Dental Journal
Majalah Kedokteran Gigi
Vol. 41. No. 3 July–September 2008

Research Report

Isolation and identification of java race amniotic membrane secretory leukocyte protease inhibitor gene

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ABSTRACT

Background*: Secretory leukocyte protease inhibitor (SLPI) has been found to facilitate epithelialization, maintain a normal epithelial phenotype, reduce inflammation, secrete growth factors such as IL-4, IL-6, IL-10, EGF, FGF, TGF, HGFand 2-microbulin. SLPI is serine protease inhibitor, which found in secretions such as whole saliva, seminal fluid, cervical mucus, synovial fluid, breast milk, tears, amniotic fluid and amniotic membrane. Impaired healing states are characterized by excessive proteolysis and often-bacterial infection, leading to the hypothesis that SLPI may have a role in the healing process in oral inflammation and contributes to tissue repair in oral mucosa. The oral wound healing response is impaired in the SLPI sufficient mice since matrix synthesis and collagen deposition delayed. The objective of this research is to isolate and identify the amniotic membrane of Java Race SLPI Gene.* ***Methods****: SLPI RNA was isolated from Java Race amniotic membrane and the cDNA was amplified by polymerase chain reaction (PCR).* ***Result****: Through sequence analyses, SLPI cDNA was 530 nucleotide in length with a predicted molecular mass about 12 kDa. The nucleotide sequence showed that human SLPI from sample was 98% identical with human SLPI from gene bank. PCR analysis revealed that the mRNA of SLPI was highly expressed in the amniotic membrane from Java Race sample.* ***Conclusion****: it is demonstrated that human SLPI are highly conserved in sequence content as compared to the human SLPI from gene.*

Key words*: secretory leukocyte protease inhibitor, amniotic membrane, cloning, recombinant, wound healing*

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INTRODUCTION

Gingival recession occurs at all ages varied from 65 years old or higher (88%) and 18–64 years old (50%).[1] Surgical treatment of gingival recession cause fairly large muco gingival injury due to gingival retraction toward coronal. Tension will lead to excessive thin gingival and laceration.[2] In the oral cavity, bacterial free environment is difficult to achieve. Further complication would occur if immediate treatment is not carried out since it would be easily to be contaminated by bacteria, leading to cellulites, abscess or osteomyelitis. Various method have been developed to achieve injury recovery.[3,4]

The present concept of treatment and recovery has been developed simultaneously with dental biomaterial progress. Biological material is currently considered to be applied due to the supporting component of treatment and recovery. One of the bio material expected to be able to accelerate injury recovery is amniotic membrane that effective in burn injury treatment as well as in ophthalmology.[5,6,7] Secretory leukocyte protease inhibitor (SLPI) has been found to be an active material of amniotic membrane expected to be influential to injury recovery.

SLPI is one of the inhibitors of serine in addition to α1-antitrypsin (α1-PI) and leafin. The main target of SLPI is to inhibit serine protease-the human netrophil protease (HNE) including chemotripsyn super-family member of serine protease expressed in monosite and mast sell especially netrophil. The function of this intracellular enzyme is to degrade foreign extra cellular matrix (ECM), remodeling damaged tissue and facilitating netrophil migration into other tissue.[8]

Additional function of SLPI is to inhibit leukocyte proteinase, to maintain immunity against infection, regulating leukocyte function, producing matrix and improving tissue. SLPI also facilitates several functions: inhibiting protease, controlling leukocyte activity, reducing TGF-beta, anti inflammatory, anti bacteria, anti retroviral (human immunodeficiency virus). Another function of SLPI are to control synthesis of intracellular enzyme, and to suppress MMP production, to prevent the formation of scar tissue.[9]

The study was conducted to analyze the character of amniotic membrane SLPI molecular protein and cloning of amniotic membrane SLPI protein to achieve amniotic membrane SLPI protein recombinant in large amount which would be beneficial as biomaterial candidate to achieve fast recovery of gingival injury.

MATERIAL AND METHOD

Identification of amniotic membrane SLPI gene used the following materials: amniotic membrane, proteinase K, RNAse, RNA later, TBE buffer, ethidhium bromide, bromophenol blue, marker X 174x174 RF DNA/Hae III fragments RNAa later, RT buffer, ribonuclease, random primer, reverse transcriptase, 2% agarose, ethidium bromide, loading dye, primer (sense): 5' ACT CCT GCC TTC ACC ATG AA 3' and primer (anti sense) 5' ATT CGA TCA ACT GGA CTT 3' (invitogen). 100 bp DNA Marker (fermentas), distilled water, TTH buffer reverse, cDNA mineral oil, enzyme TTH DNA polymerase, binding buffer (PB), washing buffer (buffer PE), 200 µl buffer PE, elution buffer (EB), sodium acetate, 100% alcohol, 70% alcohol, pure DNA, template suppression reagent.

Amplification, purification and sequencing of amniotic membrane SLPI gene using the following materials: amniotic membrane SLPI, primer : 5' CAC CAT GAA GTC CAG CGG CCT CTT CC 3' and 5' AGC TTT CAC AGG GGA AAC GCA GGAT 3', Rneasy mini kit (Qiagen), 100 pb DNA marker, nuclease free water (MP Bio), ethidhium bromide (MP Bio), Agarose (Fermentas), 6x loading dye solution (Fermentas), Tris Bufferc EDTA (TBE) buffer 10× ilt (MP Bio), gel extraction purification kit (Qiagen), low melting agar (Ssigma), DNA sequencing kit.

RNA isolation method as the following: 3 × 3 mm (30 µl) fresh amniotic membrane sample was put into RNA later and kept at 40° C in refrigerator until requested. 30 µl amnion membrane sample was added to 300 µl buffer RLT followed by 590 µl distilled water into 20µl eppendorf tube, then, 10 µl proteinase K was added. The mixture was incubated at 55° C for min. Next, centrifugation was done in 3 min at 10.000 rpm at 20–25° C. 1.5 µl other eppendorf was filled with 900 µl supernatant added 450 µl (1/2 vol) ethanol 100% mixture of sample was done using pipette into RNA tube, centrifugation at 10.000 rpm, the lower part of the solution was evacuated. Centrifugation was repeated for 15 second at 10.000 rpm, lower part of the solution evacuated added by 700 µl buffer RW, centrifuged for 15 second at 10.000 rpm. New collective tube was filled by 500 µl buffer RPE, following by 2 minute 10.000 rpm centrifugation, repeated to throw out the lower part of the solution. 1.5 ml eppendorf tube was filled by 50 µl RNeasy free water, then centrifugation was done was done in 1 minute at 10.000 rpm. The result of the procedure was RNA continued by cDNA isolation.

Isolation of cDNa using the following procedures: 10µl RNA was incubated at 65° C for 3 minute, added by 4µl (5X) buffer RT, 4 µl dNTPs mix, 0.5µl ribonuclease, 0.5µl random primer, 0.5 µl reverse transcriptase (19.5 µl). The mixture was incubated at 42° C for 1 hour. Electrophoresis was performed to identify whether cDNA was formed in 2% agarose gel containing ethidium bromide. 5 µl cDNA was added by 2 µl loading dye and put into well agarose, then, it was run in 100 volt for ± 30 minute, followed by detection with UV-transilluminator and documented by Polaroid camera.

Polymerase Chain Reaction (PCR) was done in the following procedure: 70 µl distilled water mixed with 9 µl buffer reaction TTH, 8 µl dNTP mix, 1µl primer reverse CAT TCG ATC AAC TGG CAC TT and 1 µl primer forward ACT CCT GCC TTC ACC ATG AA 1 µl cDNA (total 99 µl), next, added by 102 µl mineral oil denaturation was done at 72° C for 30 second and 94° C for 5 minutes, continued by adding 1 µl TTh DNA polymerase, then inserte d into PCR machine. Initially denaturation was performed at 940 C for 40 second, annealing at 52° C for 40 second, extension was done at 72° C for 40 second, extra extension at 72° C for 10mm. PCR was done in 35 cycles analysis of PCR product with electrophoresis agarose gel. The result of PCR could be seen through electrophoresis in 2% agarose gel containing ethidhium bromide. 5 µl cDNA added by 2 µl loading dye put into well agarose. The mixture was run in 100 volt for 30 min. next, detection was conducted by UV-transuminator, then, the result was documented using Polaroid camera.

Purification of PCR product was performed using purification kit (qiagen). 100µl PCR product was added by 500 µl buffer binding (PB) and mixed to release mineral oil. The mixture was moved into column, nest step, centrifugation was performed at 14.000 rpm at room temperature for 1minute. The solution in the lower part was removed and rinsed by 750 µl washing buffer, the mixture was centrifuged at 14.000 rpm at room temperature for 1 minute, the fluid was thrown out, added by 200µl buffer PB centrifugation was performed again at 14.000 rpm at room temperature for 1 minute. Pure DNA which was purified put into eppendorf, added by 50 µl buffer elution (B.E) and it was left for 5 minute. Centrifugation was done at 14.000 rpm at room temperature for 1 min.

Electrophoresis was done to identify the result of DNA purification in 2% agarose gel containing ethidhium bromide. 5 µl PCR product had been purified and mixed

with 2 µl loading dye into well agarose. Next, it was run in 100 volt for about 30 minute, detected using UV-transluminator finally documented by Polaroid camera.

Sequencing process is 5 µl sodium acetate 3M was added into sample, followed by 50 µl alcohol 100% and the eppendorf was agitated. The mixture was than incubated at room temperature for 15 minute and continued by centrifugation at 15.000 rpm at 40 C in 15 minute. Supernatant was evacuated again, and drying process in vacuum 15 min. the result was ready to kept or directly sequenced.25 µl template suppression reagent (TSR) was added into pure DNA and dried at 950 C for 5 minute, then, put into the ice for 10 minute, moved into 0.5 ml (500µl) tube, finally placed into sequencing machine for one hour (10 nucleotide for 1 minute).

Amplification, purification and sequencing Gen encoding SLPI amniotic membrane: Gen encoding SLPI could be amplified after RNA isolation was previously done from amniotic membrane using Rneasy mini kit. 50µl sample was put into Eppendorf tube (1.5 ml), added by 550 µl β –ME/RLT and 600 µl ethanol 70% into the sample, then, mixed by agitating the tube. 600µl solution was moved into Rneasy spin column and kept in collective tube 2ml, followed by centrifugation at 12.000 rpm for15sec. The lower part of the solution was removed and the remain was repeated. 700 µl RW1 solution was added into Rneasy Column followed by centrifugation at 12.000 rpm for 15 sec. and the lower part of the solution was removed. Rneasy Column was moved to a new tube (2 ml), added by 500µl RPE buffer and centrifuged at 12.000 rpm for 15 sec. the lower part of the solution was removed and repeated. Rneasy column was moved into another tube then centrifuged at 13.000 rpm for 2 minute (to release the ethanol). Rneasy column was moved again into 15ml tube, next, added by 30 µl Rneasy free water in the middle part of the column, centrifugation was done at 12.000 rpm for 2 minutes. Reverse transcriptase to obtain cDNA product was done on the lower part of the solution containing collective RNA.

This present study applied two step PCR method and primer was designed to fulfill the criteria of Gen insertion in pET 101/D-TOPO by adding CACC prior to ATG (at initial Gen) i.e. primer (sense): 5' CAC CAT GAA GTC CAG CGG CCT CTT CC 3' and primer (anti sense)5' AGC TTT CAC AGG GGA AAC GCA GGA T 3'. Amplification was done using reagent mixture consisting of : 70µl distillated water mixed with 9 µl reaction buffer TTH, 8 µl dNTP mix, 1µl primer forward and 1µl primer reverse, 10µl CDNA (total 99 µl) and added by mineral oil. The process was done according to the following stages: predenaturation at at 72° C for sec. and 94° C for 5 min. the mixture was then added by 1µl TTh DNA polymerase enzyme, put into PCR machine with denaturation process at 94° C for 40 sec. followed by annealing at 52° C for 40 sec. and extension at 72° C for 40 sec, next final step extra extension at 72° C for 10 sec. PCR was done in 35 cycles. The result of PCR could be seen by electrophoresis in 2% gel agarose containing ethidium bromide, 5µl cDNA added by 2µl loading dye put into well agarose and operated in 100 volt approximately 30 min, continued by UV-transluminator detection, and documentation was taken using Polaroid camera.

Preparation for sequencing and cloning, the PCR product should be previously purified. The product was run in 1.5% low melting agar, DNA fragment was cut from gel agarose with scalpel. Under UV light, gel cutting containing DNA fragment added by 3 volume of QG buffer in 1 volume gel (100 mg ± 100 µl) the ext step, incubation was performed at 50° C for 10 min until all gel was dissolved and a mixture of gel and vertex was done in tube every 2–3 minute during the incubation. After all gel was dissolved, the color of the solution would be yellow similar to QG buffer prior to dissolving gel. 1 volume of isopropanol was mixed with the sample, then, QIA quick spin was placed column in 2 ml collective tube and sample was added in column, then it was centrifuged at 13.000 rpm for 1mm. the lower part of the solution was removed, then, column was put back into tube. 750µl PE buffer was added in column, followed by centrifugation at 13.000 rpm for 1 min. the solution in the lower part of centrifugation was thrown out and centrifugation was performed again at 13.000 rpm for 1 min. column was placed in 1 ml tube of new micro centrifuge. Next, it was added by 50 µl EB buffer (10 mm Tris-cl, pH 8.5) or H2O, centrifugation was done at 13.000 rpm for 1min. a part of DNA could be sequenced and the remaining was cloned.

RESULT

Identification of encoding SLPI gene was initiated by obtaining DNA of SLPI, sample preparation of amniotic membrane was previously done to extract RNA, than, cDNA SLPI was achieved. Amplification was done using Two Step PCR (Qiagen) method with primer sense: 5'ACT CTT GCC TTC ACC ATGAA 3' and 5'ATT CGA TCA ACT GGA CTT 3', producing fragment of DNA 530 bp. Visualization of PCR product was performed using agarose

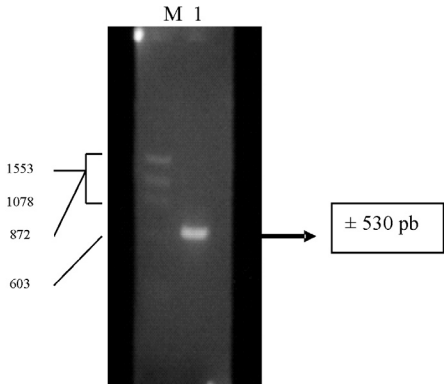

Figure 1. Electrophoresis of PCR product to identify SLPI gene.
M: Marker × 174 RF DNA/Hae III fragments;
1: SLPI.

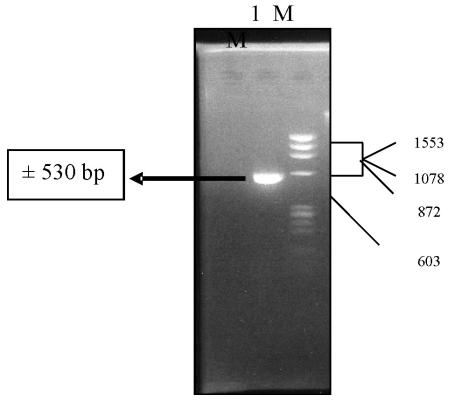

Figure 2. Electrophoresis of purified PCR product to identify SLPI gene.
M: Marker × 174 RF DNA/Hae III Fragments;
1: SLPI.

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  1   cggccnttcc cttcctggtc tgcttgccct gggaactctg gcaccttggg ctgtggaagg
 61   ctctqqaaaq tccttcaaaq ctqqaqtctq tcctcctaaq aaatctqccc aqtqccttaq
121   atacaaqaaa cctqaqtqcc aqaqtqactq qcaqtqtcca qqqaaqaaqa qatqttqtcc
181   tgacacttgt gggcatcaaa tgcctggatc ctgttgacac cccaaaccca acaaggagga
241   agcctgggaa gtgcccagtg acttatggcc aatgtttgat gcttaacccc cccaattttct
301   gtgagatgga tggccagtgc aagcgtgact tgaagtgttg catgggcatg tgtgggaaat
361   cctqcqtttc ccctqtqaaa qcttqattcc tqccatatqq aqqaqqctct qqaqtcctqc
421   tctgtgtggt ccaggtcctt tccaccctga gacttggctc cccactgata tcctcctttg
481   gggaaaggct tggcacacag caggctttca agaagtgcca gtgnnnccca atgggnnnnn
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Figure 3. Sequensing of SLPI gene using forward primer.

electrophoresis supported by UV-transilluminator and documented by Polaroid camera, as shown on figure 1.

In this present study identification of amniotic membrane SLPI gene of PCR product should be done through purification prior to sequencing and using qiaquuick PCR purification kit (Qiagen), followed by UV-transluminator detection, documented using Polaroid camera (Figure 2).

Amplification of sequence of amniotic membrane SLPI gene encoding protein with primer reserve CAT TCG ATC AAC TGG CAC TT and primer forward ACT CCT GCC TTC ACC ATG AA (Zhang et al, 2001) producing about 530 bp amplicont as shown on figure 3.

Amplification of amniotic membrane SLPI cDNA is required for analysis of amniotic membrane SLPI gene using two-step PCR method i.e RNA isolation method was previously done and continued by cDNA isolation. cDNA amplification was done in thermal cycler. Primer was designed in accordance with the criteria of gene insertion on pET 101/D-TOPO adding CACC prior to ATG (At the early gene) presented on figure 4.

5' CACC-ATGAAGTCCAGCGGCCTCTTCC 3'
5' AGCTTTCACAGGGGAAACGCAGGAT 3'

Figure 4. Primer design for SLPI gene amplification.

Amplification of SLPI Gen producing DNA fragment in ± 530 bp positions can be shown on figure 5.

PCR products of amniotic membrane were purified using TOPO primer prior to sequencing.. Band contaminants was found during visualization of PCR product through electrophoresis, therefore prior to purification, running of PCR product was done using 1.5% lomelting agar. Under UV light, the required band was cut and mixed with QG buffer containing pH indicator. Optimal binding between DNA and silica gel requested pH Ý 7.5 and would produce yellow appearance, while, purple appearance would occur, pH was too high. After DNA was bound with the membrane continued by DNA rinsing process by releasing unnecessary primer and other contaminants. PE buffer additional containing ethanol would make contaminants. Flow out from matrix column through centrifugation stage. DNA bound with membrane eluated by adding elution buffer exactly in the middle of membrane and partly purified DNA from electrophoresis to identify the existence of purification, followed by UV-transluminator detection, finally, documented by Polaroid camera shown on figure 6.

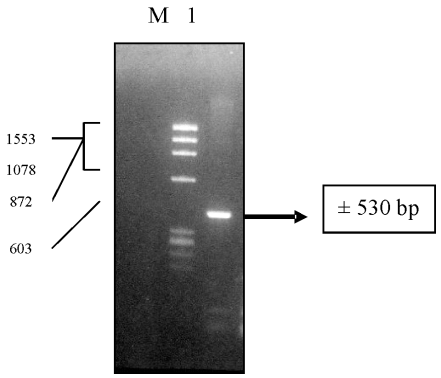

Figure 5. The result of electrophoresis SLPI Gen PCR Product with TOPO primer:
M: Marker × 174 RF DNA/Hae III fragment,
1: SLPI

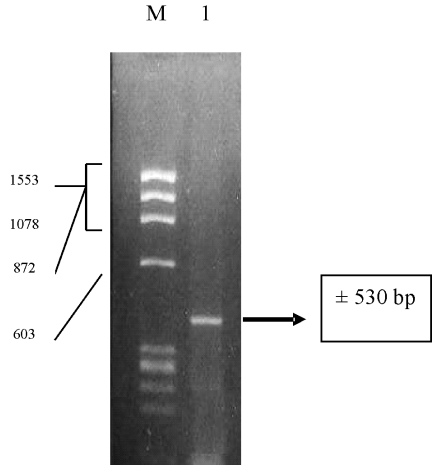

Figure 6. Result of SLPI gene purification.
M: Marker × 174 RF DNA/Hae III fragment,
1: SLPI

Table 1. Gene bank's list of SLPI size

No	Accession number	Gen size (Pb)	Source of SLPI
1	X04502	2657	Parotid tissue
2	M74444	1472	Epithelium cell
3	BC020708	625	-
4	NM003064	598	Endometrial cell
5	CS106326	594	-
6	X04470	594	Cervical uterus
7	X04503	573	Parotid tissue
8	DQ891365	439	Synthesis
9	DQ894545	439	Synthesis
10	AF114471	399	Intestinal epithelium cell

DISCUSSION

The existence of amniotic membrane SPLI gene was detected using PCR method to obtain amplification of DNA segment limited by synthetic oligonucleotide (primer).

This study used specific primer of human SLPI (5-'ACTCCTGCCTTCACCATGAA3'/5'-ATTCGATCAACTGGACTT-3'). The result of amplification obtained 530 bp DNA. Primer selection based on the study done by Zhang[9] that successfully isolated SLPI gene from human amniotic fluid by RT-PCRmethod obtaining 570 bp SLPI gene.[10] Homology analysis of gene bank shows human homology SLPI indicating 98–100% homology, based on this indication therefore primer was applied.

Some studies showed SLPI gene has various size. The size of SLPI in parotid gland is 580 bp,[11] in cervical mucus is 570 bp, and in endometrial epithelial cell is 451 bp.[9] Various size of SLPI according to gene bank shown on Table 1. Alignment was done on some sequence of nucleotide obtained from gene bank showed similar homology and it is proven that the identify of amniotic membrane SLPI gene is similar to human SLPI registered in Gene bank.

Amplification of SLPI gene was conducted using PCR technique i.e in vitro enzymatic method producing specific DNA in a large amount and in very short time through the stage of denaturation, annealing and extension at different temperature. Prior to detection of SLPI existence, isolation of DNA genome was initially conducted by extracting cDNA and PCR method was applied to achieve amplification of a certain DNA segment limited by synthetic of 2 oligonucleotide called primer. This technique was applied due to the ability to purify DNA polymerase and to synthesize chemically DNA oligo nucleotide therefore; it would be possible to conduct cloning in specific DNA sequence that is amniotic membrane SLPI gen.

Zhang[8] suggested the production of human amniotic membrane SLPI gene and regulating concentration of amniotic fluid through PCR amplification stated that the length of nucleotide of amniotic membrane SLPI gene is 570 pb. However, sequence data of amniotic membrane SLPI nucleotide is not found at gene bank. It was reported that expressed SLPI in parotic gland is 399 pb, while, in this study, SLPI gene is 530 pb.[1] If the alignment between sequence result of this study and the result of study done by Stedler,[9] 98% homology was found. If the alignment was done only CDS and the alignment found 100% it shows SLPI gene has the same conserve area but the gene size is not the same.

The conclusion of this study, human SLPI are highly conserved in sequence content as compared to the human SLPI from gene.

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