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ISOLATION & CHARACTERIZATION OF PROTEASE INHIBITOR GENES FROM POTATO *SOLANUM TUBEROSUM* (L.)

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ABSTRACT

Small molecule also involved in biotic resistance gene in plants and belongs to a pathogen related protein (PR protein) are known as Protease inhibitors (PI), and thereof in this investigation PIs-II isolated from potato Solanum tuberosum (L.) varieties. In this present investigation PI gene isolated from seven variety of potato. Kufri bahar varieties of potato wound-inducible PI-II the length of this gene sequence was 743 bp containing a 237 bp open reading frame (ORF) that coded 78 amino acids at code region and In silico Characterization the sequence analysis by BLASTn showed 89% homology with protease inhibitor-II mRNA of solanum phureja (Acession no. AY517498.1). Using BioEdit program find out analysis of signal peptide was predicted by SignalP-4.1 and analysis of the predicted potato PI protein sequence by TargetP 1.1 server, Computation of various physical and chemical parameters by using of ProtParam Package of Expasy web server, Deduced hydrophobic index by using ProtScale package of Expasy web server, Sequenced data were translated to protein sequences, and translated sequences were modeled to secondary structure prediction by PSIPRED software.

Key words: *Solanum tuberosum*, proteinase inhibitor-II, wound-inducible, ORF, In-silico characterization.

Introduction

Low productivity is due to various biotic and abiotic stress and worldwide crop production losses without the use of pesticides and other non-chemical control strategies is estimated to be about 70% of crop production (Lawrence *et al.*, 2002 ; Shivanna *et al.*, 2014). Pre-harvest losses due to insect pest is account 15% of total production (Lawrence *et al.*, 2002; Amy Maxmen, 2013), post-harvest losses caused by insect pest is account 30%.

The control of losses through agrochemicals is costly and resistance breeding is also limited. Although the heavy use of pesticides farmers still lose 11-40% of their crop due to pest damage. In future global population is likely to reach 7 billion by 2025 and 10 billion by 2050. The agricultural production need to be increased from these lands that are under stress; with the help of insect resistance crop. The agriculture is posed with a great challenge of meeting the ever-increasing food demand. Many crop varieties which have been developed in the past under modern high intensity agriculture included high yielders, nutritionally rich, adapted to environmental conditions and with low mammalian toxicity resulting in tremendous increase in food production for feeding the ever-growing world population.

It is necessary to develop more efficient and environmental friendly agriculture, which will have decreased inputs in energy and chemicals and will not generate harmful outputs such as pesticides residues (Jouanin *et al.*, 1998). The molecular biology and genetic engineering approaches allow harnessing and development of insecticidal molecules in crop plant in a safe and sustainable way. Most of these approaches have potent effects on insect pest, low mammalian toxicity, lack of neuro-toxic activity, low persistence in the environment and biodegradability (Jacobson, 1989). Which approach will keep away from the tones of chemical, pesticides used globally in agriculture, These chemicals and pesticides are effect directly or indirectly plant, animal and human also, so the application of such type of chemicals and pesticides should be avoided.

The scientist used *(Bt)* genes for producing insect resistant transgenic plants in several crops some are commercialized as *Bt* cotton, maize and soybean. The production of transgenic crops has seen rapid advances during the last decade with the commercial introduction of *Bt* transgenics, but the major concern with these crops has been the development of resistance by pest and public acceptability. Hence, there has been a need to discover new effective plant genes which would offer resistance/protection against these pests. Protease inhibitors (PIs) are one of

the prime candidates with highly proven inhibitory activity against insect pests and also known to improve the nutritional quality of food, so there is a need to discover new effective plant genes, which would offer resistance or provide protection against insect pests. In a co-evolving system of plant-insect interactions, plants synthesize a variety of toxic proteinaceous and non-proteinaceous molecules for their protection against insects. They have evolved various complex chemical weapons of defense which include antibiotics, alkaloids, terpenes as well as proteins such as enzyme inhibitors and lectins (Rhodes, 1979; Baldwin and Semultz, 1983). These inhibitors also have the properties to prevent uncontrolled proteolysis within the cells, organelles or fluid and PIs are large, ubiquitous family of small proteins with diverse functions both in plants and animals (Ryan, 1980; Ryan, 1990, clynen, schoofs and salzet 2005). Protease inhibitor and α-inhibitors serve as one of the defense mechanisms in plants against invading pests. Protease inhibitors are generally low molecular weight protein that make complexes with proteases and reduce their proteolytic activities. The potato is the 3rd most important crop in the world after rice and wheat, keeping in view the potential of the potato in the food security of developing nations, FAO has declared it as the "food for future" (MISBD, 2012). In potato (*Solanum tuberosum*), PIs are the most abundant tuber protein (Bauw *et al.* 2006) Evidences indicate that the presence of protease inhibitor proteins in plant leaves can reduce predation by insects.

Insect resistance PI genes are of plant origin; and have practical advantages that they are eukaryotic in nature and are non-injurious to pollinators, predators, economic insects, natural enemies of the pests. The PI has four classes as 1.serine, 2.cysteine, 3.aspartic acid 4.metallo protease inhibitor, basic mechanism of action of the PI are the secretion of protease in insect guts depends upon the midgut protein content rather than the food volume. The secretion of proteases has been attributed to two mechanisms, involving either a direct effect of food components (proteins) on the mid gut epithelial cells or a hormonal effect triggered by food consumption and mode for synthesis and release of proteolytic enzyme in the midgut of insect follow this phenomenon, the ingested food protein triggered the synthesis and release the enzyme from the posterior midgut epithelial cells. The enzyme are released from membranes associated forms and sequestered in vesicles that are in turn associated with the cytoskeleton. By transferring single defensive gene from one plant species to another with higher expression using their own wound inducible or constitutive promoters would impart resistance against insect pest (Boulter, 1993;

Gatehouse *et al,* 1997) and can be used as bio control option in an Integrated Pest Management (IPM) strategy.

The insect resistant transgenic crops not only eliminate the use of chemical pesticides but also provide a season long protection to crop against insects pests. It is worthwhile to identify and isolate the inhibitor proteins encoding genes from tomato/potato, so that these genes will be free from IPR issues and transgenic crops can be developed and commercialized (Kondal *et al.,*2003). Numerous genes encoding potato inhibitors I and II (*PI-1* and *PIN2*), and KTIs have been characterized (Heibges *et al*. 2003, Hermosa *et al*. 2006, Turra *et al*. 2009). Protease inhibitor (PI) proteins are major constituents of seeds and storage organs including legumes, cereals, beans and potatoes (1-10% of total protein) which have insecticidal activity for various insect pests (Ussuf *et al*.,2010). So, the PI genes can be used for the development of insect resistant crop plants through principles of genetic engineering.

Introduction of genetically engineered insect resistance crops is one of the major advances in agriculture. The specificity of PIs in targeting definite groups of insects can help in generating transgenic plants with particular PIs that have inhibitory actions against specific pests. Transformed white poplar (*populous alba* L.) plants developed using the *Arabidopsis thaliana* cysteine PI gene were resistance against *Chrysomela populi* beetle. Research data demonstrated that biotic stress such as insect chewing results in the expression of plant defensive proteins. Approximately 100 genes in lima bean, *Phaseolus lunatus* L., can be expressed in response to the chewing of the 2-spotted spider mite, *Tetranychus urticae* (Munir *et al.,* 2013), potato inhibitor I (PI-1) and potato inhibitor II (PI-2), type inhibitors are widespread in the Potato family (Obregón *et al.,* 2012).

Fig 1:- Nucleotide sequence of the potato protease inhibitor-II gene

GACGGTTTTTTTCCTCCTTAAAACAACCTGACGACGGGTTGTTTCCTGCAAAAGAAC
AAAGATTGGTGGTTTACCGGTAGTACCGCATCGGGTAGAGCTAATAGCACGCACGC
ACCCGAAGGGCCGAAAGCAACGACACAGTCGCCGGGGGGAGCGGTGGGCGCCCAA
TGCGCCAACCGCCTCTCCGCGCGCGTTGTCCGATTCAAGAATGCAAAAATTTTTTTTT
CAGAAGGAAGTCGGACAAATCCCCCCCGACCCCCCCGTTGTTCAGGTTATAAGGGTT
GTAATTATTATAGTGCATTTGGGGGTTTTATTTGCGAAGGAGAATTTGACCCAAAAA
ACCCAAATGCTTGCCCCCTAAATTGTGATCCACTTATTGCCTATTCAAAATGTCCCCG
TTCAGAAGGAAAATGAGTACAATCCCACCGGATGTACCACCTGCTGCACAGGGTAC

AAGGGTTGCTACTACTTCGGTAAAAACTGCAAAGATCCTATTTCCTGGTTACATTTTT
CCTTCCCCCCCCCCCCTTGCCATTTTTCCCGAAATAGTAGCAACCCTTGTAGGCCTGT
GCAGCATGTGGTACTCCGGTGGGATAAATAGCGATTTTCCTTTACGGGACTTTGATC
CCCAAGTTTCACAATTCGGGCTAAATTAGGGGTGGAAAAAAAATCTCCTTCGCAAAT
GAATAGCCCCAAAGCACTATAATAATTACAACCCTATACCTCAGCTACGACTTTTCC
GG

MATERIAL AND METHODS

Plant material

7 varieties of *Solanum tuberosum* L. have been collected from CPRI, Modipuram, Meerut (U.P) and crop maintained to collect the leaf sample for RNA isolation. After 35 day the young leaf samples were collected in the sterile polybags and the packed samples were stored in deep freeze -80^{0}C. Under field condition stress were given as artificial wounding with the help of steel razor blade (He *et al.* 1983). Took leaf sample at the time 72h after given treatment and froze the samples in liquid nitrogen quickly and preserved them at the temperature of -80 ^{0}C (Li *et al.* 2007b).

List of potato (*Solanum tuberosum* L.) genotypes

S. No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Varieties	Kufri Bahar	Kufri Pukhraj	Kufri Khyati	Kufri Garima	Kufri Himsona	Kufri Surya	Kufri Chipsona-3

Extraction of total RNA

The selected potato varieties along with their treatment plants were subjected for total RNA extraction using Genei Pure Total RNA Isolation Kit-for Plants. Care should be taken that all the tubes tips used in the process must be treated with DEPC. For RNA extraction, take 50mg of fresh leaf sample and crushed in liquid nitrogen in ice chilled mortar and pestle followed by the instruction given by the manufacturer of the kit. Crushed samples were transferred to 1.5ml tubes and added 500µlof lysis buffer and 5µl of β-mercaptoethanol to the samples and centrifuge them at 10,000rpm for 4 min. The supernatant was taken in green filtration column provided in the kit and centrifuge for 1 min. Discard the column and add 300µl of absolute ethanol to the filtrate

and mix gently by repetitive pipetting. Transfer the solution to the blue filtration column tube provided in the kit and centrifuge for 1 min. Discard the filtrate and add 500µl (250µl wash buffer 1 + 250µl of absolute ethanol) to the column and centrifuge for 1 min. Discard the filtrate and wash the column again two times by adding 500µl (400µl absolute ethanol + 100µl wash buffer 2) wash buffer 2 for every wash and centrifuged them at 10,000 rpm for 1 and 2 min. Add the 50-70µl of pre-warmed (60-70^{0}C) elution buffer to the column, incubate for 2 min. at room temperature and centrifuge at 10,000 rpm for 4min for the elution of pure RNA. Added 1µl DNase I and incubated at 37^{0}C for 15 min to degrade the genomic RNA in the sample. The samples were kept at 75^{0}C for 5 min to inactivate DNase otherwise it will degrade the product of reverse transcription and then store at -20^{0}C or -80^{0}C further use.

Table 1:- List of cDNA amplification components for gene specific Primers

Components	**Volume (µl)**
10X Assay Buffer with 15.0 mM $MgCl_2$	1.0
1.0 mM dNTPs Mix	2.0
Taq DNA Polymerase (1U/µl)	0.5
5.0µm Primer (Forward)	0.75
5.0µm Primer (Reverse)	0.75
cDNA (25ng/µl)	2.0
Water (Milli Pore)	3.0
Total	**10µl**

Preparation of cDNA from RNA

For the preparation of cDNA, take 1 to 8µg of total RNA and add 1µl of oligo dT first strand primer to the nuclease free tubes, incubate it at 65^{0}C for 5min. and then allowed to cool down on ice. Thereafter, remaining reagents were added i.e. 2µL of dNTP mix (10Mm), 4µl Reaction buffer (5X), 2µl of M-MLV reverse transcriptase (20U/µl), 1µl of Ribolock RNase inhibitor (20U/µL) and incubate at 37^{0}C for 60 min. To terminate the reaction incubate the samples at 70^{0}C for 5 min. The samples containing the cDNA were stored at -20^{0}C or -80^{0}C for isolation of PIs gene.

RT-PCR amplification

Amplification with gene specific primers **F-GCCTTGGGTTCATCACTCTCT, R-TTCAGAAGGAAGTCCGACAAA (**Primer sequences previously described by Lin et al., 2003) were performed in a total of 10µl reaction volume as given in **(Table-1)**. All components were mixed gently in 0.2ml thin walled PCR tubes. A master mix except template cDNA and primers was prepared for certain number of tubes to avoid pipeting error. Master mix was mixed by spinning for a short time and distributed in each tube and finally template cDNA of all 14 samples (7 control and 7 treatment) was added to each tube and placed in (Geni Master Cycler Gradient) for amplification. The amplification was performed by using the thermal profile as described in **(Table-2)**.

Table 2:- Reaction condition for PCR.

Step	Temperature (^{o}C)	Time
Initial denaturation	95.0	10 min
Denaturation	94.0	30 sec
Annealing	60.0	30 sec
Extension	72.0	45 sec
Cycles	35	------
Final extension	72.0	10 min
Final hold	4.0	------

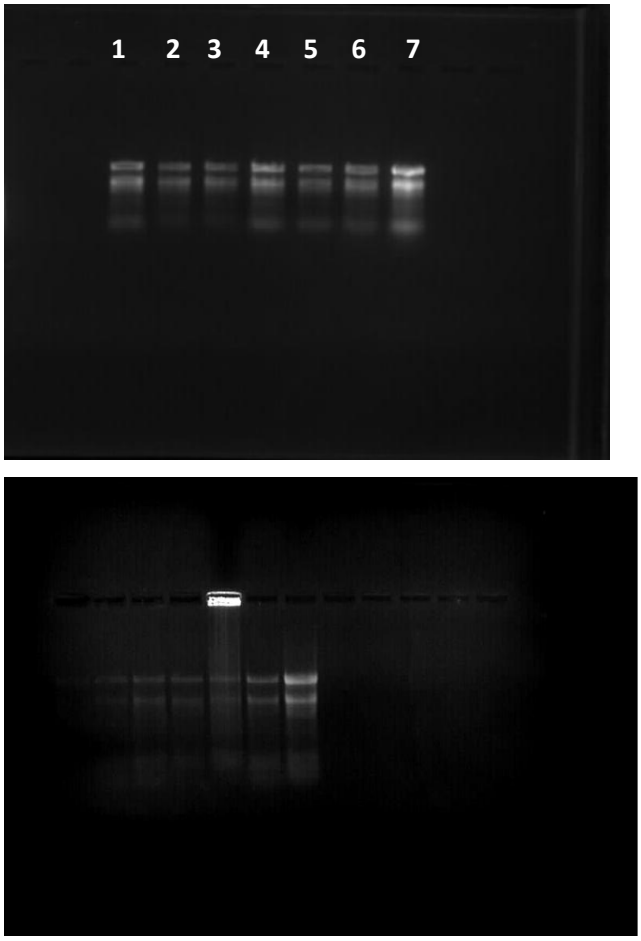
Agarose gel electrophoresis of RT-PCR products

1.6% of agarose gel was used to resolve obtained small size bands using Midi submarine electrophoresis unit (Tarson, India). Gel was prepared by dissolving appropriate amount of agarose in TBE (1X) buffer. A low range DNA ladder of known molecular weight (100bp) was also loaded at one end. Electrophoresis was done at 50 volts for 1 hrs in 1X TBE. The gel was then visualized and photographed using Alpha Innotech (Alphaimager) System.

Electrophoresis

The amplified DNA samples were mixed with a loading dye (50% glycerol containing 0.1% xylene cyanol and 0.1% bromophenol blue) in 5:1 proportion and were electrophoresis on 1.6% agarose gel in 1x TAE buffer at 3-5 volt/ cm for 1 hours

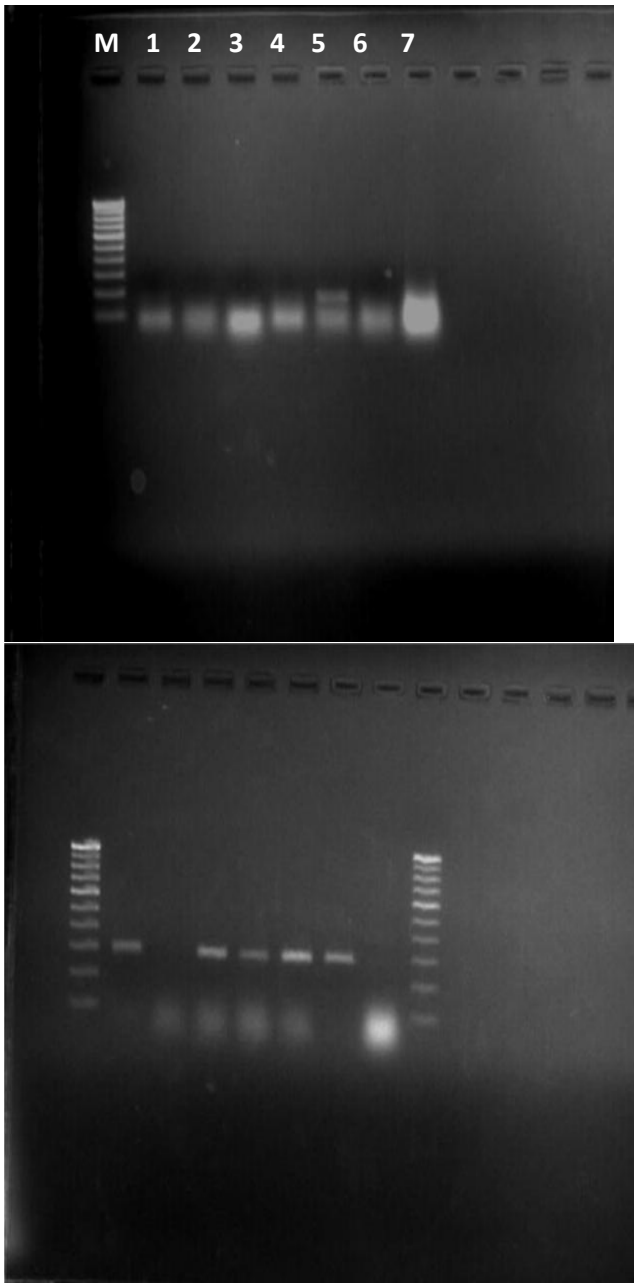
RESULTS AND DISCUSSION



(Control) **(Stress)**

Fig 2.1:- 1.6% Agarose gel electrophoresis showing 2 promonent bands (28 S and 18 S) of seven different genotypes of potato (1-7)

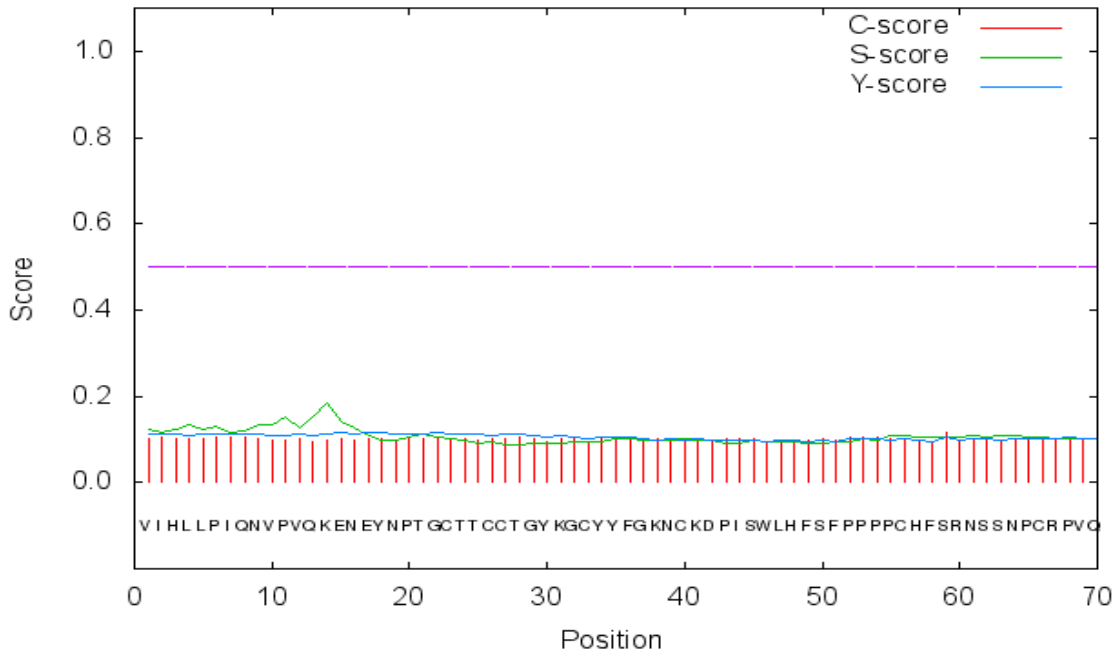
Lane details: 1-Kufri Bahar, **2-** Kufri Pukhraj, **3-** Kufri Khyati, **4-** Kufri Garima, **5-** Kufri Himsona, **6-** Kufri Surya, **7-** Kufri Chipsona-3



(Control) **(Stress)**

Fig 2.2:- 1.6% Agarose gel showing PCR amplification with gene specific primer of seven different genotypes of potato (1-7)

M- Marker (100 bp) **Lane details: 1**-Kufri Bahar, **2-** Kufri Pukhraj, **3-** Kufri Khyati, **4-** Kufri Garima, **5-** Kufri Himsona, **6-** Kufri Surya, **7-** Kufri Chipsona-3



#	Measure	Position	Value	Cutoff	signal peptide?
max.	C	59	0.116		
max.	Y	22	0.116		
max.	S	14	0.183		
mean	S	1-21	0.126		
	D	1-21	0.122	0.450	**NO**

Figure 2.3:- signal peptide prediction of potato protease inhibitor Kufri Bahar (PPinIIKB) using Expasy software

TargetP 1.1 Server

Name	Len	cTP	mTP	SP	other	Loc	RC
PPinIIKB_78aa	**88**	**0.191**	**0.284**	**0.042**	**0.436**	**-**	**5**

Figure 2.4:- Predicted subcellular localization of potato protease inhibitor (PPinIIKB) protein by TargetP program of Expasy web server

Analysis	Whole Protein
Molecular weight	8995.34
Theoretical pI	8.76
Total number of negatively charged residues (Asp + Glu):	4
Total number of positively charged residues (Arg + Lys):	8
Formula:	$C_{405}H_{603}N_{111}O_{109}S_7$
Total number of atoms:	1235
Extinction coefficients:	17335
Instability index:	53.48
Aliphatic index:	57.31
Grand average of hydropathicity (GRAVY):	-0.514

Figure 2.5:- Computation of various physical and chemical parameters for potato protease inhibitor (PPinIIKB) using ProtParam Package of Expasy web server

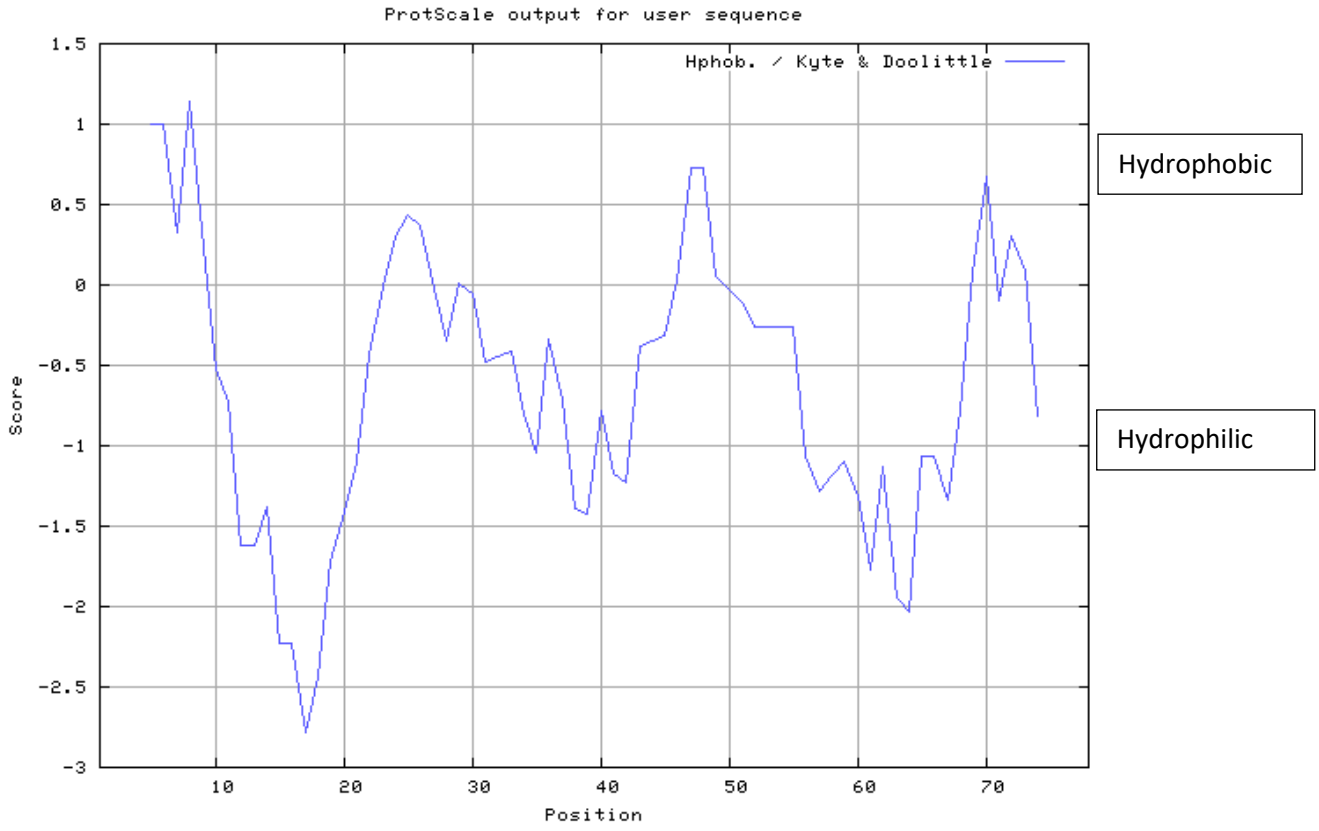


Figure 2.6:- Deduced hydrophobic index of potato protease inhibitor (PPinIIKB) protein using ProtScale package of Expasy web server

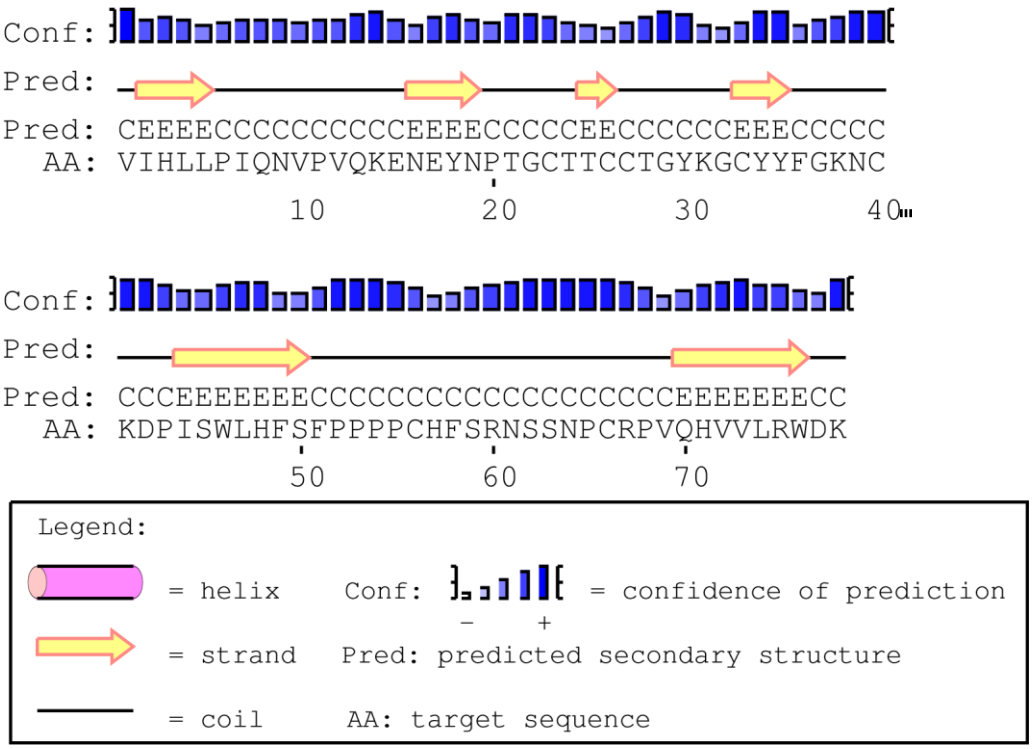


Figure 2.7:- Secondary structure

Potato is third most imported vegetable crop of the world. India is the largest producer and consumer of the potato. The recent advances in Biotechnology have accelerated the protease inhibitor (PI) gene isolation in potato. The present investigation entitled **"Isolation & characterization of protease inhibitor genes from potato *(Solanum tuberosum* L.)"** was carried out with seven genotypes subjected to isolate PIs gene and characterized analysis. PI can inhibit the catalytic activity regulating factor in plant itself and plays an important role in the regulation of the metabolism and a variety of physiological activity of all kind of protease. With the help of PIs gene plant resistant to disease and insect by combining the transgenic technology. Under normal condition the content of PI in plant leaf is very low or not present, but can increase within a few hours when it is wounded mechanical damage.

In this study, PI gene was isolated and characterized. It was found that in this potato family there is **no signal peptide** presents (Figure 2.3). Analysis of the predicted potato PI protein sequence by TargetP 1.1 server revealed secretion pathway score is zero because there is no signal peptide, that indicate the inhibitor was likely to be secreted where it is present in the cell (Figure 2.4). The whole protein atomic composition analysis of PI also was done by using

ProtParam Package of Expasy web server (Figure 2.5), the computation of various physical and chemical parameters of PI protein was carried out. The predicted MW of the PI protein was **8995.34 kDa** with a theoretical pI of **8.76.** A total no. of negatively charged **(4)** and positively charged **(8)** residues was predicted for PI protein under study. Aliphatic index of this protein was **57.31** while the Grand Average of Hydropathicity was **-0.514** which confirmed the hydrophilic nature of the inhibitor was predicted by using ProtScale package of Expasy web server (Figure 2.6) and secondary structure of this protein was predicted with the help of Psipred protein structure prediction server http://bioinf.cs.ucl.ac.uk/psipred/ which provided the information about Alpha helix, Beta strand and coils (Figure 2.7) and resulted high level of **beta strand** present in this protein.

Conclusions

Following conclusions could be drawn from the present investigation:

1. Study of seven potatoes (*Solanum tuberosum* L.) genotype was analyzed via agarose gel electrophoresis with the help of gene specific primer.
2. RNA was isolated from leaf taken from each of the seven genotypes of potato following using Genei Pure Total RNA Isolation Kit-for Plants and preparation of cDNA from total RNA for molecular studies.
3. The isolated PI genes can be used for the development of insect resistant crop plants through principles of genetic engineering.
4. It will help in minimizing biotic stress which cause a big loss in crop production.
5. By development of insect-pest resistance crop plants, we can save pre and post harvest losses from the insect-pest and which will help in enhancing crop production

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