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OMICS Group has organized 500 conferences, workshops and national symposiums across the major cities including San Francisco, Las Vegas, San Antonio, Omaha, Orlando, Raleigh, Santa Clara, Chicago, Philadelphia, Baltimore, United Kingdom, Valencia, Dubai, Beijing, Hyderabad, Bengaluru and Mumbai.

Molecular cloning, expression, purification and functional characterization of 37 kDa Serine Protease from Bombyx mori

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School of Environmental Science
Bharathidasan University
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Tamilnadu, India

Origin of this study

➢Agriculture is one of the most important sectors and its back bone of Indian economy.

➢ India has achieved self sufficiency in food grains but there is an important need to produce additional to 5 - 6 million tonnes of food grains for every year.

➢The modern day agriculture plays a integral role in order to feed the ever expanding population and crop protection (**Paroda.,1999**).

➢Currently, the pesticides are widely used to protect crops from insects; however the conventional usage of pesticide results in developing resistance in insect, damage to the environment and agricultural product thereby affects the human health (**Pimentel., 1997**).

➢To overcome these drawback few alternative methods has been emerged such as Transgenic plant contain Protease Inhibitor, Bt, etc. (**Harsulkar *et al.*, 1999**) and Irradiation techniques (Gamma radiation, X ray irradiation, UV irradiation, Electron Beam irradiation and microwave irradiation, etc) to control the insect pests (**Hyun-Na Koo *et al.*, 2011; Valizadegan *et al.*, 2009; Nelson .,1996**).

Classification

Kingdom : Animalia
Phylum : Arthropoda
Class : Insecta
Order : Lepidoptera
Family : Bombycidae
Genus : *Bombyx*
Species : *mori*

Life cycle of the *Bombyx mori*

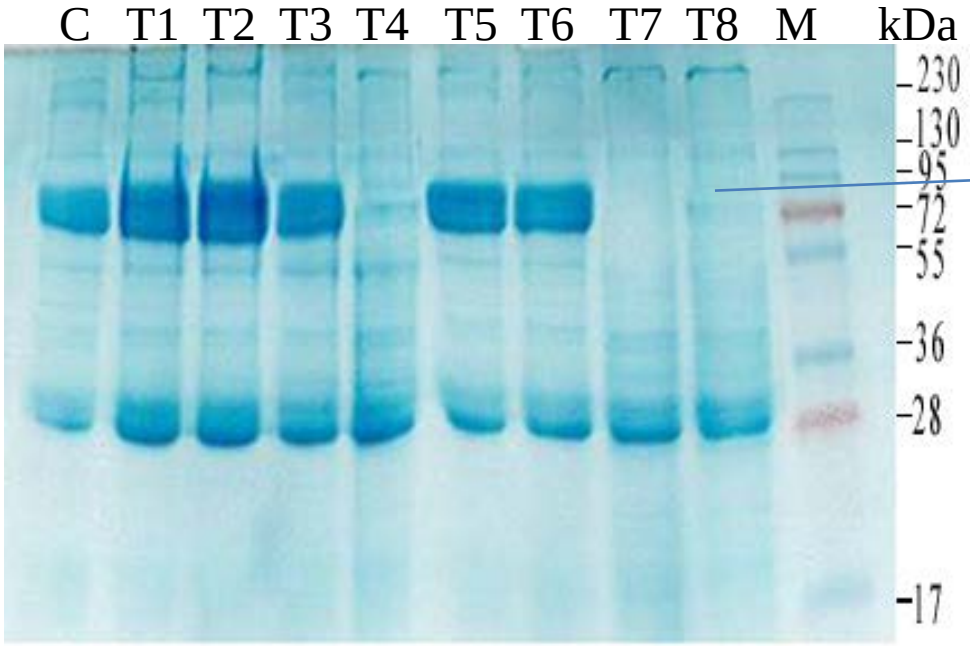
Effect of Microwave irradiation on the larvae and heamolymph protein of lepidopteran model insect silkworm, *Bombyx mori*

A **B**

A. Untreated V Instar larvae of silkworm, *B.mori*

B. Microwave irradiated day 2 of V Instar larvae , silkworm, *B.mori* at 100 W for 7'seconds

SDS-PAGE analysis of Microwave irradiated larval heamolymph



Disappearance of storage protein was observed

Lanes -C for control larval heamolymph

Lanes –T1,T2,T3 and T4 for microwave irradiated larval heamolymph at 100 W for 1,3,5 and 7'seconds respectively.

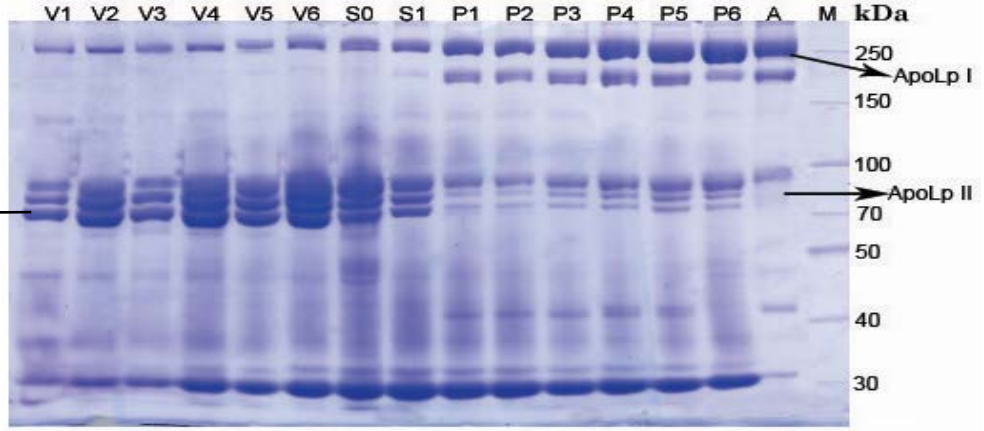
Lane - T5, T6 , T7and T8 for microwave irradiated larval heamolymph at 180 W for 1,3,5 and 7' seconds respectively.

Lane -M stand for molecular weight marker

Importance of Storage protein (Sp)

➢ Storage proteins are synthesized by perviceral fat body tissue and sequestered in perviceral fat body tissue for further developmental (Chandrasekar and **Krishnan**, 2008 ; **Krishnan** and Konig, 2010).

Storage protein ←

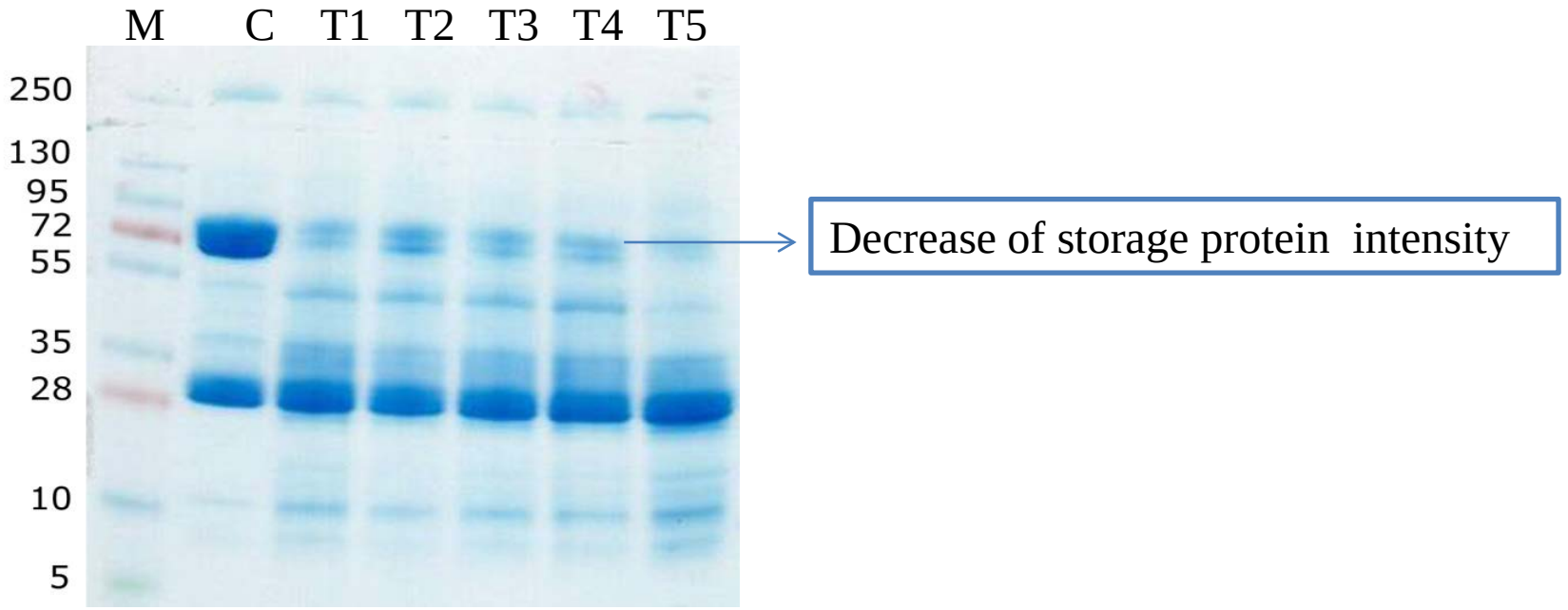


➢These storage proteins are stored as a crystal in fat body tissue and utilized during pupal to adult transformation (Chandrasekar and **Krishnan**, 2008).

➢ The storage protein has three subunits (Sp1, Sp2 and Sp3), Sp3 is female specific protein (Vanishree and **Krishnan**,2005, Chandrasekar and **Krishnan**, 2008)

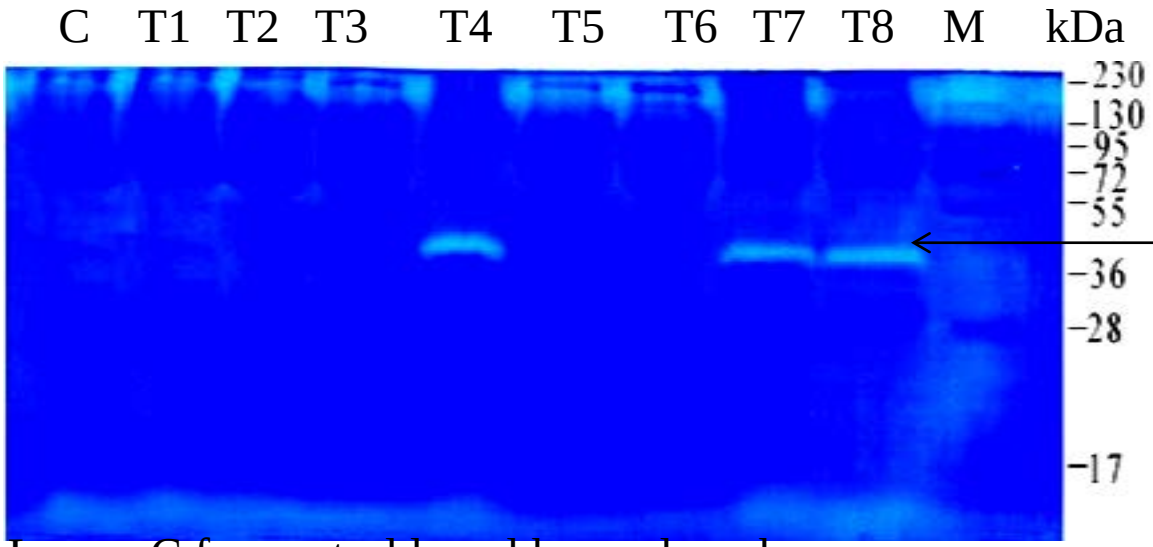
➢The unavailability of storage protein in the microwave irradiated larval heamolymph leads to lack of nutrients which act as a barrier for larval to pupal and pupal to adult development.

SDS-PAGE analysis for Mixture of Control and Microwave irradiated larval heamolymph



Lane M- Protein molecular weight marker
Lane C- Control larval heamolymph (Negative control)
LaneT1 - Control larval heamolymph Vs microwave irradiated larval Heamolymph(100 W for 7's) at 4°C for 1 hour
Lane T2 - Control larval heamolymph Vs microwave irradiated larval Heamolymph(100 W for 7's) at 4°C for 2 hour
Lane T3- Control larval heamolymph Vs microwave irradiated larval Heamolymph(100 W for 7's) at room temperature for 1 hour
Lane T4 - Control larval heamolymph Vs microwave irradiated larval Heamolymph(100 W for 7's) at room temperature for 2 hour
Lane T5 - Microwave irradiated larval heamolymph (Positive Control)

Zymogram analysis of Microwave irradiated larval heamolymph



Lanes -C for control larval heamolymph

Lanes –T1,T2,T3 and T4 for microwave irradiated larval heamolymph at 100 W for 1,3,5 and 7'seconds respectively.

Lane - T5, T6 , T7and T8 for microwave irradiated larval heamolymph at 180 W for 1,3,5 and 7' seconds respectively.

Lane -M stand for molecular weight marker

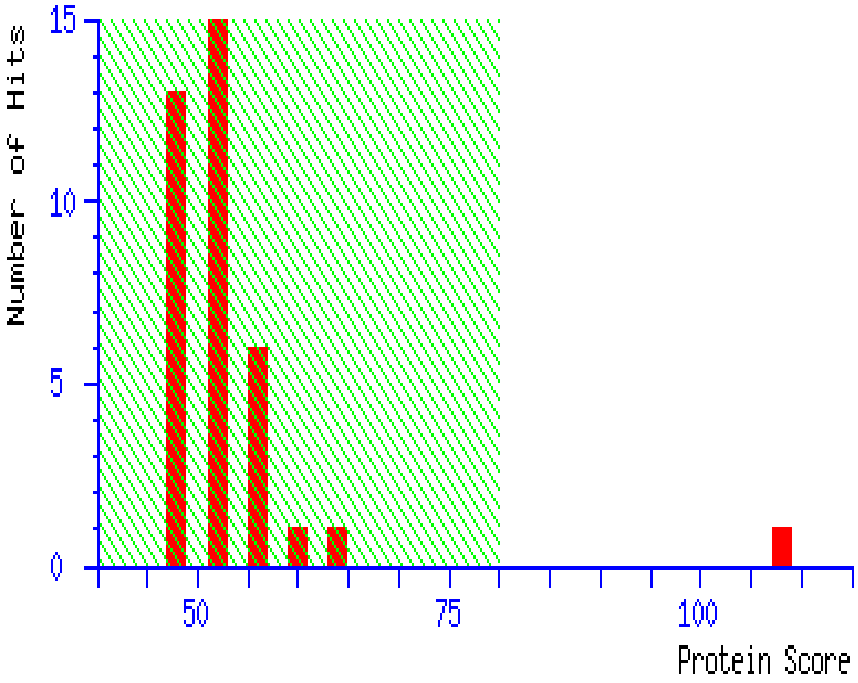
- Similarly, Kaji et al.,(2009) reported that the 37 kDa serine protease was synthesized as a zymogen at larval stage and activated upon pupation for midgut remodeling process.
- We suggest that the microwave irradiation activated the p37k and acted on the storage protein disappearance during larval stage.

Identification of expressed protease using MALDI-TOF-MS

MATRIX SCIENCE

The resulted peptides of 37kDa protease from Mass spec analysis showed match with 37 kDa serine protease

Mascot Search Results
User : M.Kannan
Email : ahilkannanbdu@gmail.com
Search title : p37k
Database : NCBInr 20140323 (38032689 sequences; 13525028931 residues)
Taxonomy : Metazoa (Animals) (5354817 sequences)
Timestamp : 28 Jul 2014 at 06:02:53 GMT
Top Score : 108 for gi|206725503, 37-kDa protease precursor [Bombyx mori]
Mascot Score Histogram
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 80 are significant (p<0.05).

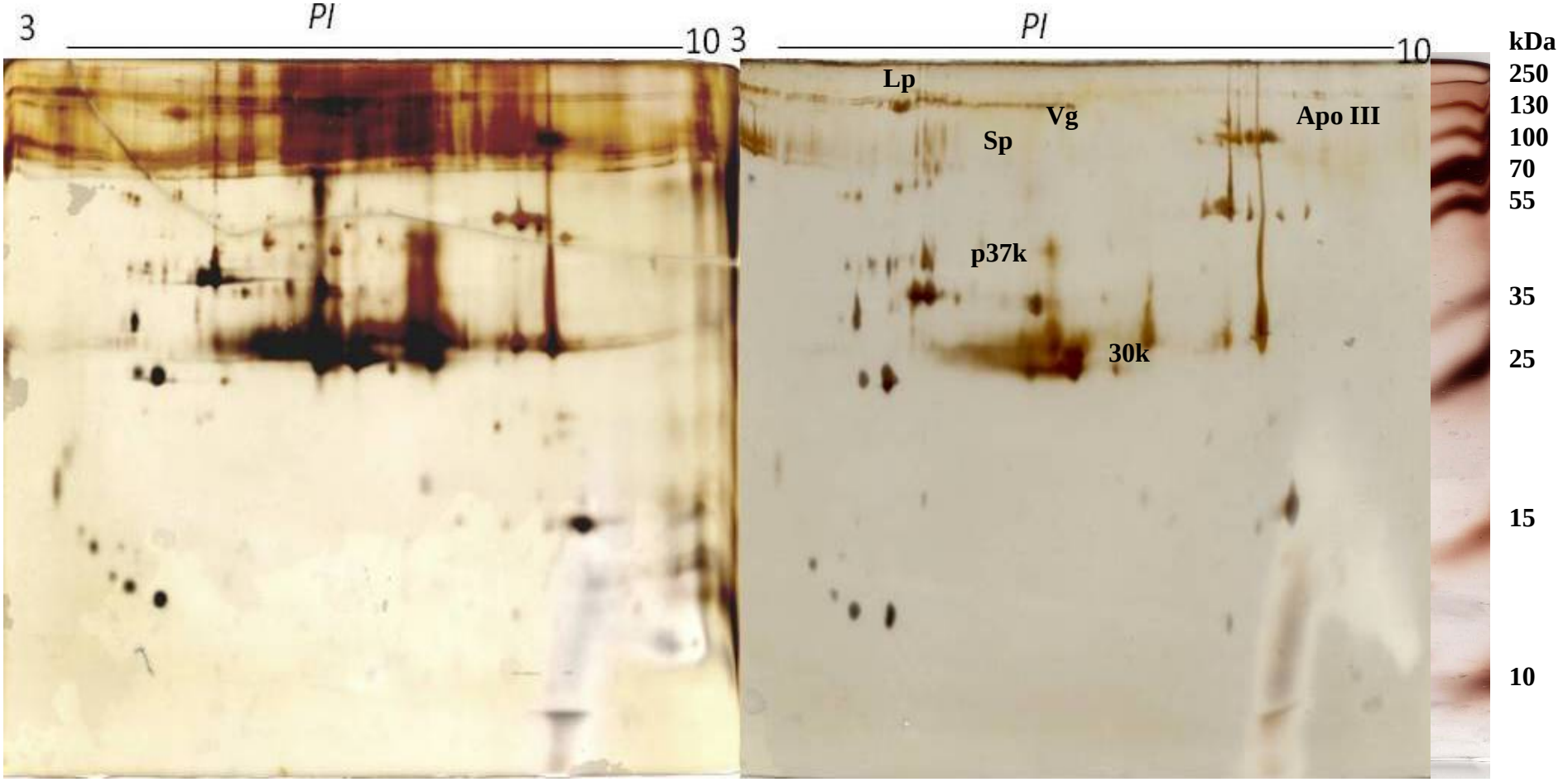


Protein sequence coverage: 28%
Matched peptides shown in ***bold red***.

1	MKWPVIMICL	VGWSSCYTQR	PIGQKDKGFI	DWINNLLGGT	TTTTTLRPID
51	DPPEDCPSCQ	CGIARTRRR**I**	**VGGYETKETE**	**YPWMAALLYG GR**FYCGGALI	
101	SDLYVLTAAH	CTSGFRKERI	TVR**FLEHDR**S	K**VNETKTIDR**	K**VSDIIR**HLR
151	**YNPGTYDSDI**	**ALLK**LAER**VD**	**LSSALK**RVR**S**	**EGDNGTATDD**	**DK**DVGLRPVC
201	LPSSGLSYNN	YTGVVTGWGT	TEEGGSVSNA	LQEVKVPIVT	NEECRK**GYGD**
251	**R**ITDNMICAG	EPEGGRDACQ	GDSGGPMHVL	EMETSKYSEV	GVVSWGEGCA
301	RPNKPGVYTR	VNR**YLTWIKQ**	**NTR**DACNCQ		

2D PAGE analysis of Control and Microwave treated larval Heamolymph of *B.mori*

Control larval heamolymph | **Microwave treated larval**



Lp- Lipophorin (*PI*: 4.91); Vg- Vitellogenin (*PI*: 6.85); Sp- Storage protein1&2 (*PI*:6.04, 6.78); 30k- Anti-apototic protein (*PI*: 7.64); Apo III- Apo-lipophorin III subunit(*PI*: 9.04); p37k-37kDa serine protease (*PI*: 6.46)

Why Serine Protease is important?

➢Serine proteases are major insect gut enzymes involved in the digestion of dietary proteins (Lehane et al., 1998; Paskewitz & Gorman, 1999; Gorman et al., 2000; Yan et al., 2001; Barrett et al., 2003).

➢Serine protease are also known to play critical roles in several biological processes such as blood coagulation, immune responses, signal transduction, hormone activation and development (Nakajima et al., 1997; Gorman et al., 2000; Barrett et al., 2003; Herrero et al., 2005; Jiang et al., 2005).

➢The 37 kDa Serine protease are synthesized during Laval stage as a zymogen and activated after pupation for the growth and midgut tissue remodelling of model insect Silkworm, *B. mori* (Kaji et al.2011).

➢In addition, an insect digestive enzyme, *Bombyx mori* serine protease, showed antiviral activity against *B. mori* nucleopolyhedrovirus (NPV) at the initial site of viral infection (Nakazawa et al., 2004).

➢Eventhough, the involvement of serine protease on various purpose has been poorly understood in lepidopteran model insect silkworm, *B. mori*.

Bombyx mori 37-kDa protease (P37k), mRNA

NCBI Reference Sequence: NM_001135203.1

GenBank Graphics

>gi|206725502|ref|NM_001135203.1| Bombyx mori 37-kDa protease (P37k), mRNA

TGCGAGCATCGCGGTGGTCAAAGTCGCTCGCCGTCCTTGATTTCGGGCCTTGAATACGTACGCGTTGGTGTTATA
GATCTCTGCATATCGTCAATCGATATTTTGTTATCAACAATGAAATGGCCAGTGATTATGATCTGCCTGGTTGGTTG
GTCGAGCTGCTACACCCAGCGGCCCATCGGTCAGAAGGATAAAGGATTTATAGACTGGATCAACAATCTCCTTGG
CGGCACAACGACTACCACGACTTTAAGACCTATAGACGACCCGCCCGAGGACTGCCCAAGCTGTCAATGCGGCA
TAGCACGCACTCGTCGGCGCATCGTGGGCGGATATGAAACGAAAGAGACGGAGTACCCCTGGATGGCCGCTCTT
TTGTACGGCGGAAGATTCTATTGTGGTGGTGCACTTATCAGTGATCTGTACGTTTTGACAGCTGCTCATTGTACTT
CAGGATTCCGCAAGGAACGGATTACAGTTCGGTTCTTGGAGCACGATCGTTCTAAAGTAAACGAAACTAAAACG
ATAGACAGAAAGGTGTCTGACATCATTCGTCATCTGCGGTATAATCCCGGAACTTACGACAGTGATATCGCCCTTT
TAAAACTAGCTGAGAGGGTAGACCTCAGCAGTGCATTGAAGCGAGTTCGCAGTGAAGGAGACAATGGCACTGC
CACGGATGACGACAAGGACGTCGGGCTAAGACCGGTCTGTTTACCCAGTTCTGGACTCTCCTATAACAATTACA
CGGGTGTTGTCACAGGCTGGGGAACTACAGAGGAAGGTGGCTCTGTATCCAATGCATTACAGGAGGTGAAAGTA
CCGATTGTGACAAATGAAGAATGTCGTAAAGGCTACGGTGATCGGATAACAGATAATATGATTTGCGCTGGGGAG
CCAGAGGGCGGCCGTGACGCTTGTCAGGGAGACTCGGGTGGACCGATGCATGTTCTTGAAATGGAGACATCAA
AATACTCTGAAGTCGGTGTCGTGTCTTGGGGCGAAGGGTGCGCGCGACCAAACAAACCAGGCGTTTATACCCGT
GTCAATCGATACCTCACTTGGATTAAACAGAACACTCGCGATGCCTGCAATTGTCAATAAAAACACTGCTATGGT
TTAAACATCGCACCCTGGTTCCAGATTTTCGTAGGAGGCTTTTAACTAATTGTTCAAATGACAAATTGTACACCTT
TGTTTTACTTCTTGGTGCCTCGTCGTTATCGGAGGTTGACTGATATGTTCTACAAATTGTTTCTCAGATAAATTTAA
TTAATGTTTAATATATTATACGATGTATGAAATTTTAATTTATTAAG

Primer used for amplification of 37 kDa protease gene

p37k F: 5′-CCGGAATTCACAATGAAATGGCCAGTG-3′
p37k R: 5′-CCCAAGCTTTTATTGACAATTGCAGGC-3′

37kDa serine protease precursor

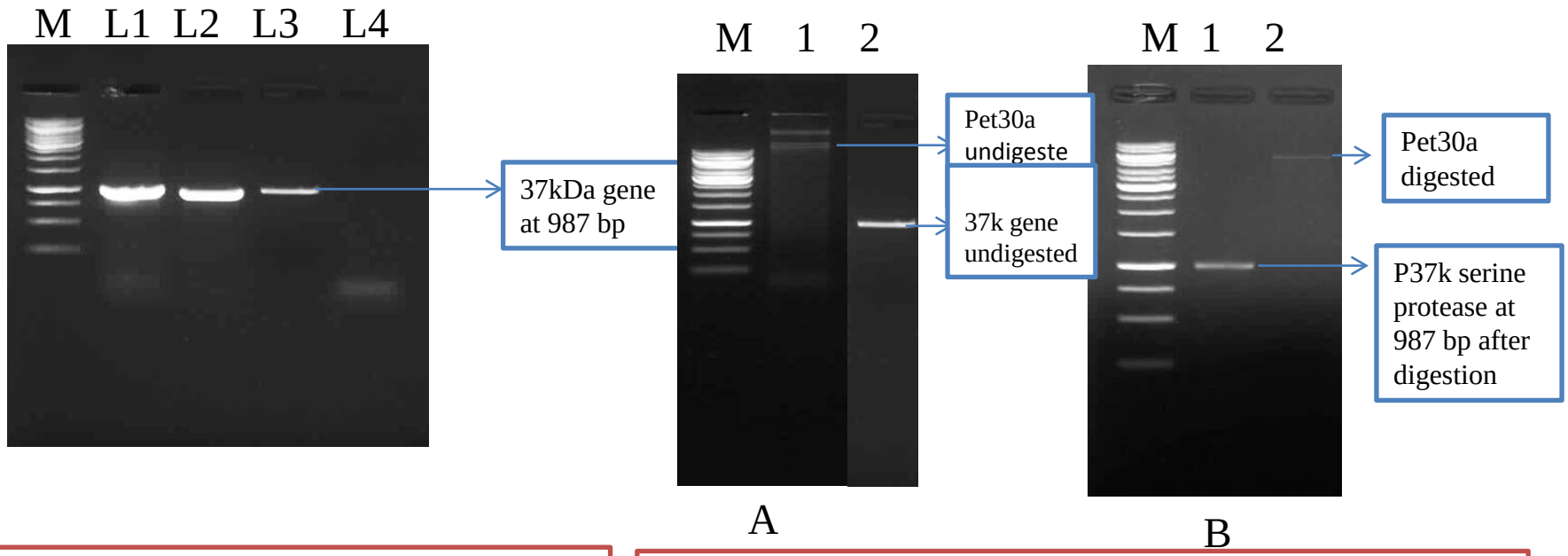
Signal peptide	Pro-peptide	Mature protein
1-18	19-68	69-329

MKWPVIMICLVGWSSCYTQRPIGQKDKGFIDWINNLLGGTTTTTTLRPIDDPPEDCPS
CQCGIARTRRRIVGGYETKETEYPWMAALLYGGRFYCGGALISDLYVLTAAHCTSGFR
KERITVRFLEHDRSKVNETKTIDRKVSDIIRHLRYNPGTYDSDIALLKLAERVDLSSA
LKRVRSEGDNGTATDDDKDVGLRPVCLPSSGLSYNNYTGVVTGWGTTEEGGSVSNALQ
EVKVPIVTNEECRKGYGDRITDNMICAGEPEGGRDACQGDSGGPMHVLEMETSKYSEV
GVVSWGEGCARPNKPGVYTRVNRYLTWIKQNTRDACNCQ

See blue word mean for cleavage site of furin like enzyme

Role of peptide:
Signal/Leader peptide: Protein Transport
Pro peptide: Maintain serine protease as a zymogen
Mature molecule: Serine protease active site

PCR amplification and Cloning of 37 kDa serine protease gene into pET30a vector

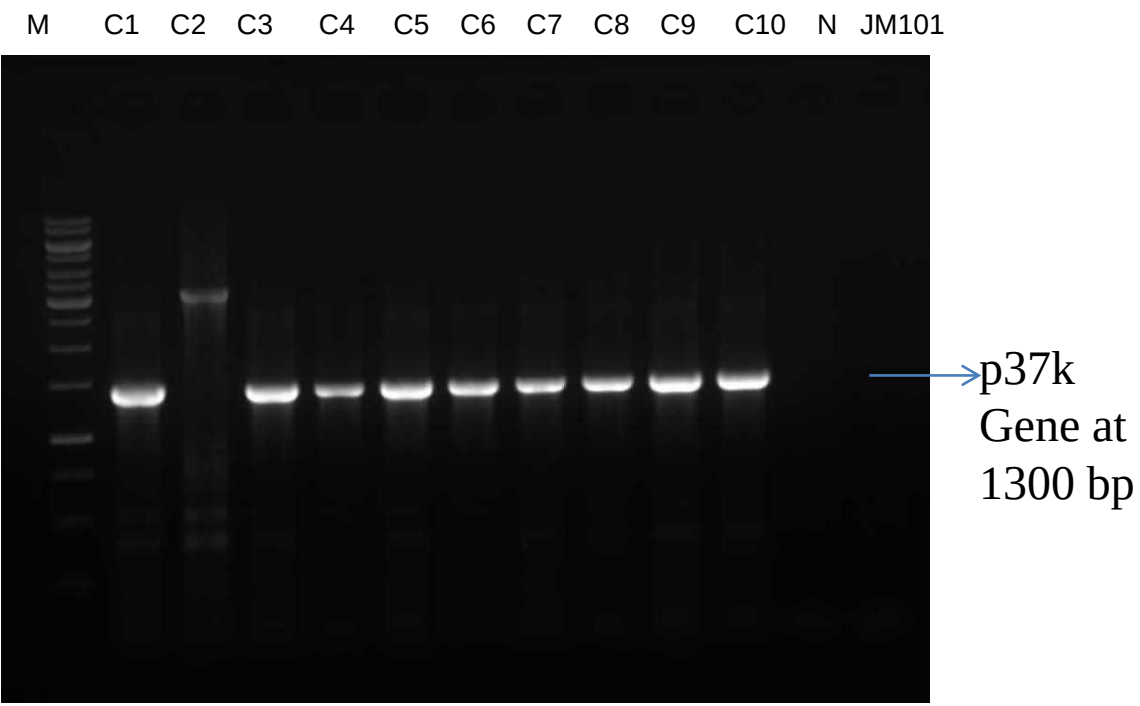


M sands for 1 kb marker
L1 -Amplified from 37 kDa gene from EST
L2- Amplified 37kDa gene from EST clone in JM101
L3 -Purified 37 kDa gene (987bp)
L4- Negative control (Water as template)

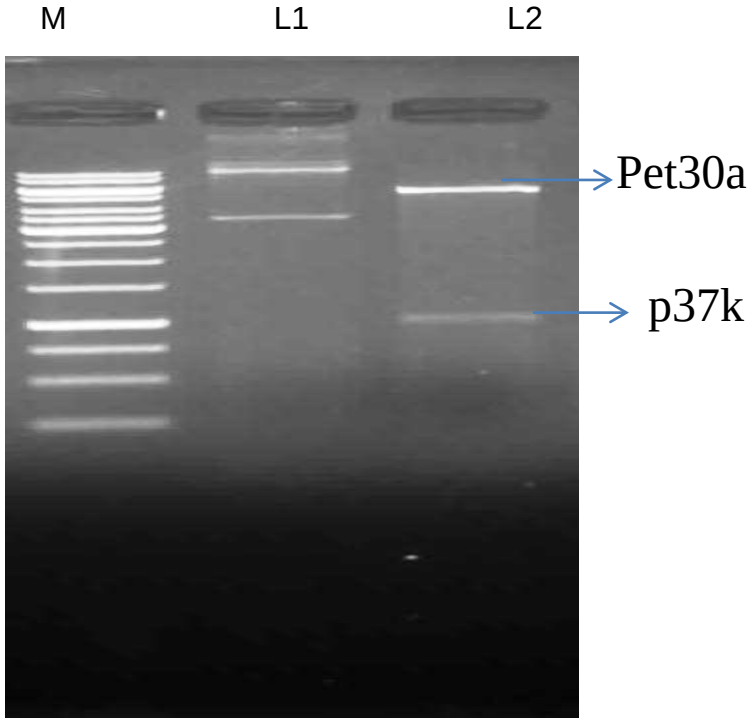
A : Before Digestion
M for DNA ladder
Lane 1 for pET30a Vector undigested
Lane2 for p37k insert undigested
B: After Digestion with Restriction enzyme *ECO-R1* and *HIND III*
M for DNA ladder
Lane 1 for pET30a Vector digested
Lane2 for p37k insert digested

Confirmation of p37k clone by PCR (A) and Restriction digestion (B)

A



B

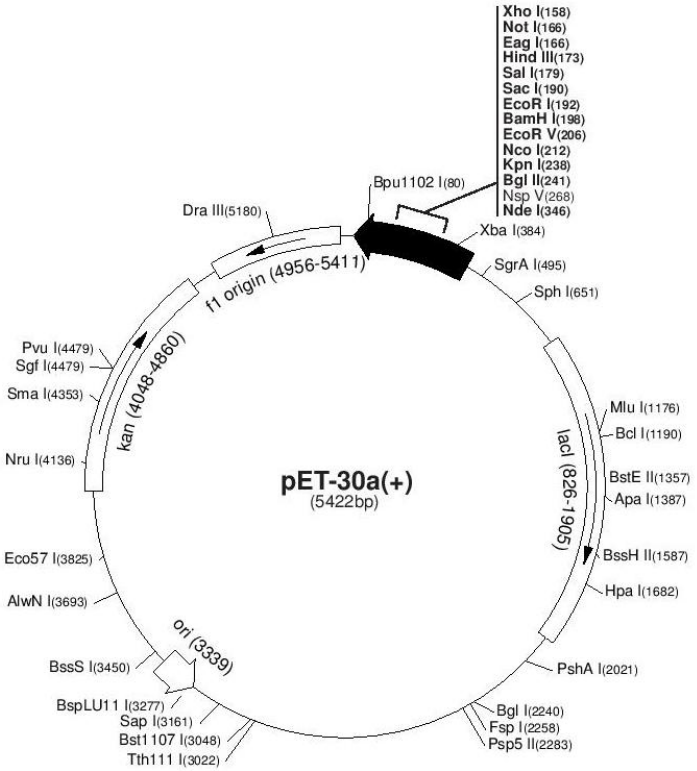


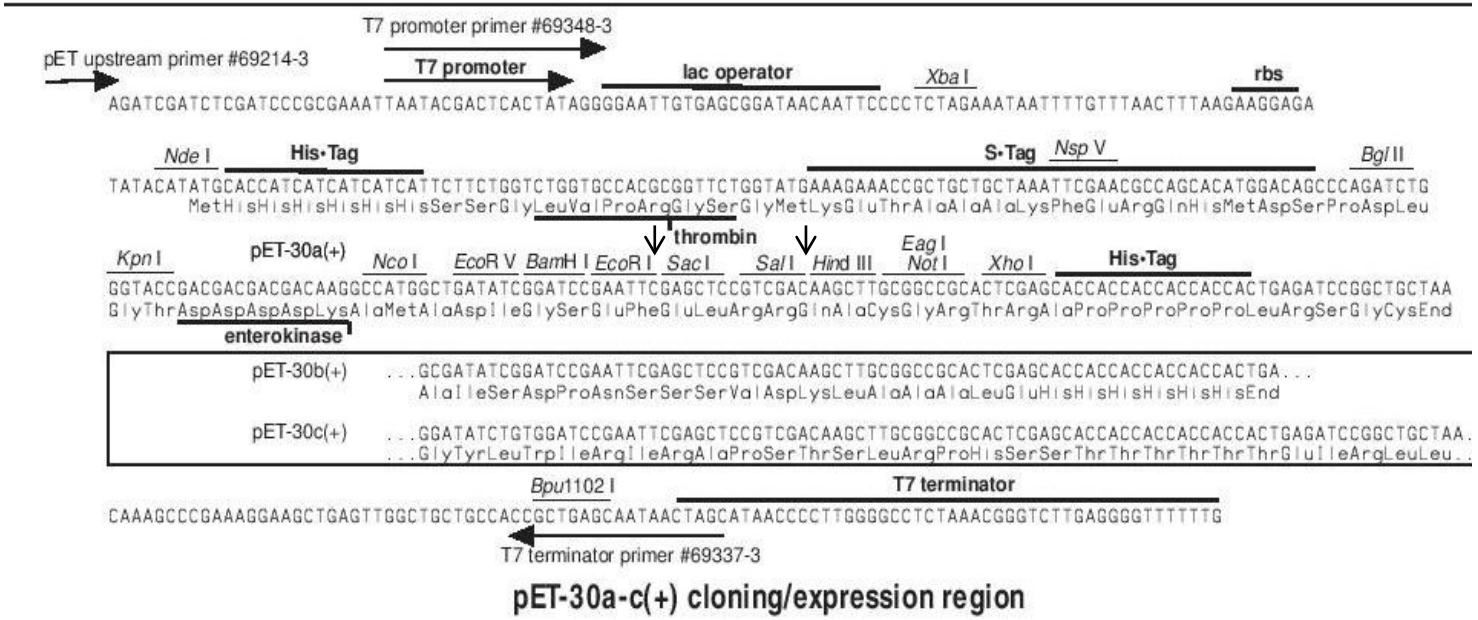
P37k gene was amplified using T7 Promotor and Terminator Primer
M stands for DNA size marker (1 kb, Biotool, Spain)
C1, C3 to C10- p37 k clone

M stands for DNA size marker (1 kb, Biotool, Spain
L1- uncut pet30b
L2-The pet30b digested by Eco-R1 & Hind III

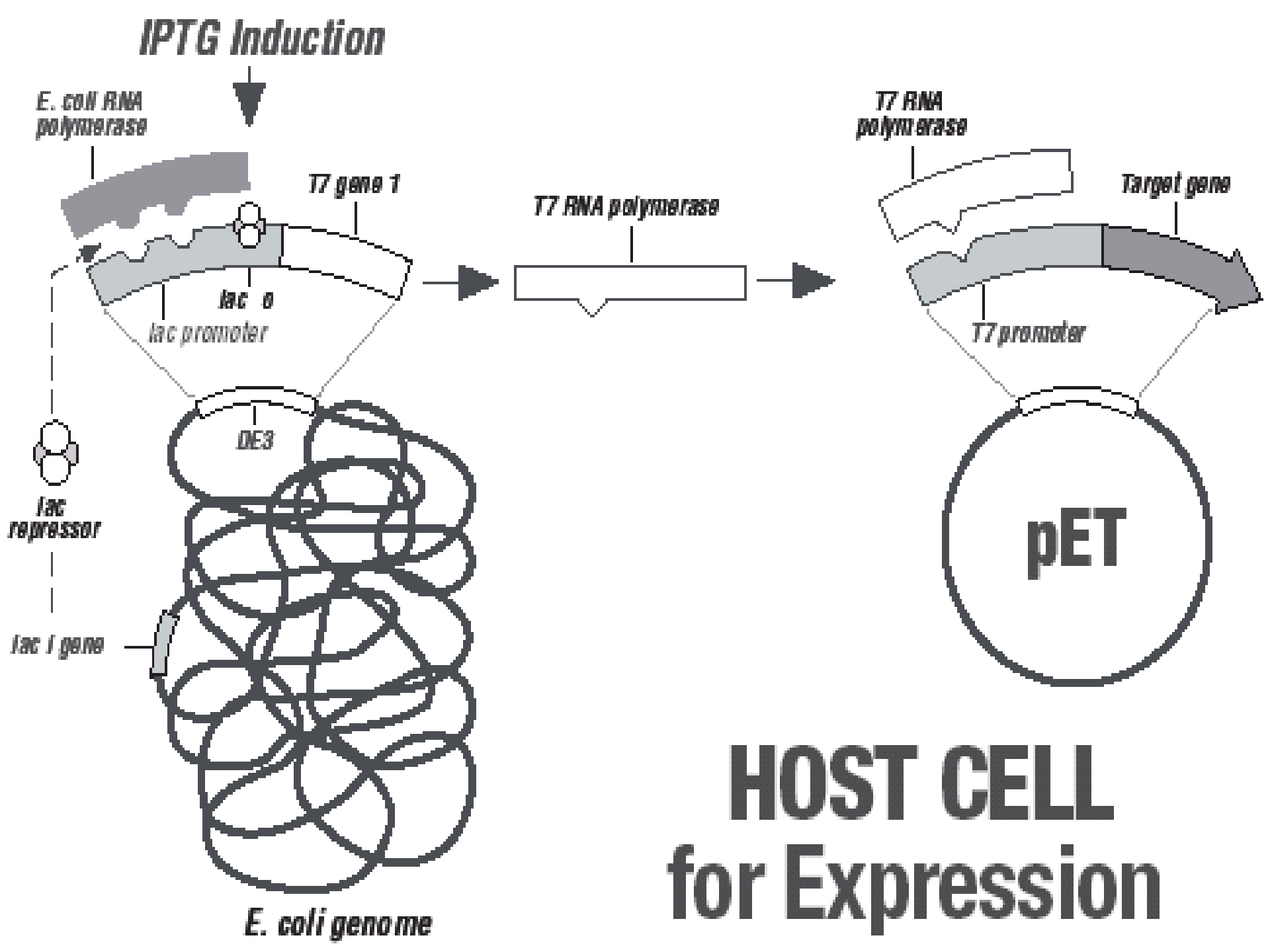
➢ **After confirmation, the construct was transformed in to *E.coli* BL21 (DE3) expression strain**

pET30a Vector map

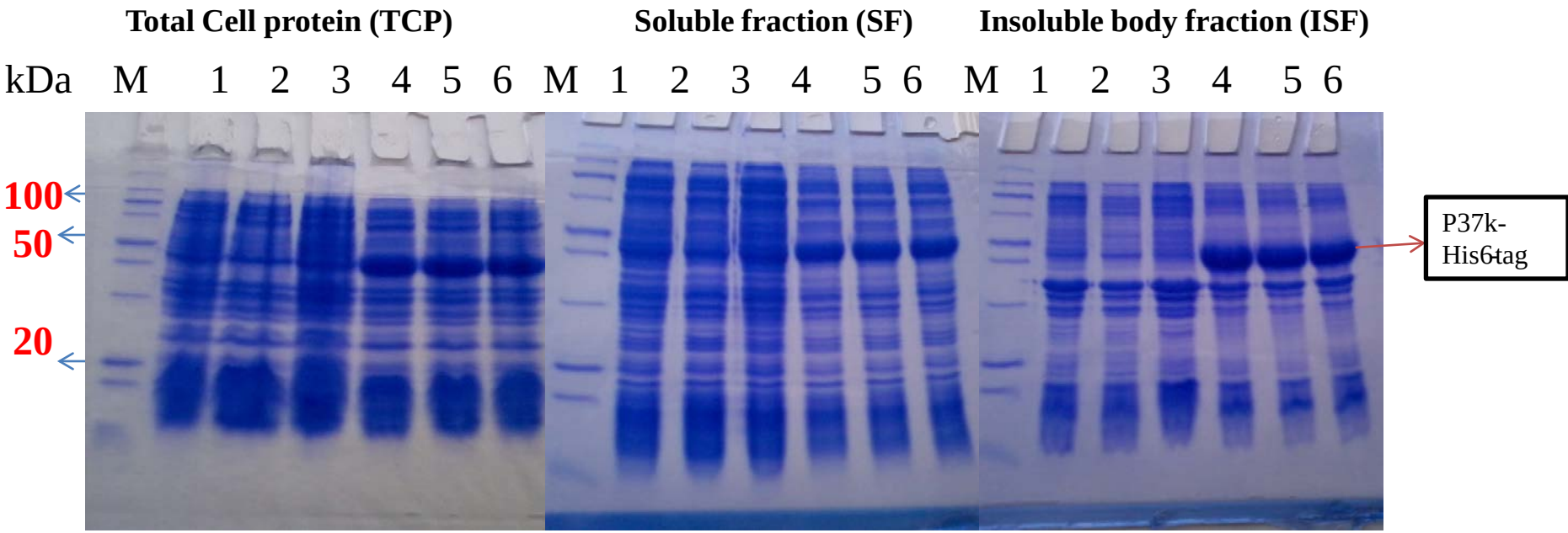




pET-30a-c(+) cloning/expression region

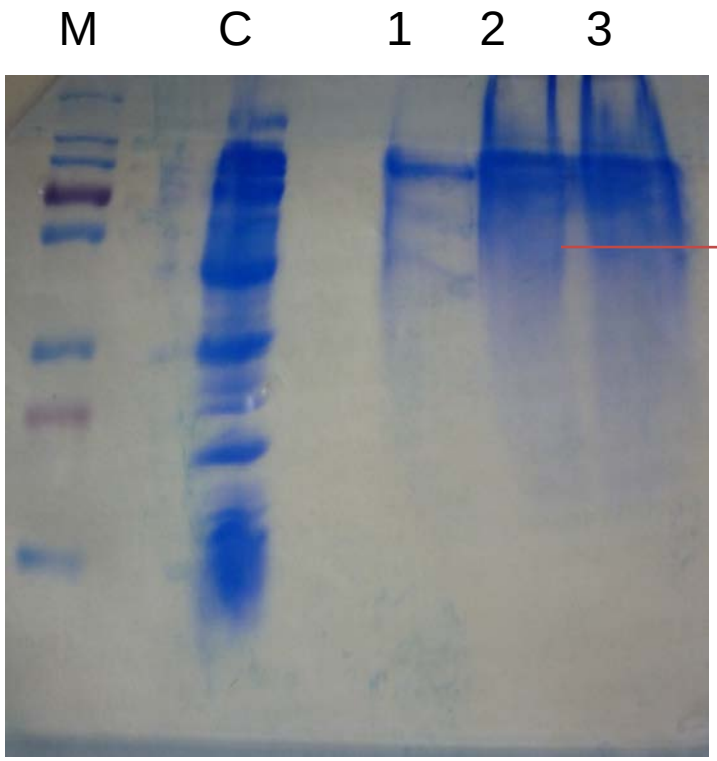

IPTG Induction
E. coli RNA polymerase
T7 gene 1
lac o
lac promoter
DE3
lac repressor
lac I gene
E. coli genome
T7 RNA polymerase
T7 RNA polymerase
Target gene
T7 promoter
pET
HOST CELL
for Expression

SDS-PAGE profile for Expression of p37k with His-tag in TCP, SF& ISF



M :Stands for Molecular weight marker
(Purchased from Thermo scientific)
Lane 1: PET Uninduced
Lane2:PET Induced
Lane 3: p37k Uninduced
Lane4: p37k Induced (0.5mM)
Lane 5: p37k Induced (1 mM)
Lane 6: p37k Induced (1.5 mM)

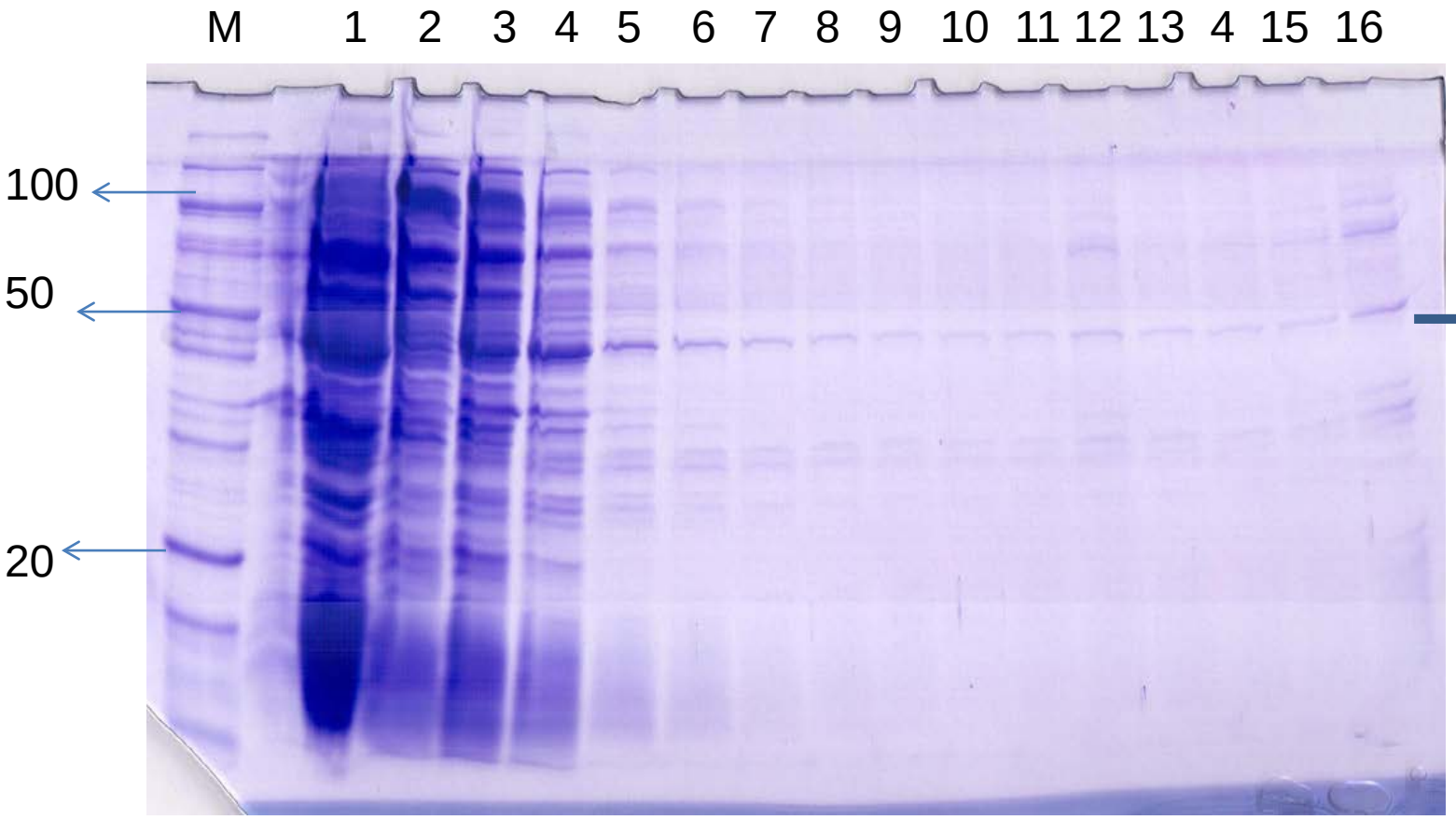
SDS-PAGE analysis of extracellular protein from p37k clone after Ammonium sulphate precipitation



Absence of p37k expression as extracellular protein

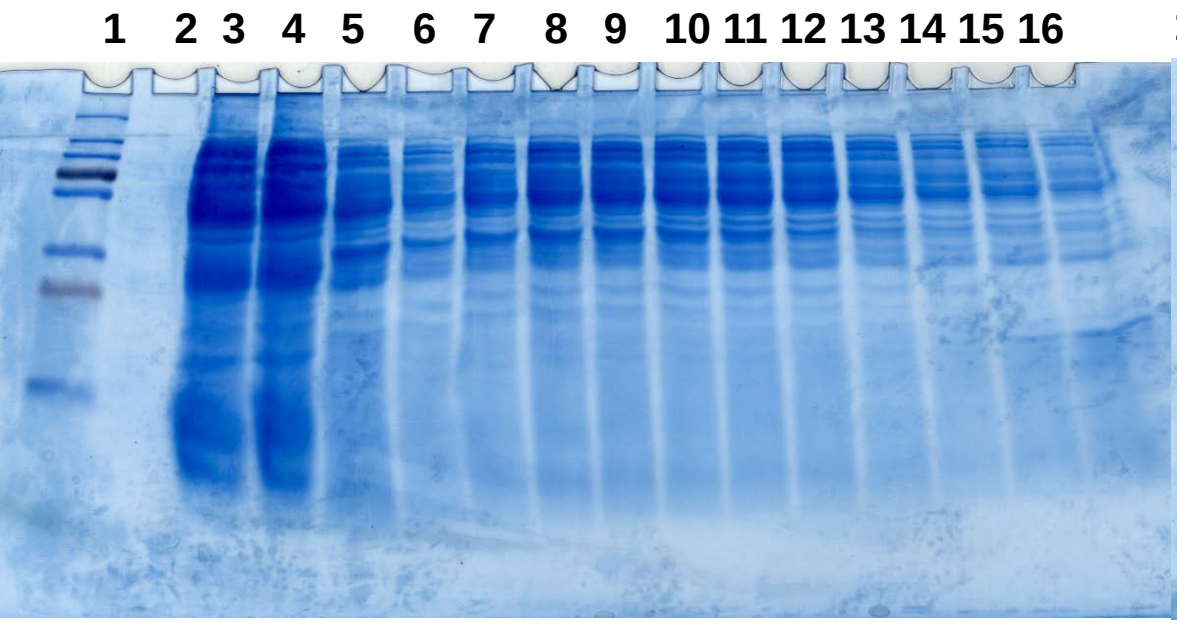
M Stands for Protein marker
C stands for whole cell protein
1 Stands for extracellular protein precipitated with 30 % NH_2SO_4
2 Stands for extracellular protein precipitated with 50 % NH_2SO_4
3 Stands for extracellular protein precipitated with 70 % NH_2SO_4

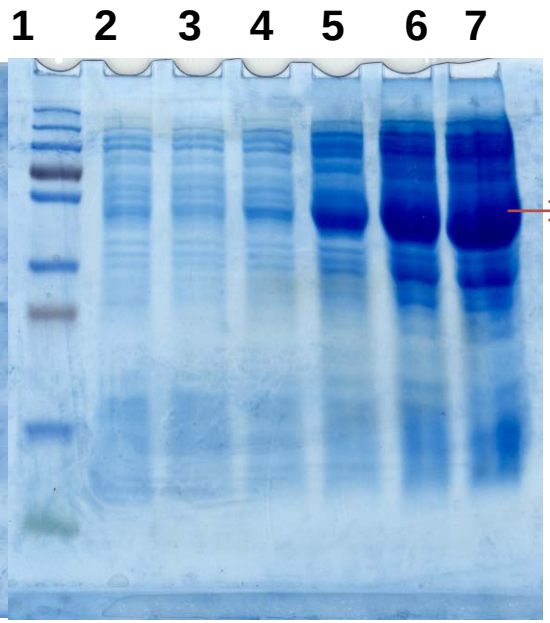
Purification of p37k under native condition



M Stands for protein marker
L1- Crude
L2- Flow through
L3 to L8-Wash1-9
L9 –Drops 1,2&3
L10-Drops 4, 5 &6
L11-Drops 7,8 &9
L12- Drops16,17 &18
L13- Drops10,11&12
L14-Drops 13,14 &15
L15- Drops19,20&21
L16-Drops 22,23 &24

Purification of p37k under denaturing conditions





Elution of recombinant P37k protease with His-tag

Lane 1: Marker
Lane 3: Crude
Lane4 : Flow through
Lane 5 – 7: Wash
Lane 8-11: Elution 1
Lane 12-16: Elution 2

Lane 1: Marker
Lane 2: Crude
Lane 2-4: Elution 2
Lane 5-7: Elution with 300mM imidazole

The purified His-Tagged 37kDa serine protease was also confirmed by Mass Spec analysis

Domain identification of 37k serine protease of *B.mori*

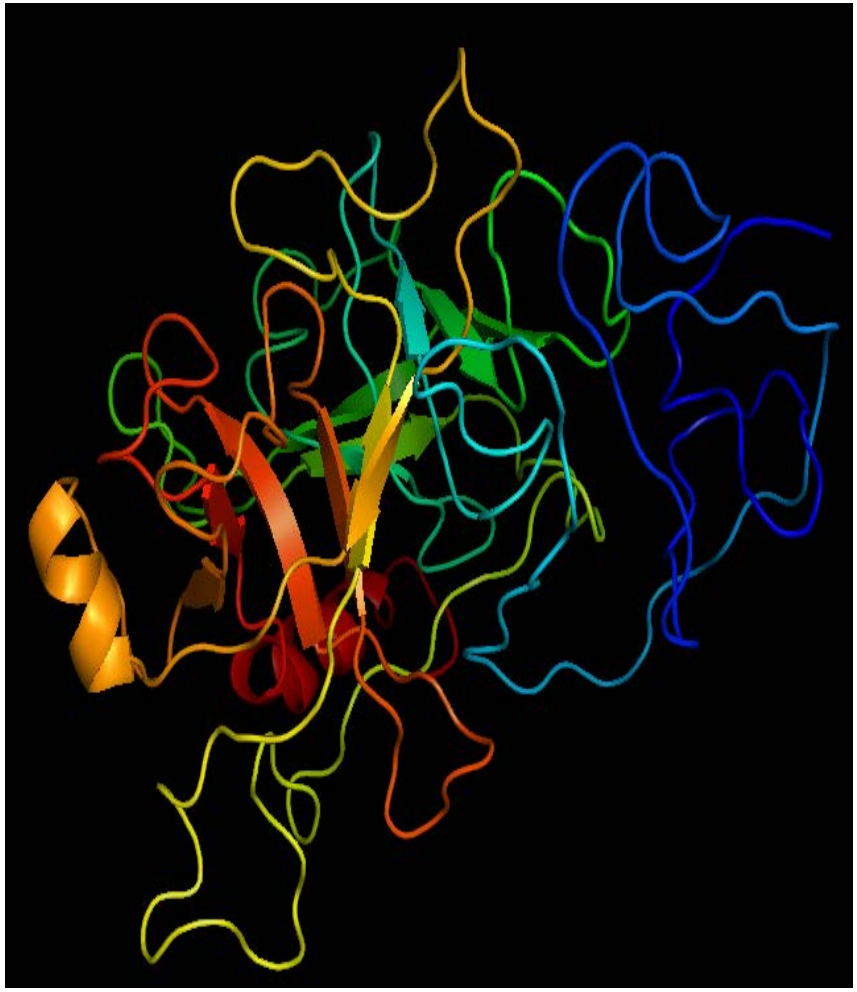
Name	Start	End	E-value
signal peptide	1	18	N/A
low complexity	36	46	N/A
Tryp_SPc	69	318	3.75e-88

MKWPVIMICLVGWSSCYTQRPIGQKDKGFI
DWINNLLGGTTTTTTLRPIDDPPEDCPSCQC
GIARTRRRIVGGYETKETEYPWMAALLYGG
RFYCGGALISDLYVLTAAHCTSGFRKERITV
RFLEHDRSKVNETKTIDRKVSDIIRHLRYNP
GTYDSDIALLKLAERVDLSSALKRVRSEGD
NGTATDDDKDVGLRPVCLPSSGLSYNNYTG
VVTGWGTTEEGGSVSNALQEVKVPIVTNEE
CRKGYGDRITDNMICAGEPEGGRDACQGD
SGGPMHVLEMETSKYSEVGVVSWGEGCAR
PNKPGVYTRVNRYLTWIKQNTRDACNCQ

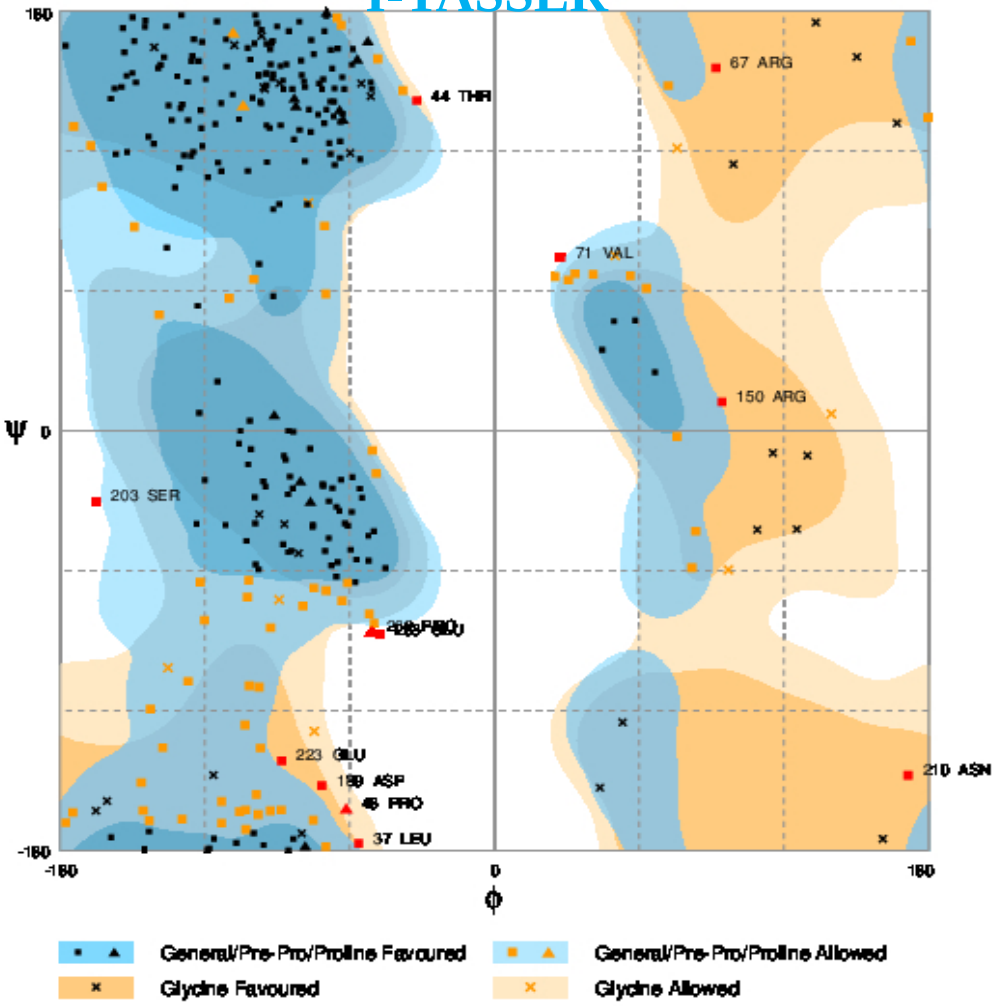
➢Motif pattern analysis through PROSITE for 37-kDa serine protease revealed the trypsin like domain at 70-323 aa.

➢SignalP analysis clearly showed that active site of p37k upon minimal proteolytic cleavage by pro-protein convertase.

3D structure of 37kDa serine protease by I-Tasser

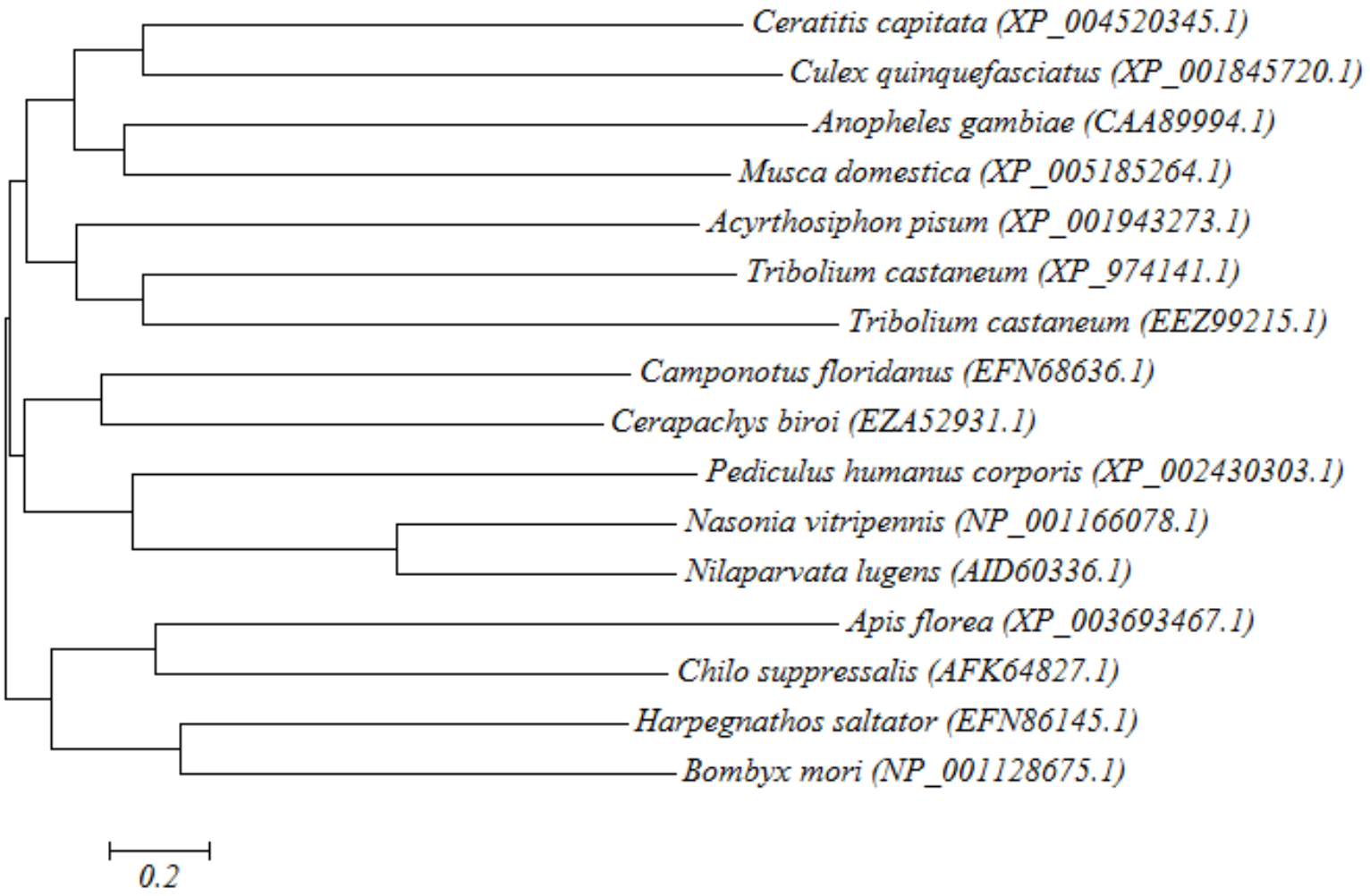


Ramachandran plot of 37kDa serine protease structure model predicted by I-TASSER



Number of residues in favoured region	(~98.0% expected)	:	242 (74.0%)
Number of residues in allowed region	(~2.0% expected)	:	73 (22.3%)
Number of residues in outlier region		:	12 (3.7%)

Phylogenetic tree of 37k serine protease in various insects



Glide Scores, Glide energy, H Bonds and ligand structures from databases docked to 37k serine protease

S.No	Database	No of Compounds	Glide score	Energy	H bond
1	Binding	34,401	-10.825	-37.842	4
2	NCI	2,60,071	-10.588	-49.327	2
3	Zinc	4 Million	-10.215	-39.476	2

Conclusion

➢Design a novel inhibitor for the active site of p37k serine protease will prevent the protease activation during pupation. So, the absence of active protease or inactivation of 37 kDa serine protease during pupal stage leads to developmental barrier.

➢Therefore identification or design of an effective inhibitor can be used as drug in future.

My Unit

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THANK YOU

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