

Indirect Genetic Characterization of *Serratia marcescens*

A. Mohankumar (Corresponding Author)

Department of Zoology, Chikkanna Govt. Arts College,

Tirupur- 641 602, Tamilnadu, India

E-mail: moniver@satyam.net.in

R. Hari Krishna Raj

Department of Microbiology, Sengunthar Arts and Science College

Tiruchengode- 637 205, Namakkal Dt, India

E-mail: harikrishraaj@yahoo.co.in

Received: June 10, 2012 Accepted: June 24, 2012

doi:10.5296/jbls.v3i1.1935

URL: <http://dx.doi.org/10.5296/jbls.v3i1.1935>

Abstract

The pure culture isolate of *Serratia marcescens*, obtained from a clinical specimen was maintained in nutrient agar medium at 4°C. It was genetically characterized by performing 16s rRNA sequence analysis, neighbour tree exposition, phylogenetic tree construction and peptide mass fingerprinting of serratiopeptidase, obtained from it. In order to obtain high purity of serratiopeptidase from the fermentation broth of pure culture isolate of *Serratia marcescens*, some separation and purification techniques were also explored. The results demonstrate that the combination of bio separation processes of ammonium sulfate precipitation with ion exchange chromatography is suitable for the separation and purification of serratiopeptidase from culture broth of *Serratia marcescens*. Peptide mass finger printing was performed and the amino acid sequence of serratiopeptidase was elucidated, compared and confirmed with clustal pair wise alignment database tool to confirm that the product produced and purified was serratiopeptidase from *Serratia marcescens*. Thus, indirectly *Serratia marcescens* has been completely genetically characterized using its therapeutically important product serratiopeptidase and the 16s rRNA sequencing of the bacteria.

Keywords: Genetic characterization, *Serratia*, Serratiopeptidase

1. Introduction

Serratia marcescens was, for many years, believed to be non virulent but is now known to be an important opportunistic pathogen capable of causing a large variety of infectious diseases in humans (Lyerly and Kreger, 1979; Dorwart *et al.*, 1975; Graber *et al.*, 1965). In addition to the toxic lipo polysaccharides in its cell wall, the bacterium produces extracellular proteases. *Serratia* protease preparations of undemonstrated homogeneity have been reported to elicit skin, cornea and lung damage (Conroy *et al.*, 1975). Various investigators have described procedures for obtaining purified preparation of *Serratia* proteases (Aiyappa and Harris, 1976; Lyerly and Kreger, 1979). Many of these isolates do not form pigment and are resistant to various antibiotics. The potential role of the protease produced by this organism was proteolytic destruction of corneal tissue and may be a cause of corneal lesion. A number of strains of *Serratia marcescens* have been reported to produce one to four proteases (Liu, 1961).

The present study is concerned with the indirect approach to genetically characterize *Serratia marcescens* by the production and purification of serratiopeptidase enzyme and its characterization apart from its 16s r RNA sequencing.

2. Materials and Methods

The strain of *Serratia marcescens* culture used in this study was isolated from a clinical specimen (corneal lesion).

2.1 Genotypic characterization

2.1.1 DNA Extraction, PCR Amplification and Agarose Gel Electrophoresis

From the overnight culture of pure culture isolate *Serratia marcescens*, the genomic DNA was extracted as per the procedure given by Helini Biomolecules, Chennai, India. Then the extracted DNA was amplified by the polymerase chain reaction. The amplified DNA was loaded into 2% agarose gel along with 10 µl of 100bp DNA ladder. The band pattern was observed by viewing the gel in UV transilluminator.

2.1.2 16s rRNA Sequence Analysis

The PCR amplified product was subject to 16s rRNA sequence analysis at Helini Biomolecules, Chennai, India using applied biosystems sequencer and an electropherogram data was obtained. The FASTA sequence obtained was compared with the database to confirm the identity of the *Serratia species*.

2.1.3 Clustal Sequence Allignment for Neighbour and phylogenetic tree construction

The clustal sequence alignment was done with the FASTA sequence obtained, comparing it with other species of *Serratia sps*. The clustal alignment tool helped in construction of a neighbour tree and phylogenetic tree to assign the isolated bacteria to a particular species in the taxonomical classification. Thus the sequence establishes the complete genetic identity of the organism.

2.1.4 Production and Purification of Serratiopeptidase

The identity of the organism was also indirectly achieved with the help of the serratiopeptidase produced by it. Hence the isolated pure culture of *Serratia marcescens*, was inoculated in trypticase soy broth (pH7.2). Then the broth was incubated at 37° C for 20 to 24 hours. The supernatant fluids were obtained by centrifugation of the production medium at 10000 rpm for 10 minutes and was used as the source of the enzyme.

2.2 Dialysis

Ammonium sulfate was added slowly to the culture supernatant fluids, with gentle stirring by using magnetic stirrer, to a final concentration of 60% saturation (420g/liter). After 24 hours, the precipitate was obtained by centrifugation. Then the precipitate was dissolved in 0.02M potassium phosphate buffer (pH7.0). This precipitant mixture was dialyzed overnight against phosphate buffer (pH7.0).

2.3 Column Chromatography

The dialysed sample was applied to a column of DEAE-Cellulose equilibrated with 10mM Tris Hydrochloride buffer (pH8.3). A linear gradient consisting of 2 liters of 10mM Tris HCL buffer (pH8.3) and 2 liters of the same buffer with 0.3m NaCl was then used.

Elute was collected for the measurement of absorbance at 280nm and enzyme activity. The active fractions were pooled, dialyzed against double distilled water at 4°C. The dialyzed sample was stored in refrigerator at 4°C.

2.4 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of the purified Serratiopeptidase. This purified serratiopeptidase was denatured by boiling for 2mins in 2% SDS (Sodium dodecyl sulfate) and 1% mercaptoethanol and then the denatured sample was loaded in the SDS-PAGE. Then the gel (30% acrylamide) was stained with 0.04% coomassie brilliant blue and it was destained with 7% acetic acid.

2.5 Peptide Mass Finger Printing

An artificial structure or an analogue design of the enzyme can be assigned and the serratiopeptidase identity can be confirmed only when the amino acid composition of the enzyme is known. Hence the enzyme was subject to peptide mapping by MALDI – TOF (Time Of Flight) – LCMS (Liquid Chromatography Mass Spectrophotometry) at TCGA research, New Delhi, India.

Pair Wise Alignment

The amino acid sequence was compared with the Serratiopeptidase in database to confirm and characterize the serratiopeptidase produced with the standard serratiopeptidase from *Serratia marcescens*.

3. Results

3.1 Agarose Gel Electrophoresis and PCR Amplification

The isolated DNA was electrophoresed and demonstrated in plate: a and the PCR amplified DNA was electrophoresed and the PCR amplified DNA product is shown in plate:b .

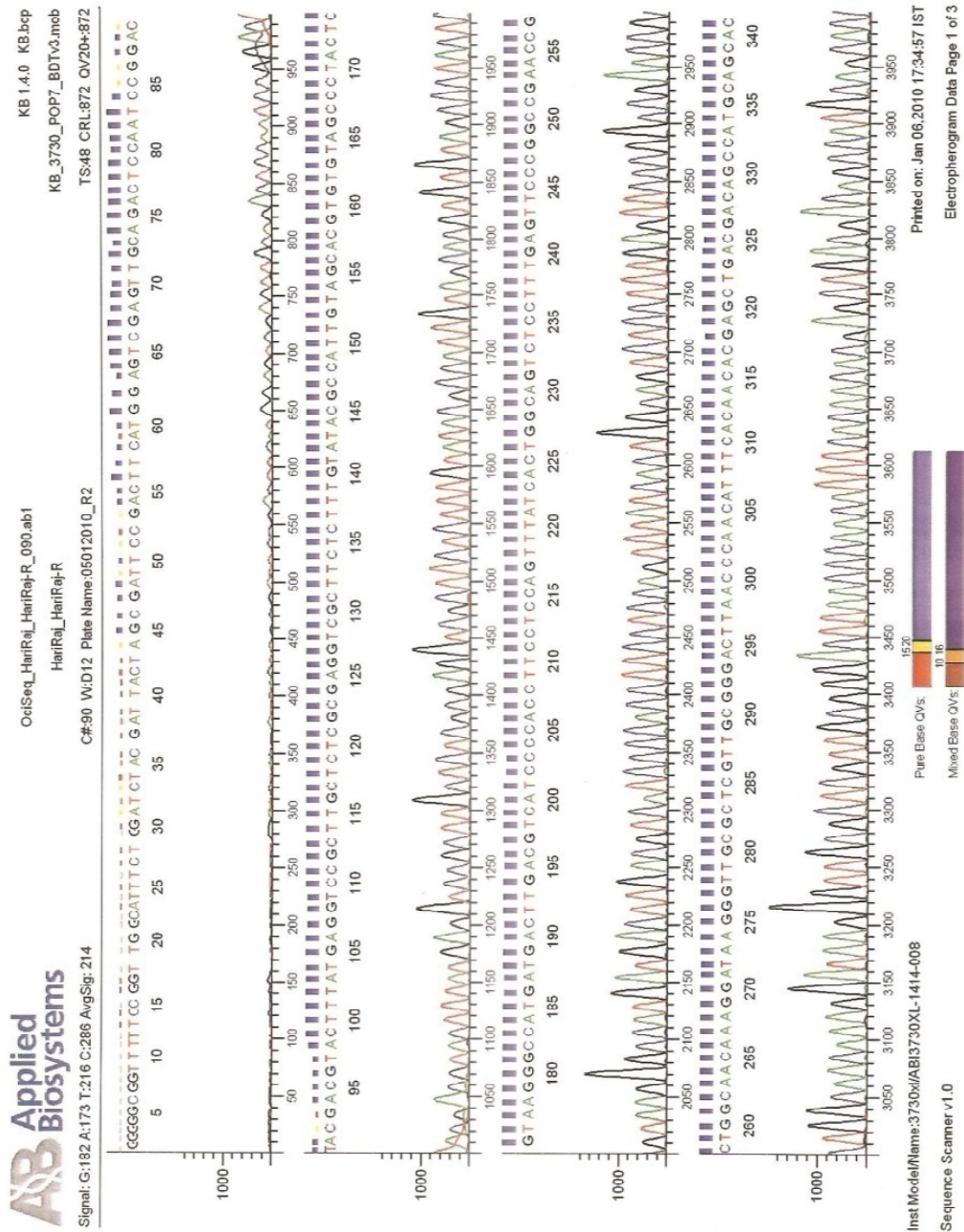


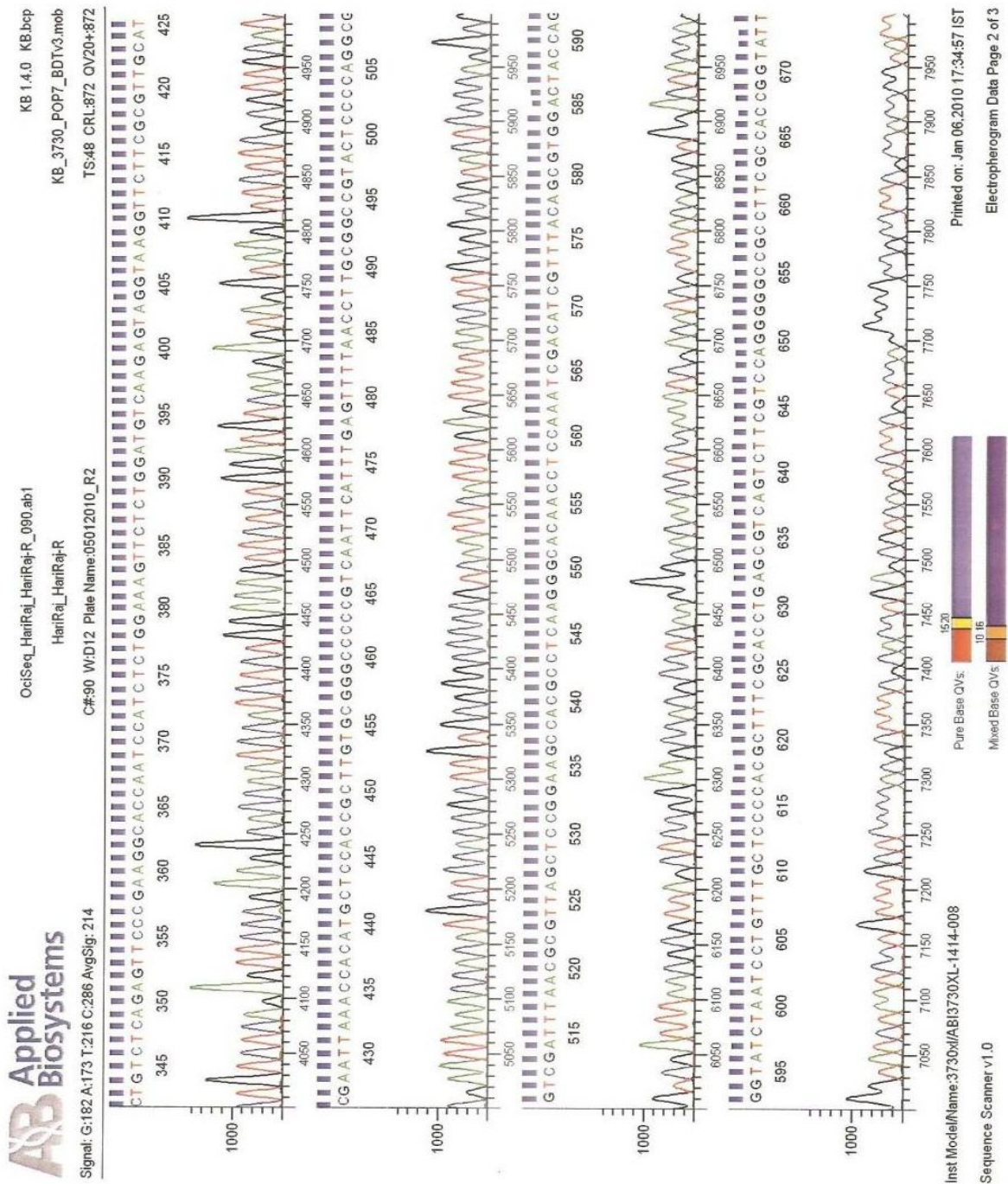
Plate a&b

3.2 16s rRNA Sequence Analysis and phylogenetic tree

The isolate (pure culture) obtained from the clinical specimen was subject to 16s rRNA sequence analysis. From the sequence analysis chromatogram (fig 1.0) the isolate was identified as *Serratia marcescens*. The sequence was compared with closely related sequences of other *Serratia sps* and a neighbour tree(fig. 2.0) was designed. The FASTA

sequence (fig. 3.0) obtained was used for its gene submission in gene databank and structural analysis. Using the sequence obtained and the neighbour tree a “phylogeny tree” was constructed as shown in the figure4.0. Finally from this analysis, it was proved that the isolate was found to be a *Serratia marcescens*, confirming its identity.





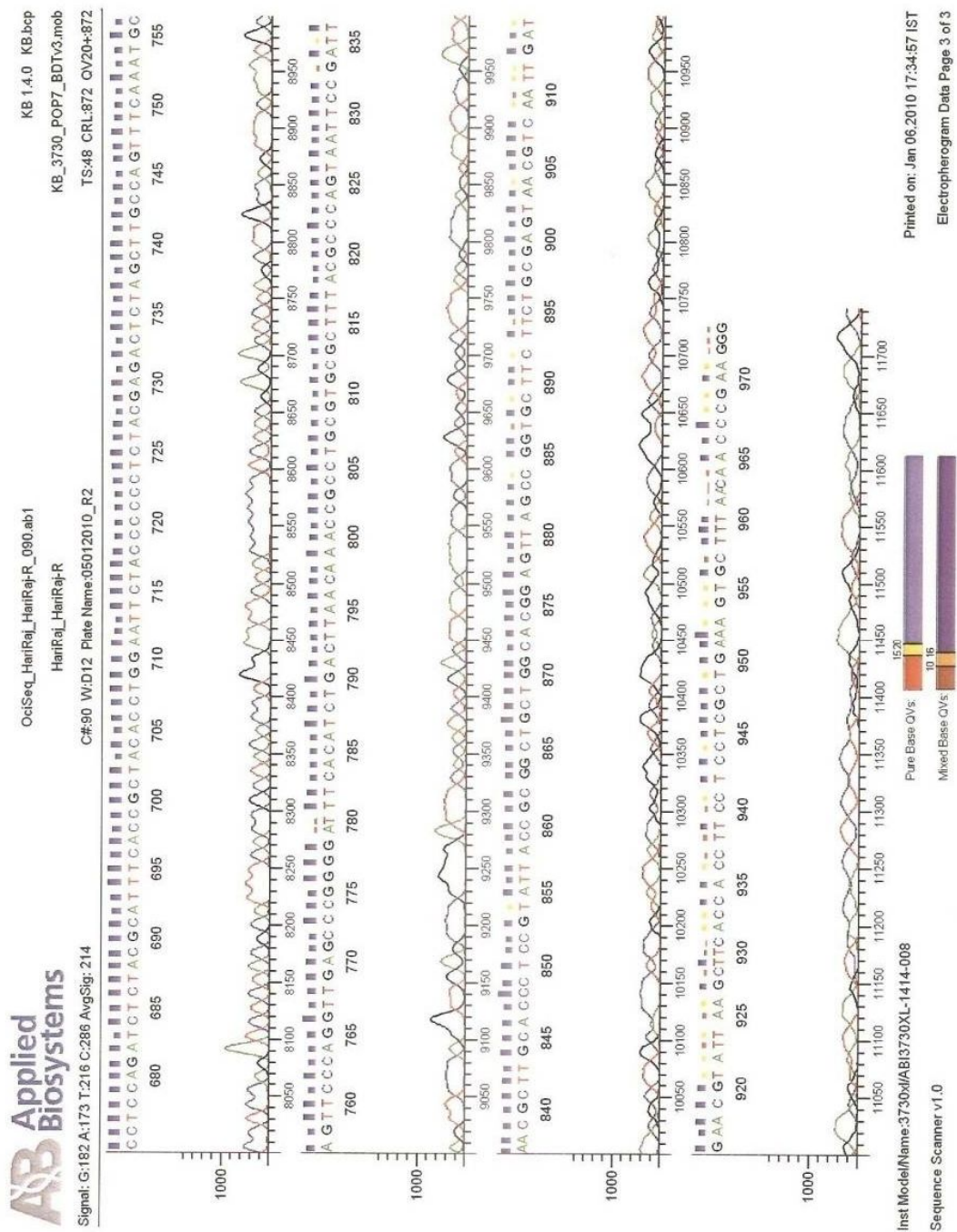


Figure 1. Chromatogram

Populations
Neighbor-Joining/UPGMA method version 3.6a2.1
Neighbor-joining method
Negative branch lengths allowed
+*Serratia nematodiphila*
! +*Serratia nematodiphila* strain P36
!! +*Serratia marcescens*
2-3 +-1
!! +-4 +*Serratia marcescens*
! +-5 +*Serratia marcescens* strain CS265
! +*Serratia marcescens* strain MH6
+-----HariRaj
this is an unrooted tree!

Figure 2. Neighbour Tree

GGGGGCGGTTTTCCGGTTGGCATTCTGGATCTACGATTACTAGCGATTCCGAC
TTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGTACTTTATGAGGTC
CGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATACGCCATTGTAGCACGTGTGTAG
CCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTAT
CACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATTTACAACACGAGCTGACGACAGC
CATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTC
TCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAACCACATG
CTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGT
ACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGC
ACAACCTCCAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTG
TTTGCTCCCCACGTTTCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTC
GCCACCGGTATTCCTCCAGATCTCTACGCATTTACCGCTACACCTGGAATTCTA
CCCCCTCTACGAGACTCTAGCTTGCCAGTTTCAAATGCAGTTCCAGGTTGAGC
CCGGGGATTTACATCTGACTTAACAAACCGCCTGCGTGCCTTTACGCCAGTA
ATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAG
CCGGTGCTTCTTCTGCGAGTAACGTCAATTGATGAACGTATTAAGCTTCACCACC
TTCCTCCTCGCTGAAAGTGCTTTAACAACCCGAAGGG

Figure 3. FASTA Sequence

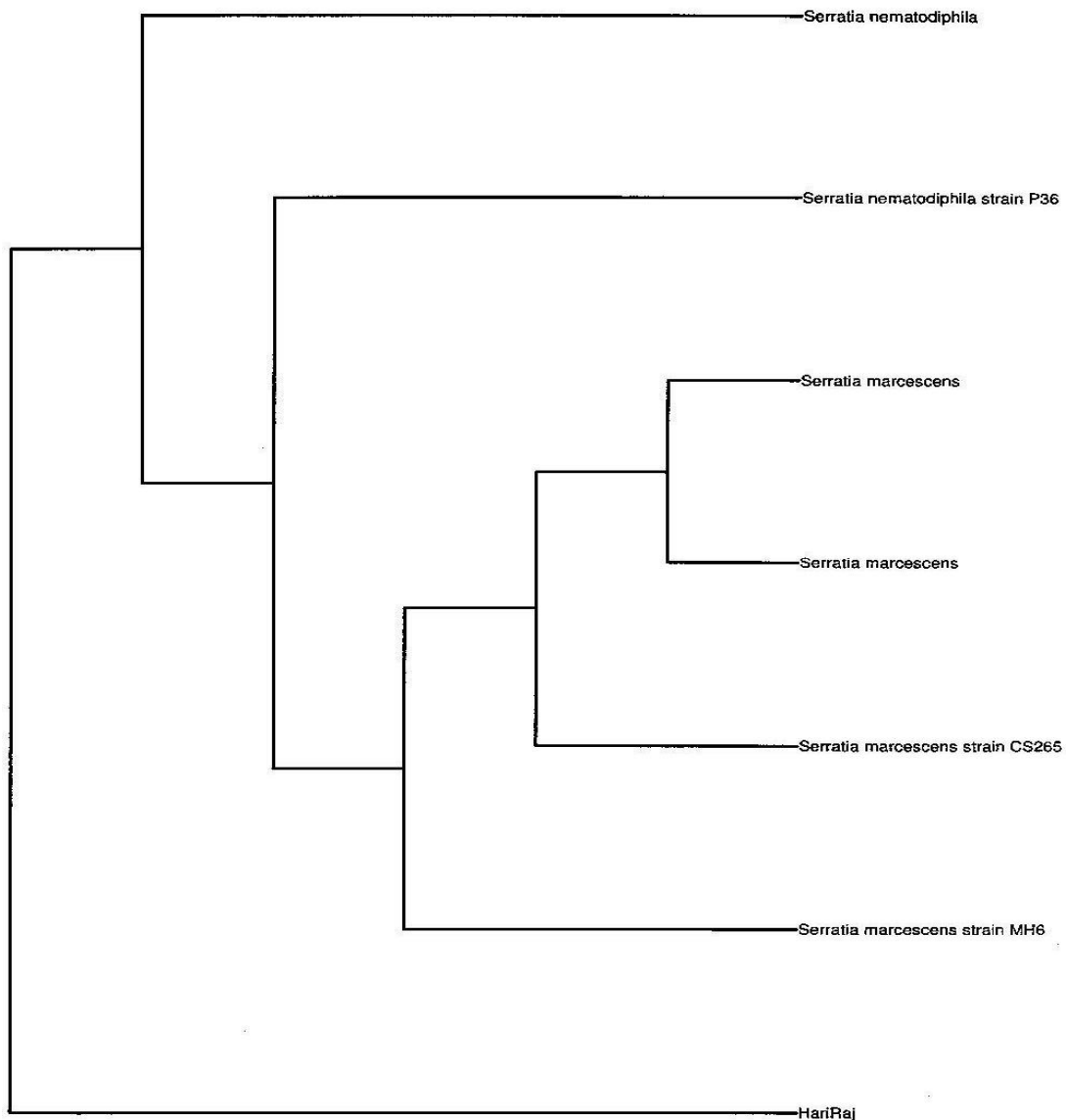


Figure 4. Phylogeny Tree

3.3 Production and Purification of Serratiopeptidase Enzyme

The isolated organism *ie., Serratia marcescens* was inoculated in trypticase production medium for the production of serratiopeptidase enzyme. Then the fermentation broth was centrifuged at 10000rpm for 10-15 mins. After centrifugation, the pellet was discarded and the supernatant broth was stored in refrigerator for further use. The enzyme present in the supernatant broth was concentrated by performing ammonium sulfate precipitation. For the purification, the concentrated enzyme was dialyzed against phosphate buffer for the removal of impurities. Then for further purification the dialyzed enzyme was allowed to run in the DEAE cellulose pack. The purified sample was allowed to check the purification effect by performing SDS-PAGE Analysis.

3.4 Characterization of the Serratiopeptidase Enzyme

The purified enzyme solution was characterized by SDS-PAGE analysis. In this analysis, the molecular weight of the purified enzyme sample was found to 51KDa, when compared to that of the standard protein marker (Plate: C). From the results of SDS-PAGE analysis it was confirmed that “*Serratia marcescens*” produced the purified serratiopeptidase and also it was to examine the effect of purification, and the results indicated that the final product obtained is homogenous.

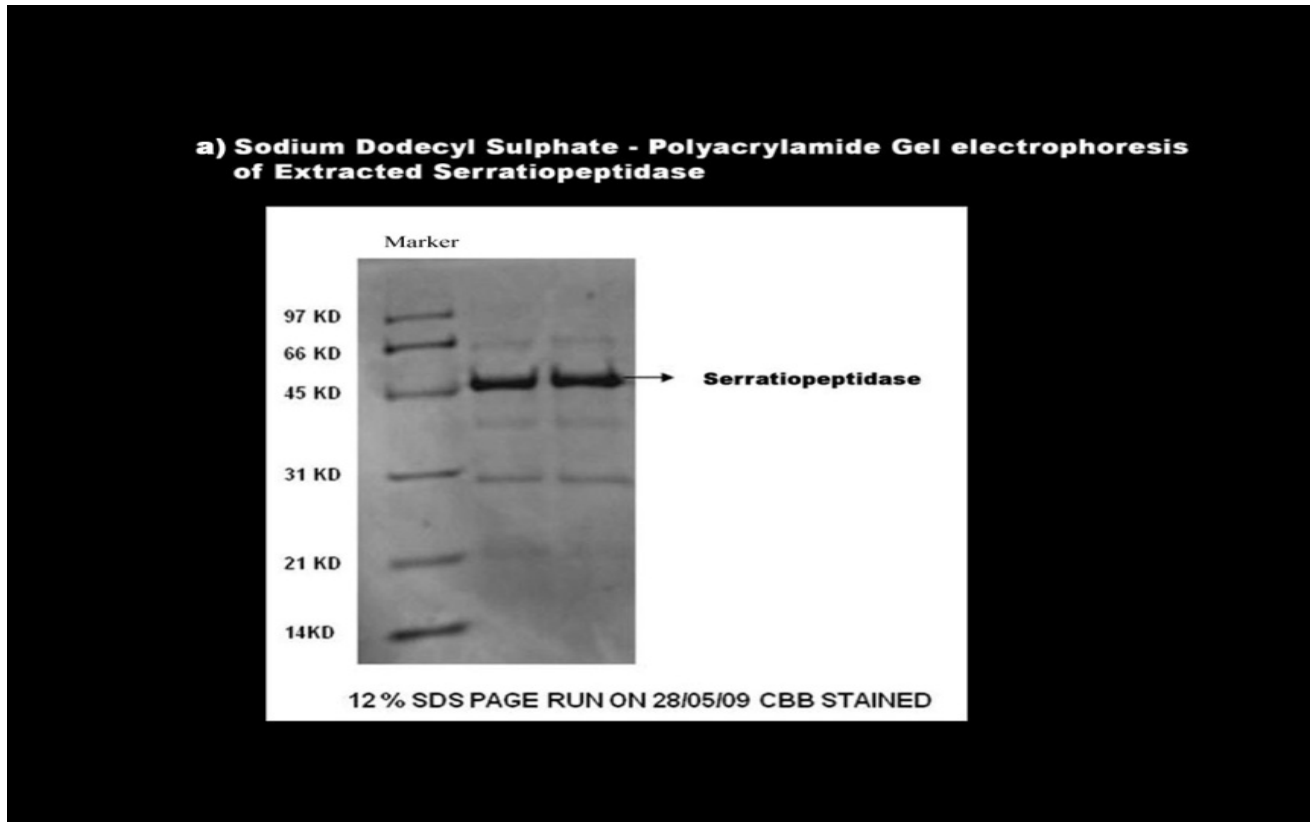


Plate C.

3.5 Peptide Mass Fingerprinting

A mass spectrum of the peptide mixture resulting from the digestion of a protein by an enzyme provides a fingerprint of great specificity. So specific, that it is often possible to identify the protein from this information alone.

This method of identification is much more reliable than using fingerprints based on PAGE migration patterns or HPLC retention times. However, peptide mass fingerprinting is limited to the identification of proteins for which sequences are already known, it is not a method of structural elucidation.

3.6 Mascot Protein View

The peptide sequence of STP obtained matched to PRZN_SERSP of NCBI database with a score of 530. The score 530 indicates high probability match and hence the enzyme can be

confirmed as serratiopeptidase. The mass of the protein was determined to be 52203 Daltons. The enzyme also aids the taxonomical identification of *Serratia marcescens*. The protein sequence is stored in the database of matrix science as a database file (ID MSDB 20060831). This database file, reviews show 3239079 sequence and 1079594700 residues.

Match to: **PRZN_SERSP** Score: **530**

Serralysin precursor (EC 3.4.24.40) (Extracellular metalloproteinase) (Zinc proteinase) (Serrapeptase) (Serrapeptidase) (Serratiopeptidase).-Serratia sp. (strain E-15) (Serratia marcescens (ATCC 21074)).

Found in search of J:\DATA_DELIVERY\LCMS FILES

JUNE2009\AGI\TIRUCHENGODE-HARI KRISHNA RAJA-PROTEIN.mgf

Nominal mass (M_r): **52203**; Calculated pI value: **4.64**

NCBI BLAST search of PRZN_SERSP against nr

Unformatted sequence string for pasting into other applications

Taxonomy: *Serratia sp.* E-15

Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **27%**

Matched peptides shown in **Bold Red**

1 MQSTKKAIEI TESNFAAATT GYDAVDDLLH YHERGNIGIQI NGKDSFSNEQ
51 AGLFITRENQ TWNGYKVFQGV PVKLTFSFPD YKFSSTNVAG DTGLSKFSAE
101 QQQQAKLSLQ SWADVANITF TEVAAGQKAN ITFGNYSQDR PGHYDYGTQA
151 YAFLPNTIWQ GQDLGGQTWY NVNQSNNVNHKHP ATEDYGRQTF THEIGHALGL
201 SHPGDYNAGE GNPTYRDVTV AEDTRQFSLM SYWSETNTGG DNGGHYAAAP
251 LLDDIAAIQH LYGANLSTRT GDTVYGFNSN TGRDFLSTTS NSQKVIFAAW
301 DAGGNDTFDF SGYTANQRIN LNEKSFSDVG GLKGNVSIAA GVTIENAIGG
351 SGNDVIVGNA ANNVLKGGAG NDVLFGGGGA DELWGGAGKD IFVFSAAASDS
401 APGASDWIRD FQKGIDKIDL SFFNKEAQSS DFIHFVDHFS GAAGEALLSY
451 NASNNVTDLS VNIGGHQAPD FLVKIVGQVD VATDFIV

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
44 - 57	792.9500	1583.8854	1583.7580	0.1275	0	
K.DSFSNEQAGLFITR.E (<u>Ions score 95</u>)						
74 - 82	559.3500	1116.6854	1116.5492	0.1363	0	K.LTFSFPDYK.F
<u>(Ions score 33)</u>						
83 - 96	692.4000	1382.7854	1382.6678	0.1177	0	
K.FSSTNVAGDTGLSK.F (<u>Ions score 65</u>)						
97 - 106	582.8300	1163.6454	1163.5571	0.0884	0	K.FSAEQQQQAK.L
<u>(Ions score 66)</u>						
107 - 128	1175.1000	2348.1854	2348.2012	-0.0158	0	
K.LSLQSWADVANITFTEVAAGQK.A (<u>Ions score 109</u>)						
270 - 283	744.8400	1487.6654	1487.6641	0.0013	0	
R.TGDTVYGFNSNTGR.D (<u>Ions score 30</u>)						
295 - 318	875.4500	2623.3282	2622.1776	1.1506	0	
K.VIFAAWDAGGNDTFDFSGYTANQR.I (<u>Ions score 55</u>)						
319 - 333	541.0100	1620.0082	1619.8519	0.1563	1	
R.INLNEKSFSDVGGLK.G (<u>Ions score 28</u>)						
475 - 487	688.4200	1374.8254	1374.7395	0.0859	0	
K.IVGQVDVATDFIV.- (<u>Ions score 33</u>)						
475 - 487	688.4300	1374.8454	1374.7395	0.1059	0	
K.IVGQVDVATDFIV.- (<u>Ions score 49</u>)						

>P1;PRZN_SERSP

Serralysin precursor (EC 3.4.24.40) (Extracellular metalloproteinase) (Zinc proteinase) (Serrapeptase) (Serrapeptidase) (Serratiopeptidase).-*Serratia* sp. (strain E-15) (*Serratia marcescens* (ATCC 21074)).

C;Species PRZN_SERSP: *Serratia* sp. (strain E-15) (*Serratia marcescens* (ATCC 21074)).

C;Family: Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;

Enterobacteriaceae; *Serratia*.

C;Accession: P07268;

C;EMBL; X04127; CAA27738.1; ALT_FRAME; Genomic_DNA.

C;SRCDB SWISSPROT

3.7 Protein Hits

The sequence when subject to matrix science protein hit, the enzyme method. Serralysin precursor protein (EC 3,4,24,140) (Extracellular metalloproteinase) (Zinc proteinase) (serratiopeptidase) from *Serratia sp* (strain E-15), *Serratia marcescens* (ATCC 21074) with a score of 530. Hence the protein is more similar to serratiopeptidase.

Protein hits : PRZN SERS Serralysin precursor (Ec 3.4.24.40) (Extracellular Metalloproteinase) (Zinc proteinase) (Serrapeptase) (Serrapeptidase) (Serratiopeptidase).-*Serratia sp*. (Strain E-15) (*Serratia marcescens* (ATCC 21074)).

AB2633 conserved hypothetical protein Atu0461 [imported]-*Agrobacterium tumefaciens* (Strain C58, Dupont)

Q3N3U3 9DELTA Rubrerythrin.-*Syntrophobacter fumaroxidans* MPOB.

AB2166 hypothetical protein alr2881 [imported]-*Nostoc sp*. (Strain PCC 7120)

Q8EMU2 OCEIH Hypothetical protein OB2748.-*Oceanobacillus iheyensis*.

The enzyme also matched to some other proteins such as Atu 0461 from *Agrobacterium tumefaciens* with a score of 39, Rubrerythrin from *Syntrophobacter fumaroxidans* (score 37), also 2881 (inparted)-*Nostoc sp* (score 36), OB 2748 from *Oceanobacillus iheyensis* (Score 35), and also to some other un assigned proteins .The protein hits helps to identify the role of STP as enzymes that matched the sequence. Hence multifunctional activity of the STP enzyme is exposed.

• Mascot Search Results

User : REENA
Email : arorareena1@rediffmail.com
Search title : TIRUCHENGODE-HARIKRISHNARAJ-PROTEIN A-LCMS
MS data file : J:\DATA_DELIVERY\LCMS FILES
JUNE2009\AGI\TIRUCHENGODE-
HARI KRISHNA RAJ\A-PROTEIN.mgf

Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Taxonomy : Bacteria (Eubacteria) (1478092 sequences)
Timestamp : 11 Jun 2009 at 06:12:10 GMT

4. Discussion

This paper is to describe the identification of *Serratia marcescens* by 16s RNA sequence analysis and indirect approach towards the identification of the organism by the serratiopeptidase enzyme production and its characterization by peptide sequencing.

The red pigmented pure culture isolated from clinical specimen was given to the Helini Biomolecules Research institute, for the 16s r RNA sequencing analysis. This was done for the genetic identification of isolated pure culture. The DNA was isolated from the given culture and amplified by polymerase chain reaction with the help of forward and reverse primer. Then the amplified DNA was sequenced and the sequence was presented in the graphical form (chromatogram) which is shown in the figure1.0. The obtained sequences were compared to the closely related sequences of other bacteria and it was presented in the form of neighbour tree and phylogenetic construct (dendogram). These types of sequence analysis, studied by other researchers were not represented elaborately in their research, as described in this paper. Hence there is little or no scope for a comparative discussion, in this way this work represents a novel approach towards genetic characterization and identification of an organism.

Various investigations have reported the isolation and characterization of *Serratia* proteases (Liu, 1961; Maeda, 1979; Miyata *et al.*, 1970). In this study, the isolation of pure culture from the clinical specimen was identified for the production of serratiopeptidases. This enzyme was purified by ammonium sulfate precipitation, DEAE cellulose column chromatography. (Aiyappa and Harris, 1976; Lysterly and Kreger, 1979; Decedue *et al.*, 1979; Miyata *et al.*, 1970). After purification the enzyme was subject to SDS-PAGE analysis and peptide mass finger printing.

An enzyme of low specificity, which digests proteins to a mixture of free amino acids and di- and tri- peptides, is not a good choice. So the choice of enzyme for MALDI-TOF analysis was very important. A complex mixture containing large numbers of components of similar mass will result in a lot of overlapping peaks. A further consideration for MALDI analysis is that the low mass region, below ~500 Da, is obscured by the presence of matrix peaks. In general, it is best to use enzymes of specificity equal to or greater than trypsin.

Setting the number of allowed missed cleavage sites to zero simulates a limit digest. If the digest is perfect, with no partial fragments present, it will give maximum discrimination and the highest score. If the digest mixtures include some partials that is, peptides with missed cleavage sites, a setting of 1, or may be 2 is chosen for missed cleavage sites. With all these considerations the peptide sequencing was performed to the fullest possibility and was very accurate in identifying serratiopeptidase and subsequently *Serratia marcescens*. Thus a complete indirect and direct genetic identification of *Serratia marcescens* was achieved.

Acknowledgement

We are grateful to the management of Sengunthar Arts and Science college, Tiruchengode, India, for providing the necessary facilities to carry out this research.

References

- Aiyappa, P. S., & J. O. Harris. (1976). The extracellular metalloprotease of *Serratia marcescens* its purification and characterization. *Mol.cell. Biochem.*, 13, 95-100.
- Conroy, M. C., N. H. Bander & I. H. Lapow. (1975). Effects in the rat of intradermal injection of purified proteinases from *Streptococcus and Serratia marcescens*. *Proc. Soc. Exp. Biol. Med.*, 159, 801-806.
- Decedue, C., E.Broussard, A. Larson & H. Braymer. (1979). Purification and Characterization of the extracellular proteinase of *Serratia marcescens*. *Biochem. Biophys. Acta.*, 569, 293-301.
- Dorwart, B., B. E. Abrutya & H. R. Schumacher. (1975). *Serratia* arthritis: Medical eradication of infection in a patient with rheumatoid arthritis. *J. Am. Med. Assoc.*, 225, 1642-1643.
- Graber, C. D., L. S. Higgins & J. S. Davis. (1965). Seldom-encountered agents of bacterial meningitidis. *J. Am. Med. Assoc.*, 192, 114-118.
- Liu, P. V. (1961). Observations on the specificities of extracellular antigens of the genera *Aeromonas* and *Serratia*. *J. Gen. Microbial.*, 24, 145-154.
- Lyerly, D., & A. Kreger. (1979). Purification and Characterization of *Serratia marcescens* metalloprotease. *Infect. Immun.*, 24, 411-421.
- Maeda, H. (1979). Array of proteolytic enzymes by fluorescence polarization. *Anal. Biochem.*, 92, 222-227.
- Miyata, K., K. Maejima, K. Tomoda & M. Isono. (1970). *Serratia* protease.Part I. Purification and general properties of the enzyme. *Agri. Biol. Chem.*, 34, 310-318.
www.matrixscience.com

Copyright Disclaimer

Copyright reserved by the author(s).

This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).