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Research Article

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Isolation of a Papain like Cysteine Proteases (PLCP) Gene Sequence from the Genomic DNA of Local *Carica Papaya* Plant

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ABSTRACT

Hemorrhage is spontaneous bleeding caused by a defect in clotting mechanism which is fatal to human health. Proteases have important role in human health and are helpful in metabolism and in cure of diseases like hemorrhagic disorders. Proteases have a role in heamostasis mechanism in clot formation by the regulation and production of thrombin like enzymes. These enzymes are obtained from plant sources have taken a primary importance in the medical sciences for the treatment of hemorrhagic disorders. During the last few years studies are focused on thrombin like enzymes for isolation, identification, cloning, purification, characterization and overproduction for commercialization in fields like medical sciences, detergent development and meat tenderizer. In our present research an act was made to isolate the gene of cysteine protease a thrombin like enzyme called papain like cysteine proteases (PLCPs) from the leaves of Carica Papaya grown locally. This enzyme has the ability to stop strong procoagulant action and has also been reported to be fibrinogen activator. PCR amplification of PLCPs gene sequence was done by using specifically designed primers (originally for cDNA) using genomic DNA as template that is isolated from the leaves of plant using CTAB method. The amplified product of ~1200 base pairs different from the expected size of 1023 bp was confirmed electrophoretically. The PCR optimized conditions were at 2.5 mM $MgCl_2$ and with initial denaturation at 95°C for 4 minutes and followed by 35 cycles of denaturation at 94°C for 1 minute, annealing 49°C for 1 minute, extension 72°C for 2 minutes and final extension at 72°C for 10 minutes. The PCR amplicon from genomic DNA of Carica Papaya is not reported previously. This study can further help to understand the genetic diversity of this enzyme, their characteristics, regulation and better research based understanding of the topic.

Keywords: Proteases; Papain like cysteine proteases; *Carica papaya*; Apocynacae family; Thrombin

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INTRODUCTION

Proteases are one of the most commercially important enzymes due to their multiple applications in food, pharmaceutical and detergent industries, as well as in the preparation of leather and wool [1]. Proteases are those enzymes which catalyze the degradation of peptides and proteins, and have a significant role in different physiological processes in the living beings [2]. On the basis of different functional groups present at the active site, proteases are further classified into four well-known groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases [3]. Due to high stability of temperature and specificity of broad substrate, cysteine proteases are widely used in industry and involved in various biological processes [4].

Cysteine proteases are endopeptidases and present in prokaryotes as well as in eukaryotes [5]. They have a common catalytic mechanism that involves a nucleophilic cysteine thiol in a catalytic dyad [6] contains Cys25 and His159 and an Asn175 residue important for proper orientation of the His side chain [7]. Cysteine proteases are divided into clans (that do not share sequence or structural identity and probably arose from different evolutionary lines) so it belongs clan CA which contain the papain like family (C1).

Family C1 is further divided into C1A and C1B on the basis of presence of disulfide bridges and locality in different organs. C1A has disulfide bridges and present in vesicles, the vacuole, or the apoplast but family C1B, which have been deficient in disulfide bridges and to be found in the cytoplasm [8]. PLCPs have a particular chain of polypeptide with three bridges of disulfide and a sulfhydral group necessary for the action of enzyme [9]. Plant PLCPs are phylogenetically divided into nine subfamilies. [10].

PLCPs are thermostable enzymes [9] due to this property it is found in proteolytically harsh environments such as apoplast, vacuole, and lysosomes because they all have acidic pH. Here PLCPs act as preproproteins and carry various targetting signals [11]. The papain like cysteine endoprotease has the capacity to process the glyoxysomal malate dehydrogenase precursor protein into the mature subunit *in vitro* [12]. Role of papain like cysteine proteases is in the clevage and maturation of proproteinases that are in turn involved in macromolecule degradation and remobilization during leaf senescence [13]. PLCPs play a role in disease resistance signaling, pathogen perception, and defense against insects [14]. It is used in breweries, leather, cosmetic and textile industries [15] meat tenderizers and surfactant synthesis [9]. The proteolytic enzyme papain also coagulates blood. In this case the enzyme does not activate prothrombin, but acts directly on fibrinogen to form a fibrillar gel resembling fibrin. This constitutes that thrombin, the physiological coagulant, is also a proteolytic enzyme with a specific action on fibrinogen [16]. Plants of *Asclepiadaceae* family contain PLCPs in latex. *Asclepias curassavica* latex has PLCPs which stop strong procoagulant action and possess thrombin like activity by activating human plasma factor X which activate fibrinogen and convert into fibrin that make cross linked fibrin clot [17]. Due to these properties and functions the present project was therefore designed to isolate a gene enconding a thrombin like enzyme PLCPs from a locally isolated plant of *carica papaya* that could be used in pharmacological and clinical research in future to enhance the existing knowledge of this diverse family of PLCPs.

MATERIALS AND METHODS

Selection of Plant

Carica Papaya, native of tropical and subtropical climates that do not tolerate temperature below 32°C was employed in current research. Fresh leaves of this plant were selected for the isolation of PLCPs gene from the genomic DNA. Plant material was obtained from botanical garden of the University of Agriculture Faisalabad for the isolation of the gene.

DNA Isolation

The fresh leaves of the *Carica papaya* were used for Genomic DNA isolation. The isolation of the nucleic acid (avoiding RNA by use of RNases) was according to the protocols defined by Doyle and Doyle 1999 [18]. The DNA isolation was confirmed on 1% agarose gel (w/v in 1X TAE) and stained with ethidium bromide. The DNA samples were stored at -20°C till further step. Further quantification of DNA was done by spectrophotometric analysis. The purity of the DNA was checked by finding A260/280 ratio. Quantity was determined in ng/mL.

Polymerase Chain Reaction

Thrombin like enzyme's gene i.e. papain like cysteine proteases (PLCPs) was amplified by PCR in a 50 µL reaction mixture from the genomic DNA of *Carica papaya* using specifically designed primer according to the reported cDNA sequence available by using online bioinformatic softwares like (www.JustBio.com and http://simgene.com/Primer3) [19]. The conditions to be optimized for the PCR reaction mixture were 20-100 ng of Genomic DNA, 10X PCR buffer (Fermentas), 2.5 mM dNTPs (Mixture), Forward and reverse primers 0.1-1 µM each, 1-5 mM $MgCl_2$ and 1.25 U/50 µL reaction of *Taq* DNA Polymerase using Gene Amp PCR System 2400 as thermocycler (Perkin Elmer, USA). The amplification was done using 4 minute hold for initial denaturation at 95°C followed by 35 cycles of repeated denaturation (94°C for 1 min),variable optimizeable annealing temperatures (from 45-55°C for 1-2 min) and polymerization (72°C for approximately 2 min) followed by a hold of polymerization of 10 minutes at 72°C. The designed primers were used GF F:5/-ATGAAGAGCTTTGTATTAATCCTTT-3/ and GF R:5/- TCAAACAGGATAAGAGGGCT -3. The amplicons were confirmed on 1.2% agarose gel electrophoresis. For further sequencing and sample purification step increased volume of the product was eluted in big digested wells on gel and recovered from agarose gel by the help of FavorPreP^{TM} Gel purification mini kit cat: FAGPK001 by following the manufacturer's protocol (FAVORGEN) which was also confirmed electrophoretically.

RESULTS AND DISCUSSION

Selection of Plant and DNA Isolation

Fibrinogen activator enzymes are now-a-days the major thrombin agents used extensively for therapeutical purposes for the treatment of heamorrhagic disorders that are responsible for various bleeding disorders. Thrombin like enzymes are therefore been reported from various sources including different plants also as a part of continously growing research activities. Among them, plants of *Asclepidacea* family have been studied most extensively. The fibrinogen activator enzyme PLCPs from *Carrica papaya* is of great interest as a thrombin like agent because of its use being safer, stable and efficiency in fibrin processes including thrombinogen activation. They also have an industrial application in detergent production as they are proteases. This plant present is tropical and subtropical areas. The cellular mass of this local flora plant was therefore used for DNA isolation following the method of Doyle and Doyle (1999) as Padmalatha and Prasad [20] who used the same protocol for isolation of DNA from palnts that belonged to followed families. RNA contamination was subjected to RNases treatment for its removal. The isolated DNA was confirmed by gel electrophoresis as shown in Figure 1 [2].

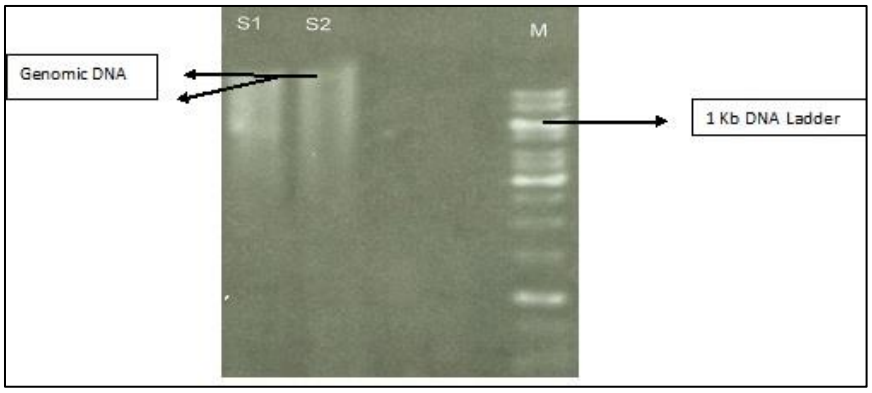

Figure 1: DNA samples extracted from *Carica papaya*

Lane 1 and 2 from left shows genomic DNA samples S1 and S2 respectively extracted by the method of Doyle and Doyle (1999) and treated with RNases to be used for PCR and lane 5 shows the marker (M) 1 kb DNA Ladder (Fermentas).

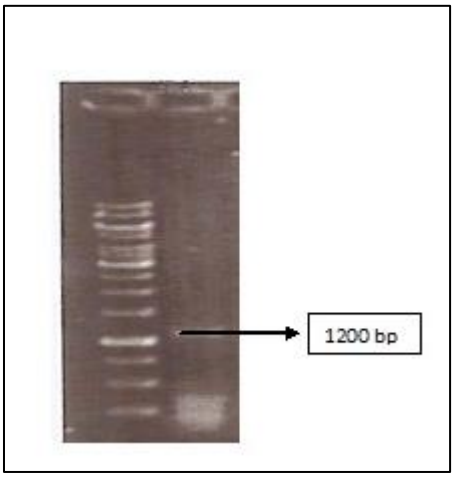

Figure 2: PCR amplified papain like cysteine proteases gene sequence (PLCPs) from *Carica Papaya*

DNA: Lane 2 shows clear amplification with more specific and expected sized band of approximately 1200bp at 2.5 mM $MgCl_2$ concentrarion.

Gene Amplification

To isolate the thrombin like enzyme gene of *Carica papaya* from the genomic DNA, a practice not made previously a set of oligodeoxyribonucleotide primers were designed from the gene sequence available from GeneBank NCBI based on deduced nucleotide sequence of *PLCPs* gene from *Asclepias fruticosa* using the CDS region encoding

asclepian f gene from cDNA and conserve sequence was choosed from different sequence for this purpose [16]. The primers were designed using online primer designing tools available on (www.JustBio.com or primer 3.0) for the PCR amplification from the genomic DNA that may possess sequence encoding PLCPs gene that was not previously practiced although some non-coding regions that are usualy present in the genome. Therefore much effort was done in optimizing the conditions for the amplification of the gene sequence on genomic DNA regarding the optimization of $MgCl_2$, Primers and DNA concentrations to be used for amplification along with PCR thermal cycling conditions specially annealing temperatures were optimized in our work for the amplification of PLCPs gene. The optimum concentrations for the amplification of PLCPs approximately 1200 bp expected gene size were $MgCl_2$ 2.5 mM, Primer 10 µM each (diluted with sterile dH_2O) and DNA 50 ng. Whereas the thermal cycling conditions optimized were initial; denaturation at 95°C for 4 minutes and 35 cycles of denaturation at 94°C for 1 minute, annealing at 49°C for 1 minute, extension at 72°C for 2 minutes. Followed by extended polymerization of 10 min at 72°C to add poly A tail and amplify completely. PCR was performed on both the DNA samples S1 and S2 isolated from leaves of the *carica papaya* plant after several tries the product achieved was electrophoretically viewed on 1% agarose gel at 80 volts for 1 hours as shown in Figure 2. Genomic DNA fragment of 1200 bp was obtained at the 2.5 mM and 3 mM $MgCl_2$ concentration but clear band was obtained at 2.5 mM concentration of $MgCl_2$ because $MgCl_2$ act as a cofactor for *Taq* polymeraese. The size of our gene is 1023 bp in cDNA [21] and the results of PCR showed approximately 1200bp fragment of gene which showed that approximately 180bp of extra fragment of DNA was amlified that could be a non-coding region like introns present within PLCPs gene. Its primers were designed against the CDS sequence of the cDNA so the location of introns and exons would have been responsible to give a extended large sequence amplified from the genomic DNA. Pereira et al. [22] in 2001 purified the cysteine proteases gene having 895 bp from genomic DNA of *Carica candamarcensis L*. It shows strong homology with chymopapain isoform IV from *C. papaya*. Other possibility reflects the possible diversity of the gene and its family as previously it has been reported in various papers like in 2011 Obregon and his coworkers found that diverse gene of Apocynaceae family exist in CIA family. The isolation and exploration of diversity was not reported before regarding the PLCPs gene on DNA from our local flora. The next step was the purification of PCR product from gel. The preferable purification was the gel extraction because only required band separated from the gel and next we preceded it by Favoprep gel purification mini kit (FAVORGEN Biotech Core cat: FAGPK001). Amplified and purified *PLCPs* gene sequence was eluted in 20 µL of deionized water and confirm electrophoretically. Trejo et al. in 2009 purified the gel by QIAEX II agarose gel extraction as well as Obregon and his coreaserchers in 2011 selected DNA bands from gel excised and purified by using a DNA extraction kit (QIAEX II Agarose Gel Extraction, QIAGEN GmbH). The product was purified by Favoprep gel purification mini kit. The expected band of approximately 1200 bp PLCPs gene can be used for further studies and analysis also in our future studies. These results are adding to the existing knowledge of the already reported data and increasing diversity of PLCPs genes of CIA family [14].

CONCLUSION

The thrombin like enzyme PLCPs gene from local isolate of *Carica Papaya* was successfully amplified from the genomic DNA. This may further help in studying genetic diversity of thrombin like enzymes for their better understandings. This gene in future will also be cloned in appropriate expression vector and will be used for enzyme characterization, hyperexpression, purification and analysis to act as a future remedy or drug. It is important from pharmacological, industrial and clinical point of view. This study will also provide amplification of the product and size of the PLCPs amplicon for future studies and thus saving optimization efforts also and may also add to the diversity of the PLCPs gene and their genetic characteristics.

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