cDNA SEQUENCING Of FRAGMENTS FROM FIBRINOLYTIC GENE (F-4) FROM *CERASTES CERASTES* VENOM GLAND

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Abstract

A fibrinolytic anticoagulant (F-4) was previously isolated by ***Daoud et al; (1986)*** from *Cerastes cerastes* venom. In this study, isolation and sequencing of gene fragments of this F-4 protein was attempted. Primers utilized in the polymerase chain reactions were obtained from two sources: **P1**, a primer that was previously used in isolating other fibrin(ogen)olytic metalloprotease. The other primers; **P2**, **P3** and **P4** were designed using doprimer program.

RT-PCR using **P1** and **P2** primers gave a band of 350 bp. **P4** and **P3** primers gave a band of 60 bp. **P1** and **P3** primers gave a band of 400 bp.

Sequence analysis and alignment using bioinformatic programs indicated that samples 1, 2 and 3 bear significant homology to the metalloprotease family of snake venom sequences deposited in the Genbank. Translation to the amino acid sequence and alignment using protein database showed strong homology with fibrinolytic metalloproteases. Conservative domain database analysis indicated that the three sequenced samples belong to reprolysin family, which is a snake venom endopeptidase requiring zinc for catalysis. Predict protein algorithm indicated one cysteine disulfide bond in sample 3. Sample 1 was described as mixed protein with 22% helix 35% extended and 41% loop structure. Sample 2 was described as all-alpha protein with 76% helix and 24% loop. Sample 3 was described as alpha–beta protein with 30% helix, 27% extended and 41% loop. The sequenced

fragments of the protein were generally described as globular and seemed to be truncated of one gene related to a fibrinolytic protein.

Keywords**:** *Fibrin(ogen) lysis, zinc metalloprotease, primer design, cDNA sequencing and conserved domain.*

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Introduction

Snake venom contains direct acting fibrinolytic metalloproteases that could have important applications in medicine, ***(Ramirez et al., 1999).***

Haemostatically active components are distributed widely in the venom of many different snake species; particularly from ***Pit-viper***, ***Viper*** and ***Elapid*** venom, ***(Markland, 1998).***

Fibrin(ogen)olytic activity has been described in a number of different snake venoms. There are two classes of fibrin(ogen)olytic enzymes in snake venom, depending on preference for degradation of either alpha or beta chains of fibrin or fibrinogen, ***(Guan et al., 1991).***

Neuwiedase is a fibrin(ogen)olytic non-haemorrhagic metalloprotease purified from ***Bothrops neuwiedi*** snake venom by ***Rodrigues et al., (2000)***. It degrades the Aα-chain of fibrinogen more rapidly than the Bβ-chain in a dose dependant manner, while γ-chain is unchanged. ***Neuwiedase*** shows also proteolytic activity upon fibrin.

Lebetase is a metalloenzyme isolated from ***Vipera lebetina*** venom that contains one mole of $\mathbf{Zn^{2+}}$ and one mole of $\mathbf{Ca^{2+}}$ /mole of protein. ***Lebetase*** degrades fibrin and fibrinogen by hydrolysis of the alpha and beta chains. It also inhibits platelet aggregation induced by ADP in a dose dependant manner, ***(Siigur et al., 1998).***

The anticoagulant action of ***Cerastase*** F-4 is due to non specific cleavage of fibrinogen, ***(Daoud et al., 1986).*** It degrades α A-chain of fibrinogen, followed by β B-chain. It also degrades prothrombin but at a

slow rate. It has very low toxicity and haemorrahgic activity, thus may be useful as thrombolytic agent. ***Daoud et al., (1987)*** reported that it is a metalloprotease, which is Ca^{2+} and Zn^{2+} dependant.

In this work three fragments of the gene for F-4 protein were sequenced. Sequence analysis using bioinformatic programs for sequence alignment was performed using **BLAST,** Basic Local Alignment Search Tool.

A detailed description was presented for the presence of conserved domains using **CDD,** Conserved Domain Database Search, **CDART**, Conserved Domain Architecture Retrieval Tool and **SMART,** Simple Modular Architecture Research Tool. Predict Protein program was used for prediction of secondary structure, prediction of the possible position of disulfide bonds, prediction of solvent accessibility, and prediction of globularity.

Materials and Methods

***Dissection of Venom Gland*:**

The gland tissue of horned Egyptian ***Cerastes cerastes*** snake was dissected in the cold room at 4ºC, minced on ice, then vortexed in SV RNA Lysis Buffer (Promega's kit, USA).

Total RNA Isolation:

Total RNA Isolation was performed using Promega's SV Total RNA Isolation kit, USA according to ***Otto et al, (1998).*** *RNase Inhibitor* (40units/µl) was added to the purified RNA prior to storage at -80ºC.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One Step RT-PCR was performed using QIAGEN One Step RT-PCR Kit, Germany; according to ***Kleiboeker, (2003)***.

Design of Primers:

P1, a primer that was previously used by ***Siigur et al, (1996)*** for amplification of cDNA of ***Lebetase*** (a fibrin(ogen)olytic metalloprotease from ***Macrovipera lebetina***) was utilized. In addition, doprimer program

(www.doprimer.com) was used to design primers from sequences of some non-haemorrhagic fibrinolytic metalloproteinases; ***Lebetase (Siigur et al., 1996)***, ***Atroxase (Baker et al., 1995)***, and ***Neuwiedase (Rodrigues et al., 2000).*** Accordingly, P2, P3 and P4 were designed respectively. Sequences used in doprimer program were chosen so as to include the catalytic site, based on the concept that there is high sequence homology in the catalytic zinc binding site between members of the metalloprotease family, (***Manning 1995).*** Sequences of primers are shown in **table (1).**

Table (1): sequences of PCR primers

Primer name	Primer sequence	Melting temp.(T_m)	Primer conc.
P1	5`GGTCACGAAATACAACGGTGAT 3`	58.4ºC	***50 Pmol/µl***
P2	5` TGCACTATGATCCTGAAC 3`	51.4ºC	
P3	5` GCACCACGAAGACACTTTT 3`	54.4ºC	
P4	5` TTGGTGGGAGTTACAATGG 3`	54.4ºC	

One Step RT-PCR was performed on:

Sample 1= P1 as forward primer and P2 as reverse primer.

Sample 2= P4 as forward primer and P3 as reverse primer.

Sample 3= P1 as forward primer and P3 as reverse primer.

The thermal cycler program:

- Reverse transcription of mRNA to cDNA at 50ºC for 30 min.
- Initial PCR activation step at 95ºC for 15 min.
- Three step cycling was repeated for 40 cycles and included:

a. **Denaturation** step at 94ºC for 1 min.

b. **Annealing** step at 46ºC for 1 min in sample 1, at 50ºC for 1 min in sample 2 and sample 3.

c. **Extension** step at 72ºC for 1 min.

Agarose Gel Electrophoresis:

The PCR products of **samples 1**, **2** and **3** were analyzed by 2% agarose gel electrophoresis, stained with ethiduim bromide (10 mg/ml). *Promega's* low molecular weight markers (catalog# G3161) were used, according to ***Sambrook et al, (1989*).** The gel was photographed using a Polaroid camera and a UV transilluminator.

DNA Gel Extraction:

Agarose DNA Gel Extraction was performed on **samples** 1, **2** and **3** using Roche DNA gel extraction kit [Roche molecular biochemicals, Germany] according to ***Vogelstein and Gillespie, (1979).***

Automated DNA Sequencing:

DNA sequencing was performed on **samples 1**, **2** and **3** after gel extraction, at VACSERA using ABI Prism 310 Genetic Analyzer according to ***Watson et al, (1992).***

Sequence analysis:

The sequenced samples were then analyzed using different ***bioinformatics programs:***

- Nucleotide-nucleotide BLAST for sequence similarity search, *(www.ncbi.nlm.nih.gov/BLAST)*
- Nucleic acid Translation program, *(www.bioinf.man.ac.uk/dbbrowser/bioactivity/proteinfrm.htm)*
- Protein-protein BLAST for sequence similarity search, (*www.ncbi.nlm.nih.gov/BLAST)*
- Conserved Domain Database search**,** (*http://www.ncbi.nlm.nih.gov/Strcture/cdd/wrpsb.cgi*)
- SMART for domain search and domain architecture analysis, *(http://smart.embl-heidelberg.de/*)
- Predict Protein program for motif search, prodomain search, multiple sequence alignment, cysteine prediction, secondary structure prediction and globularity prediction, (*http://maple.bioc.columbia.edu/pp/)*

Results

PCR Products

PCR Products were detected by electrophoresis on 2% agarose:

- **Sample 1**: gave a single clear band ~ at 350bp (fig.1)
- **Sample 2**: gave a single band ~ at 60 bp. (fig.2)
- **Sample 3**: gave a single band ~ at 400 bp (fig.3)

Automated DNA sequencing

DNA gel extraction was performed for the previously mentioned bands followed by Automated DNA sequencing.

- **Sample 1**: The 350 bp band gave 304 bp (fig. 4).
- **Sample 2**: The 60 bp band gave 57 bp (fig. 6).
- **Sample 3**: The 400 bp band gave 393 bp (fig. 8).

Sequence analysis

A nucleic acid translator program translated **sample 1** into **98 amino acid residues** [fig. 5]**, sample 2** into **25 amino acid residues** [fig. 7] since the forward primer **p4 (**19-mer) was added at the beginning of sequence prior to translation in order to increase the sequence size and **sample 3** into **128 amino acid residues** [fig. 9]**.** Similarity searches were performed through standard Nucleotide-nucleotide BLAST (BLASTN) and Protein-protein BLAST (BLASTP) to align the sequenced samples with sequences published in the GenBank.

Sample 1 alignment results revealed similarities with non haemorrhagic fibrin(ogen)olytic metalloproteases from ***Vipera lebetina*** with identity 82% [fig. 10], ***Bothrops neuwiedi*** (identity 90%), and ***fibrolase*** from ***Agkistrodon contortrix contortrix***, in addition to other metalloproteases as ***Gloyduis saxatilis*** metalloprotease, ***Bothrops jararaca*** metalloprotease, **(*Maruyama et al., 2002)***, metalloprotease from ***Crotalus molossus molossus*** metalloprotease, and ***Agkistrodon contortrix (ACLPREF)*** metalloprotease, **(*Selistre de Araujo and Ownby, 1995)***.

Sample 3 showed similarities with fibrinolytic metalloproteases as ***lebetase*** [fig. 11], ***fibrolase*** [fig. 12], ***neuwiedase***, and ***atroxase***. It also showed similarities with ***Gloyduis halys*** metalloprotease, ***Crotalus molossus molossus*** metalloprotease, and zinc-endopeptidase ***adamalysin II*** from ***Crotalus adamanteus***. As regards **sample 2**, its sequence is mainly formed of the conserved zinc catalytic region, so it had strong similarities with fibrinolytic metalloproteases from ***Bothrops neuwiedi***, ***Crotalus atrox***,

and from ***Macrovipera lebetina***, in addition to many other haemorrhagic and non haemorrhagic zinc metalloproteases.

Application of **CDD** revealed that the sequenced fragments belong to the **reprolysin** family which is a snake venom endopeptidase requiring zinc for catalysis, [fig. 13, 14, 15]. Also 3D model for reprolysin with the aligned sequences of the three fragments was also presented [fig. 16, 17, 18].

SMART program showed that **sample 1** domain starts at position 20 and ends at position 97**,** and **sample 3** domain starts at position 3 and ends at position 127**,** both domains belong to the ***reprolysin*** family.

Using **Predict Protein** program, more information about our protein **was known:**

- **Prosite motif search**: phosphorylation sites were present in **sample 1** and **sample 3,** N-myristoylation site was found in **sample 1** and **sample 2** and zinc binding site was found in **sample 3**.
- **CYSPRED** for cysteine prediction: for **sample 1** and **sample 2** no bonded cysteine residues were found, but in **sample 3**, presence of one disulfide bond at position 83 was predicted.
- As for **Solvent accessibility;**

Sample 1: 43% of the protein is exposed with more than 16% of its surface**.**

accessib type	b	e
% in protein	56.12	43.88

Sample 2: 64% of the protein is exposed with more than 16% of its surface**.**

accessib type	b	e	sub...:	accessib type	b	e
% in protein	64.00	36.00	...set:	% in subset	100.00	0.00

Sample 3: 60% of the protein is exposed with more than 16% of its surface.

accessib type	b	e
% in protein	39.37	60.63

- As regards the **secondary structure**,

Sample 1: was described as **mixed protein;** with 22% helix, 35% extended and 41% loop**.**

sec str type	H	E	L
% in protein	22.45	35.71	41.84

Sample 2: was described as **all-alpha** with 76% helix, 24% loop**.**

sec str type	H	E	L
% in protein	76.00	0	24.00

Sample 3: was described as **alpha-beta protein** with 30% helix, 27% extended and 41% loop.

sec str type	H	E	L
% in protein	30.71	27.56	41.73

- **Globularity prediction**: described **sample 3** and **sample 2** to be as compact as a globular domain, and **sample 1** may be globular but not as compact as a domain.

Comparison between sequences of samples 1, 2 and 3 using **BLAST two sequence alignment** revealed that there is no significant alignment between samples 1 and 2, while samples 1 and 3 have sequence homology (not identical) and samples 3 and 2 also have sequence homology in the region of the consensus zinc binding domain**.** These results suggest that the three fragments may be truncated from one gene.

Discussion

Fibrinolytic enzymes that directly dissolve fibrin from blood clots have been extensively studied from various sources and used clinically as thrombolytic agents. A number of these fibrinolytic enzymes have been isolated and characterized from snake venom and some of the genes encoding those enzymes have been cloned, ***(Lee and Park, 2000).***

Cerastase is an anticoagulant protease, isolated from the venom of ***Cerastes cerastes*** (Egyptian Sand Viper) by a combination of gel filtration, ion exchange chromatography, and HPLC. It is a proteolytic enzyme that hydrolyzes casein, fibrinogen and fibrin. It degrades α-A chain of fibrinogen followed by hydrolysis of the β-B chain, while the γ-chain relatively resists hydrolysis. ***Cerastase*** also degrades the three chains of fibrin at different rates. In conclusion, the anticoagulant property of ***Cerastase*** **F-4** is due to destruction of fibrinogen, ***(Daoud et al., 1986).***

In this work, molecular biology studies were performed on the venom gland tissue of ***Cerastes cerastes,*** in an attempt to identify the gene sequence of ***Cerastase*** **F-4** or its catalytic domain to be followed later on, by cloning and expression of this protein owing to its significant value as an anticoagulant and thrombolytic agent.

Primer design and choice of the appropriate primers were first performed. The PCR products were extracted from agarose gel and sequenced by the Automated DNA sequencing technique. Sequences were analyzed using BLAST then translated using the nucleic acid translator program.

Siigur et al., (1996) purified ***lebetase***, Aα, Bβ-fibrinogenase, from the crude ***Macrovipera lebetina*** venom, with fibrinolytic activity. On the basis of the sequence of a CNBr-peptide, the oligonucleotide primer 1 was synthesized for PCR reaction. The primer 2 was synthesized on the basis of conserved region of metalloproteinases. The 246 bp PCR product was used to screen the cDNA library made with the mRNA from ***Vipera lebetina*** venom glands and the clone (Le3) which was selected to be completely sequenced consisted of 2011 bp, having an open reading frame of 1434 bp. The deduced amino acid sequence from this open reading frame was 478 amino acids.

Siigur et al., (2003) deduced amino acid sequences of two anticoagulant serine fibrinogenases (alpha and beta fibrinogenases) from ***Vipera lebetina*** venom. They were named ***VLAF*** and ***VLBF*** and they exhibit significant similarities with other snake venom serine proteases.

Rodrigues et al., (2000) isolated the cDNA encoding ***neuweidase*****;** a fibrin(ogen)olytric non haemorrhagic metalloprotease purified from ***Bothrops neuweidi*** snake venom by RT-PCR from the venom gland RNA, using oligonucleotides based on the partially determined amino acid sequence of this metalloprotease. The full sequence contained approximately 594 bp which codified 198 amino acid residues with estimated molecular weight of 22,375.

Bioinformatics is the application of tools of computation and analysis to the capture and interpretation of biological data, ***(Bayat, 2002).***

The methods of bioinformatics have become essential to research progress in structural biology, genomics, structure based drug design and molecular evolution, ***(Benton, 1996).***

Different bioinformatics tools were applied to the sequenced samples for their further analysis.

Similarity searches were performed through standard Nucleotide-nucleotide BLAST (BLASTN) and Protein-protein BLAST (BLASTP) to align the sequenced samples with sequences published in the GenBank.

Results of alignment of **Sample 1** revealed similarities with non haemorrhagic fibrin(ogen)olytic metalloproteases from ***Vipera lebetina***, ***Bothrops neuwiedi*** (identity 90%) and ***fibrolase*** from ***Agkistrodon contortrix contortrix***, in addition to other metalloproteases as ***Gloyduis saxatilis*** metalloprotease, ***Bothrops jararaca*** metalloproteas, metalloprotease from ***Crotalus molossus molossus*** (identity 85%) and ***Agkistrodon contortrix (ACLPREF)*** metalloprotease with identity 84%**.**

Sample 3 showed similarities with fibrinolytic metalloproteases as ***lebetase*** (identity 86%)**,** ***fibrolase***, ***neuwiedase*** and ***atroxase***. It also showed similarities with ***Gloyduis halys*** metalloprotease**,** ***Crotalus molossus molossus*** metalloprotease and zinc-endopeptidase ***adamalysin II*** from ***Crotalus adamanteus* .**

The previous results are in agreement with sequence alignment of ***lebetase*** which showed similarity with ***fibrolase, atroxase, adamalysin II, HR1a*** and ***HR1b*** and ***Agkistrodon contortrix contortrix ACLPREF (Siigur et al., 1996)*** and in agreement with sequence alignment of ***atroxase*** which showed similarity with ***fibrolase*** and ***adamalysin II (Baker et al., 1995)*** and also agrees with sequence alignment of ***neuwiedase*** which showed similarity with ***atroxase*** and ***adamalysin II (Rodrigues et al., 2000).***

As regards **sample 2**, its sequence is mainly formed of the conserved zinc catalytic region**,** so it had strong similarities with fibrinolytic metalloproteases from ***Bothrops neuwiedi*** (identity 91%), ***Crotalus atrox*** (identity 91%) and from ***Macrovipera lebetina*** (identity 87%) and many haemorrhagic and non haemorrhagic zinc metalloproteases**.**

CDD (conserved domain database) was also used to identify the conserved structural core motif of the corresponding domain family to

which the query (sample) belonged [**sample 1, sample 2,** and **sample 3**]. Also 3D model for the aligned sequences was also presented.

Application of the previously mentioned tool revealed that our protein belongs to the **reprolysin** family which is a snake venom endopeptidase requiring zinc for catalysis.

In agreement with this result, ***Lebetase;*** fibrin(ogen)olytic protein from ***Macrovipera lebetina*** snake belongs to the ***reprolysin*** family of metalloproteinases, ***(Siigur et al., 1996).***

SMART was used for identification of protein domains and comparative study of domain architectures. When applied on **sample 1**, it showed that its domain starts at position 20 and ends at position 97**,** as for **sample 3**, its domain starts at position 3 and ends at position 127**,** both domains belong to the ***reprolysin*** family.

Predict protein program provided information about probable secondary structure of the sequenced protein fragments, with prediction of the possible position of disulfide bonds. In addition, prediction of whether the protein is globular or not, as described before.

Prediction of one disulfide bond in sample 3 at position 83 came in contrast to the majority of the reprolysin family as ***lebetase*** which contain 3 disulfide bonds and other metalloproteases as ***atroxase, adamalysin II*** and ***atrolysin c*** (haemorrhagic metalloprotease from ***Crotalus atrox***) which have 2 disulfide bonds, ***(Siigur et al., 1996).*** This could be explained by the fact that we have parts of the gene which are sequenced.

Secondary structure prediction of **samples 1** and **3** came in agreement with that of ***fibrolase*** (fibrinolytic metalloprotease from ***Agkistrodon contortrix contortrix***) which is formed of 25% helix and this is consistent with structures of members of metalloproteases ***(Manning, 1995)***.

**Relation between sample 1, 2 and 3 sequences:*

Comparison between sequences of samples 1, 2 and 3 using **BLAST two sequence alignment** revealed that there is no significant alignment

between samples 1 and 2, while samples 1 and 3 have sequence homology (not identical) and samples 3 and 2 also have sequence homology in the region of the consensus zinc binding domain**.** These results suggest that the three fragments may be truncated from one gene.

***Conclusion:**

Only parts of the cDNA encoding for the **F-4** gene isolated from ***Cerastes cerastes*** were sequenced and analyzed, showing significant similarities to members of fibrin(ogen)olytic zinc dependant metalloproteinases, in addition to other haemorrhagic and non haemorrhagic metalloproteases when compared with sequences in the database through BLAST. The sequences of samples 2 and 3 contained the zinc binding motif which is conserved in all metalloproteases and is responsible for catalytic activity. It was also found that **samples 1**, **2** and **3** contain a conserved domain similar to the conserved domain in ***reprolysin*** and thus it was suggested that our protein belong to the **reprolysin family** of snake venom endopeptidases. Presence of cysteine residues had been detected in **sample 3** with a strong probability of presence of disulfide bonds and it also appeared to be globular in structure and as compact as a domain.

Complete sequencing of the cDNA encoding for the ***cerastase*** **F-4 gene** is recomended to be followed by cloning and expression of the protein as this protein appears to have great potentials in fibrin as well as fibrinogen lysis that can be of good use in thrombolytic therapy.

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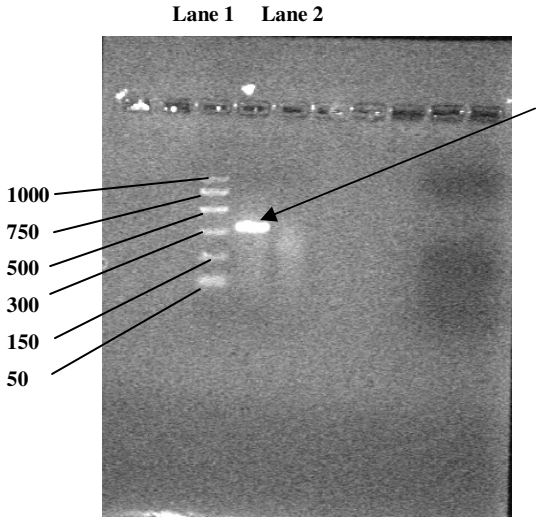
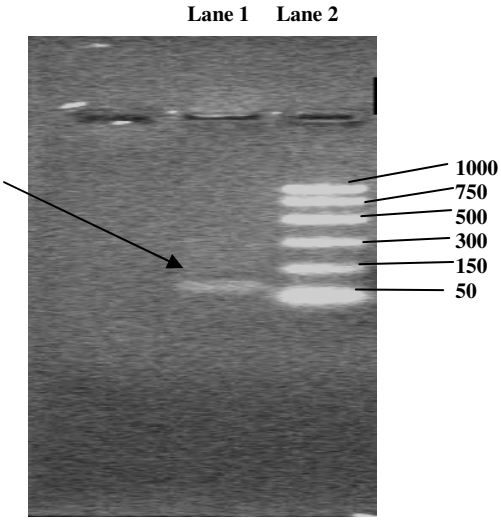


Figure (1): PCR product of **sample 1**

Lane 1: shows separation of low molecular weight markers.

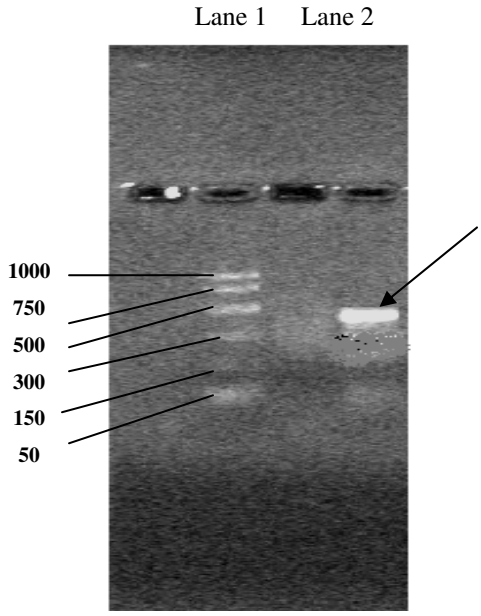
Lane 2: shows PCR product of **sample 1** appearing as a single band separated ~ at 350 bp.



Figure(2): PCR product of **Sample 2**

Lane 1: shows PCR product of **sample 2** appearing as a single band separated ~ at 60 bp.

Lane 2: shows separation of low molecular weight markers.



Figure(3): PCR product of **sample 3**

Lane 1: shows separation of low molecular weight markers.

Lane 2: shows PCR product of **sample 3** appearing as a single band ~ at 400 bp.

Figure (4): sample 1 sequence [304 bp]

```
  1 CTNTTAACAG ATTTGAATTG TTACCTTTAA TGTGACTTCA CACTTTTGAA  50
 51 NACTCATANA GCACTGGTTT GCATAAGAAA TTTGGTCCAA AGAAAGAANT 100
101 TGATTAACGT TCAATCAGCA GTGGAAGTTA CTTTGAACTC ATTTGGAGAA 150
151 TGGAGAGAGA GAGATTTGCT GAATCGGAAA AAACATGATA ATGCTCAGTT 200
201 ACTCACGGGC ATTAACCTCA ATGGAGACAC TATAGGATTT GGTTTTGTGG 250
251 GCAGCATGTG CATGCCGACG AAATCTGTAG GAATTGTTCA GGATCATAGT 300
301 GCAANNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 350
351 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 400
```

Figure (5): Amino acid sequence of sample 1

FNRFELLPLM !LHTFETHKP GLHKKFGPKK ELINVQSAVE VTLNSFGEWR ERDLLNRKKH DNAQLLTGIN LNGDTIGFGF VGSMCMPTKS VGIVQDHSA

Figure (6): sample 2 sequence [57 bp]

```
 1 TTTTTGGTGG  GAGTTACAAT  GGCCCAGGAG 30
31 ATAGGTCATA  ATTTGGGCAG  AATCAAGNNN 60
```

Figure (7): Amino acid sequence of sample 2

WWELQWFLVG VTMAQEIGHN LGRIK

Figure (8): sample 3 sequence [393 bp]

```
  1 NNAGCATGGG CACTACTGCA TGAATGGTTG TGATTTCAGA GATTTGAATA  50
 51 TCATATAATA CTGGCTAGCC TAGAAATTTG GTCCAATGGA GATTTGATTA 100
101 ACGTTCAGTC ATCAGCAAGT GTTACTTTGA ACTTATTTGG AGAATGGAGA 150
151 GAGAGAGATT TGCTGAATCG CAGAATGCAT GATAATGCTC AGTTACTCAC 200
201 AGCCATTGAC CTTGATGATA ACACTATAGG ATTGGCTTAC TTAGAGGGCA 250
251 TGTGTGATCC GAGGAATTCN TGTAGGAATT ATTGAGGATC ATAGCGCAAT 300
301 ACATCNTTTT GGTTGCAGCT ACAATGGCCC ATGAGATAGG TCATAATTTG 350
351 GGCATGAATC ATGATGGAAA AGTGNCTTTC CCGNGGGNGC AAANNNNNNN 400
```

Figure (9): Amino acid sequence of sample 3

AWALLHEWL! FQRFEYHIIL ASLEIWSNGD LINVQSSASV TLNLFGEWRE RDLLNRRMHD NAQLLTAIDL DDNTIGLAYL EGMCDPRNSV GIIEDHSAIH LLVAATMAHE IGHNLGMNHD GKVLSRGQ

Figure (10): BLAST nucleotide sequence aligment between sample 1 and lebetase

Query = sequence of sample 1, subject = sequence of lebetase

```
>gi|1502367|emb|X97894.1|VLLE3   V.lebetina mRNA for metalloproteinase
lebetase Le3
Length = 2011. Score = 79.8 bits (40), Expect = 2e-12. Identities = 115/140
(82%)
 Strand = Plus / Plus

Query: 160  tttgctgaatcggaaaaaacatgataatgctcagttactcacgggcattaacctcaatgg  219
            |||||||||||| | ||  |||||| ||||||| ||||||||||||||| ||||| |||
Sbjct: 929  tttgctgaatcgcagaatgcatgatcatgctcaattactcacgggcattgacctcgatga  988

Query: 220  agacactataggatttggttttgtgggcagcatgtgcatgccgacgaaatctgtaggaat  279
              ||| ||||||||| | ||  |  | ||||||||||   |||| | | ||||||||||
Sbjct: 989  taacattataggattggcttacgatgacagcatgtgcgacccgaggtattctgtaggaat  1048

Query: 280   tgttcaggatcatagtgcaa  299
             ||||||||||||||||||||
Sbjct: 1049  tgttcaggatcatagtgcaa  1068
```

Figure (11): BLAST nucleotide sequence aligment between sample 3 and lebetase

Query = sequence of sample 3, subject = sequence of lebetase

```
>gi|1502367|emb|X97894.1|VLLE3   V.lebetina mRNA for metalloproteinase
lebetase Le Length = 2011. Score =  285 bits (144), Expect = 3e-74
 Identities = 255/296 (86%). Strand = Plus / Plus

Query: 69    tagaaatttggtccaatggagatttgattaacgttcagtcatcagcaagtgttactttga  128
              ||||| ||||| ||||||||||||||||||||||||| || ||||||||||||||||||
Sbjct: 841   tagaagtttggaccaatggagatttgattaacgttcagccagcagcaagtgttactttga  900

Query:  129  acttatttggagaatgnnnnnnnnnnnnnttttgctgaatcgcagaatgcatgataatgctc  188
             |||||||||||||||            |||||||||||||||||||||||||| ||||||
Sbjct:  901  acttatttggagaatggagagagagagatttgctgaatcgcagaatgcatgatcatgctc  960

Query:  189  agttactcacagccattgaccttgatgataacactataggattggcttacttagagggca  248
             | |||||||| | ||||||||| |||||||||| |||||||||||||||   ||  |||
Sbjct:  961  aattactcacgggcattgacctcgatgataacattataggattggcttacgatgacagca  1020

Query: 249   tgtgtgatccgaggaattctgtaggaattattgaggatcatagcgcaatacatcttttgg  308
              |||| || |||||| ||||||||||||| || |||||||||| ||||||  || |||||
Sbjct: 1021  tgtgcgacccgaggtattctgtaggaattgttcaggatcatagtgcaataattcgtttgg  1080

Query: 309   ttgcagctacaatggcccatgagataggtcataatttgggcatgaatcatgatgga   364
              |||||| |||||||||||| ||| | ||||||||| ||||||||||||||||||||
Sbjct: 1081  ttgcagttacaatggcccacgagctgggtcataatctgggcatgaatcatgatgga  1136
```

Figure (12): BLAST amino acid sequence aligment between sample 3 and fibrolase

Query = sequence of sample 3, subject = sequence of fibrolase

>gi|26393688|sp|P83255|FIBR_VIPLE Fibrolase (VlF) (Fibrinolytic proteinase) (Non-hemorrhagic fibrinolytic metalloproteinase). Length = 202. Score = 177 bits (450), Expect = 1e-44. Identities = 84/112 (75%), Positives = 97/112 (86%)

```
Query: 9   LFQRFEYHIILASLEIWSNGDLINVQSSASVTLNLFGEWRERDLLNRRMHDNAQLLTAID 68
            ++    H  L+++E+WSNGDLINVQ  A+VTLNLFGEWRERDLLNRRMHDNAQLL  +
Sbjct: 43  FYRDLNVHFTLSAVEVWSNGDLINVQPEATVTLNLFGEWRERDLLNRRMHDNAQLLNNVA 102

Query: 69  LDDNTIGLAYLEGMCDPRNSVGIIEDHSAIHLLVAATMAHEIGHNLGMNHDG 120
            LDDNTIGLAY EGMCDP+ SVGI++DHSAI+ +VAATMAHEIGHNLGMNHDG
Sbjct: 103 LDDNTIGLAYDEGMCDPKYSVGIVKDHSAINRMVAATMAHEIGHNLGMNHDG 154
```

Figure (13): Conserved domain for sample 1

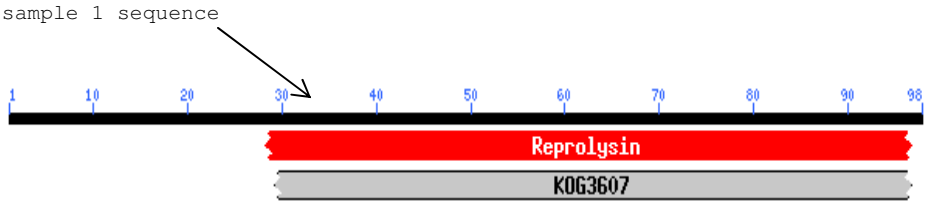


Figure (14): Conserved domain for sample 2

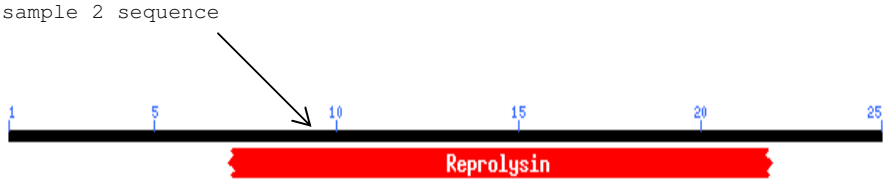
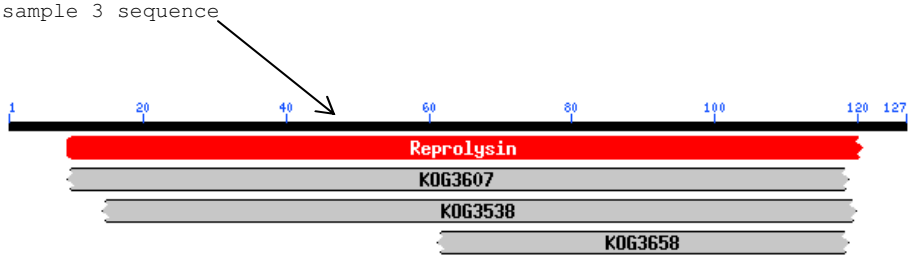


Figure (15): Conserved domain for sample 3



Three dimensional structure of Reprolysin with aligned domains of samples 1, 2 and sample 3:

Figure (16): Sample 1

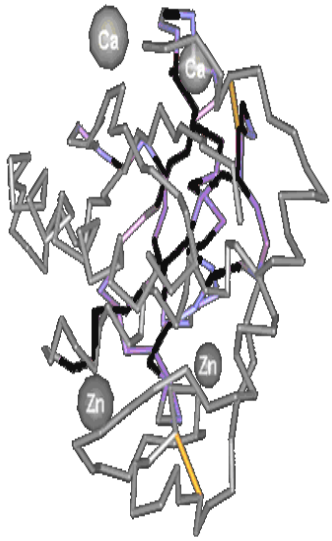


Figure (17): Sample 2

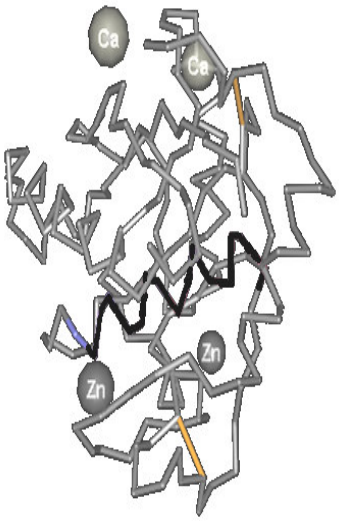


Figure (18): Sample 3