonal antibody. M: Marker rainbow broad range from Biorad 1 SLPI recombinant.

DISCUSSION

Identification of signing gene SLPI amnion membrane

The presence of sign gene SLPI amnion membrane was detected using PCR method to get the DNA amplification segmented by synthetic olygonucleotide (primer). This research used specific human SLPI (5-'ACTCCTGCCTTCACCATGAA3'/5'-ATTCGATCAACTGGACTT-3') primer. The amplification resulted DNA product of 530 pb. The primer was chosen based on Zhang's research (2001)[10] which could successfully isolate SLPI gene from human amnion liquid by RT-PCR method, resulting SLPI gene size of 570 pb. SLPI homology analysis from GeneBank showed 98-100% homology, based on this, the primer was used.

Several research showed that SLPI gene sign has various sizes, Stedler et al (1986) [11] stated that SLPI size on parotid gland was 580 pb, while Moriyama et al (1999) stated that SLPI size on cervical mucous was 570 pb. Zhang et al (2002) [9] stated that SLPI size on endometrial epithelial cell was 451 pb. SLPI size from GeneBank was various according to Table 3. Several nucleotide sequence from GeneBank on alignment resulting similar homology. This prove that the identity of signing gene SLPI amnion membrane is similar to human SLPI on GeneBank.

Table 3. SLPI size from GeneBank

No	Accessión number	Gene Size (pb)	Source of SLPI
1	X04502	2657	Parotid tissue
2	M74444	1472	Epithelial cell
3	BC020708	625	-
4	NM_003064	598	Endometrial cell
5	CS106326	594	-
6	X04470	594	Cervix uterus
7	X04503	573	Parotid gland
8	DQ891365	439	Synthetic
9	DQ894545	439	Synthetic
10	AF114471	399	Intestinal epithelial cell

Amplification, Purification and Sequencing of Signing Gene SLPI Amnion Membrane

Amplification of SLPI gene was done using PCR technique. This technique was used because of its ability to purify DNA polymerase and synthesis oligonucleotide chemically, therefore giving chance to clone specific DNA sequence, in this case for signing gene SLPI amnion membrane.

Zhang's research (2001) about the production and concentration regulation of SLPI on human amnion membrane thru PCR amplification stated that the nucleotide length of SLPI amnion membrane gene was 570 pb, but the sequence data wasn't available on GeneBank. Stetler *et al* (1986) showed SLPI expression on parotid gland with 399 pb while on this research, SLPI gene was 530 pb in size. If the sequences were paralelled with the result from Steadler's (1986), the homology was 98%, and 100% if they were paralelled on CDS. This showed that sign gene of SLPI protein has same conserve area but with different gene size.

Sign Gene SLPI Amnion Membrane Cloning

Sign Gene SLPI amnion membrane recombinant was produced by sign gene cloning into vector pET 101/D TOPO. Vector pET 101/D TOPO has 5753 pb size. Cloning on this exploration has several advantages such as less time, and ligation can be done at once (direct cloning) on PCR product without plasmid restriction. This is caused by plasmid's linear form. Primer was designed to be compatible with plasmid pET101/D TOPO. PCR product is blunt-ended to regulate the expression of *E. coli*. The insert gene was proven by cutting with endonuclease EcoRV restriction enzyme. Recombinant plasmid pET ESLPI showed similar linear band with PCR product beside band of plasmid pET TOPO itself.

The expression of recombinant protein was done after characterization of the recombinant plasmid on E.*coli* TOP10, then that recombinant plasmid was transformed back to *E.coli* BL21 star. The achieved target gene was inserted into pET101/D-TOPO the next step was transformation on *E.coli* TOP10 without *T7 promotor* to maintain the stability of recombinant plasmid and suggested for propagation and maintenance of E.*coli* TOP10. *E.coli* cell was prepared to receive plasmid with $CaCl_2$ treatment before transformation on *E.coli* TOP10. $CaCl_2$ salt was predicted to cause DNA precipitation on cell surface or probably the salt cause certain changes on cell wall that increase DNA bond. DNA movement into competent cell was stimulated by increasing temperature to 42°C in a short time, this process is known as heat stroke (Nyoman, 1999). This suspension was put into ice container immediately, to retain the plasmid inside *E.coli*. Transformant on *E.coli* after transformation was put into culture on *Luria Bertani* (LB) media containing ampicillin, pET101/D-TOPO has gene which resistent to ampicillin, while *E.coli* TOP10 host has no gene which resistent to ampicillin. The cloning process was considered successful if the transformer could grow on that LB media, showing SLPI gene sign inserted on pET101/D-TOPO by bacteria colony growth.

E.coli is often used in cloning. One disadvantage of E.coli as a procariotic organism which even though easy to grow but the target protein folding usually not match because after translation process it cannot do proper glicosylation, therefore the recombinant protein cannot function optimally. This condition in SLPI protein is advantageous because SLPI I is a non glycosylized protein so synthetic SLPI are easily produced because it can be expressed in prokaryotes (Fitch et al 2006).

E.coli BL21 star was used to see SLPI amnion rekombinant membrane after characterization on *E.coli* TOP10. The expression of recombinant gene can often happen if there is *T7 RNA Polymerase*. Procaryotic cell do not produce this enzyme, therefore *E.coli* BL21 star containing gene *T7 RNA Polymerase*, promotor *lac* and operator *lac* need to be added containing lamda DE3 lysogen which carry gene *T7 RNA Polymerase* controlled by promotor *lac UV5*, therefore IPTG is needed to induce the expression of *T7 RNA Polymerase.*

Recombinant plasmid was isolated to separate the DNA plasmid containing recombinant SLPI molecule from bacterial DNA chromosome. Recombinant DNA plasmid isolation in this research used High-speed Plasmid Mini Kit (Geneaid). This kit was designed to isolate plasmid or cosmid DNA from 1-4 ml bacterial culture. The principal of this method is based on a theory that circular DNA plasmid tend to form

supercoil, while DNA chromosome form linear fragmentation to ease denaturation. This made easier and faster plasmid isolation process.

Enzyme used as restriction enzyme was endonuclease. Three kinds of known restriction enzyme are type I, II, and III. Endonuclease type I cut unspecific, while endonuclease type III has far identification point from the cutting point. Characterization of recombinant plasmid in this research used endonuclease enzyme type II.

SLPI recombinant plasmid restriction pET-ESLPI in this research used enzyme EcoRV which would cut recombinant plasmid into two fragments. List of enzymes that do not cut SLPI gene could be seen on pET-ESLPI from isolation incubated with proper restriction enzyme, then the length of fragment from cutting was determined by agarose electrofluoresis gel. This enzyme would cut on nucleotide sequence 545 and 4775, and would result 2 bands of 4230 and 2018 giving a total of 6248.

Nucleotide sequence of recombinant gene fragment SLPI was determined using forward primer which was designed to get the entire gene. Primer selection would predict the success of PCR, and after the nucleotide sequence was known that this fragment contains 877 nucleotides, this result still describe the plasmid nucleotide, tag and signal peptide after alignment from original PCR and from Genebank resulting 495 bp.

Nucleotide sequence of protein signing gene SLPI amnion membrane from this research was registered on Genebank (htpp://www.ncbi.nib.gov) with accession number **EU116331** for nucleotide sequence, on NCBI site search Core Nucleotide with keyword SLPI synthetic on first row of 28 Core Nucleotide. Amino acid sequence was registered with accession number **ABV21606** on third row of four in amino acid sequence. The above data showed from 28 core nucleotide only 4 could be interpreted into amino acid sequence. Analysis from Genegank from the 24 accession numbers reported only genom and nucleotide primary sequence which could not be interpreted into amino acid. Four accession numbers for amino acid was SLPI amino acid sequence which consists of three accession number containing synthetic SLPI amino acid from human including result from this research while one accession number containing synthetic SLPI amino acid sequence from pig. The essence of this research was recombinant SLPI amnion membrane from Indonesia has been registered in Genebank among few registered synthetic SLPI. Sequencing resulted was single gene signing protein SLPI and was an Open Reading Frame (ORF) started from ATG as start codon and TGA as stop codon.

Based on above introduction, in the research of SLPI amnion membrane to express the SLPI gene, vector expression pET-101 DTOPO was used. This vector was added with TATA box, RBS, etc.

Sequence cDNA from cloning can show main translation product from mRNA SLPI in 132 amino acid, including 25 amino acid from signal peptide for secretion. Sequence cDNA expressed was whole area of signal protein on SLPI gene, with regard to: (1) side by side sequence with SLPI genome sequence (including amino acid metionin on N-terminal position) as in consensus with sequence cDNA for translation initiation signal (CCA/GCCAUGG); (2) no codon metionin between beginning termination codon and translation initiation side and there was no potential connector intron/exon in the area; (3) signal sequence for secretion follow the signal sequence pattern for eucariot, where an amino acid (lysine) usually follow metionin as end of sequence; and there was a hydrophobic and an amino acid praline on 6 amino acid before estimated processing side. The comparison of SLPI amino acid sequence and cDNA estimate initial peptide cutting position by peptidase signal on peptide bond between glysine and serine which is rarely found. (4) cutting side which are usually found is on peptide bound between alanin and valin which on this sequence was also found before peptide bond between glisine and serine. Therefore, the result could not be used to eliminate the possibility of cutting between alanin and valin followed by later process resulting end product of SLPI with amino acid ended without signal peptide.

This is proven with the parallel result of SLPI recombinant with designed primer Topo, shown that primer stuck on start and end of nucleotide SLPI recombinant. This primer looked complemented efficiently with the targetted DNA sequence.

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