Journal of Qassim University for Science
2023, *Vol 2* (Issue 2), pp. 95-104
Received: 23 October 2023
Revised: 25 November 2023
Accepted: 27 December 2023

JQUS

Amplification of serine protease inhibitor gen from *Rhamnus Frangula L*

Jana, Saleh, Alkharafi
Dept. Biology Department, College of Science, Qassim University, Burydah, Saudi Arabia, E-mail Jana.s.alkharafi@gmail.com

Imen M. N. Ben Abdelmalek
Dept. Biology Department, College of Science, Qassim University, Burydah, Saudi Arabia, E-mail mm.abdulmalek@qu.edu.sa

Abstract: Protease inhibitors are proteins that are found in plants, humans, animals, and most microorganisms and their function is to regulate the proteolytic activity. serine protease inhibitors have received great interest for their multiple applications in biotechnology and biomedicine and are the most prominent inhibitors in plants. Based on the amino acid information of trypsin inhibitor of kunitz trypsin inhibitor 3 Glycine max (soybean), degenerated primers were designed and a full-length cDNA sequence named RfIP1(*Rhamnus frangula* Protease Inhibitor 1) was amplified from the leaves DNA RNA by using PCR and RT-PCR respectively. Electrophoresis analysis showed a difference in the migration of the amplicons obtained on DNA and cDNA, revealing the existence of an intron of 210 nucleotides. The result of the RT-PCR proves that the sequence of cDNA contained an open reading frame (ORF) of 570 bp, encoding 190 amino acids residues. In conclusion, we successfully got DNA and synthesized cDNA of serine protease inhibitor the Kunitz-type from plant *Rhamnus frangula* L, which adds to other protease inhibitors extracted from plants.

Keywords: Serine proteases inhibitors; *Rhamnus frangula* L; gene and cDNA sequence; RT-PCR.

1. INTRODUCTION

Proteases, which are known as proteolytic enzymes, are responsible for the breakdown of proteins. They are found in plants, humans, animals, and also in most of microorganisms [1]. Proteolysis plays a major role in many processes such as physiology, defense, DNA replication, immunity, and stress responses [**Error! Reference source not found.**,2]. Proteases are classified based on depending on the nature of the functional group at the active site to four classes: serine proteases, aspartic acid proteases, cysteine proteases, and metalloproteinases [**Error! Reference source not found.**]. Although the protease is important, it can be very detrimental, especially when it over expresses and causes excessive proteolysis; as a result of this, it should be controlled with protease inhibitors (PIs). Protease inhibitors are proteins which are found in all cells and their function is to regulate the proteolytic activity [5,6].These molecules represent an efficient way to control the activity of endogenous proteases. Furthermore, in plants, (PIs) work as a modulation of apoptosis; preventing pathogens and providing protection against insects, nematodes, and microorganisms [7,8]. Besides that activity, (PIs) are directed against exogenous proteases [9]. They also contribute to numerous applications in biotechnology and medicine, such as common cancers and HIV [10,11]. In the food industry, synthetic

chemicals have become limited because of health risks; therefore, it is essential to look for natural preservatives. The protease inhibitor for instance, can be used as preservatives because of their antimicrobial properties and inhibition of proteases [11,12]. In insecticide, the protease inhibitors serve by inhibiting proteases of animal and fungal origins or proteases of insect [13]. Thus, looking for protease inhibitors from natural resources, such as plants which represent an excellent source of endogenous protease inhibitors, could be beneficial in different applications [14].

The serine proteases are the best and the most prominent among the proteases in plants. Serine proteases inhibitors are considered as defending components in plants [6] they inhibit serine proteases either partially or completely [5]. Serine proteases are mainly considered as enzymes for cancer development; thus, serine proteases inhibitors which are derived from plants can be used as drugs for diseases instead of chemicals. [14,15]. In fact, serine protease inhibitors have received a great interest for their multiple applications in biotechnology and biomedicine that focused mainly on therapeutics. Moreover, therapeutic serine protease inhibitors have proven their effectiveness in treating immune, inflammatory, respiratory diseases, and HIV. Also, they prevent pathogens and provide protection to plants by being used in pest control. Recent findings have demonstrated proteolysis control as a pharmacologically valid tool. In one of the studies, the findings suggest that serine protease inhibitors camostat can be used to treat patients with pancreatitis, and it is considered as an industrial drug, since it may inhibit the replication of influenza A/H1N1 and A/H3N2 viruses in primary human tracheal epithelial (HTE) cells through the inhibition of hemagglutinin cleavage which is essential for virus entry into the cell and the start of its replication [16]. However, to avoid the effects of chemical drugs, it is essential to search for serine protease inhibitors in natural sources. Furthermore, a novel serine protease inhibitor gene was isolated from *Hevea brasiliensis* leaves and has been used against antifungal *Trichophytonrubrum* that cause the disease Athlete's foot [17].

Rhamnus frangula L, which is known as (Glossy buckthorn) from family *Rhamnaceae*, is a shrub that grows from 3-6 meters in wetland and upland, used as laxative and can be found as a component in herbal laxative preparations. Some studies have shown that the plant is antifungal and antioxidant [18 –20]. A previous study published in 2017 by Abir et *al* [20] shows that they have purified a protease inhibitor extracted from *Rhamnus frangula*. A biochemical characterization was done and they determined its molecular weight of 22 kDA and its N-terminal sequence. They also showed that it inhibits different types of human and commercial proteases. All these results obtained pushed us to go further with a molecular characterization of the gene coding for this inhibitor. This knowledge will certainly contribute to the design of better approaches to further characterize this inhibitor in order to apply it in pharmaceutical formulations and to use it as a drug for several types of diseases.

2. MATERIALS AND METHODS

2.1Plant material

Leaves of *Rhamnus frangula* were harvested in the Qassim district. the leaves of the plant were dried for 72 hours in an oven at 40°C and subsequently ground.

2.2 Genomic DNA extraction

DNA was extracted from 300 mg of *Rhamnus frangula* by crushing leaf samples then transferred the powdered leaf sample to a 50-ml Falcon tube and added 10 ml of hot extraction buffer to the tube and incubated at 65 °C for 60–80 min, then added an equal volume of chloroform-isoamyl alcohol (24:1) we spun for 15 min at 5 200×g at room temperature then added 6 ml of isopropanol and placed the tubes at -20 °C for 10–15 min, and spun at 8 500×g for 10 min then resuspend the DNA in 200 µl 0.1× TE buffer, which was then treated with RNase and incubated at 37 °C for 2 h, then spun for 10 min at 5 200 x g and added 1/10 volume of 3 mol/L NaCl or 1/10 volume of 3 mol/L Na acetate then spun for 10 min at 8 500×g. Finally, The DNA concentration was measured by taking absorption at 260 nm, according to Sambrook et *al* [21], and by running aliquots on a 1% agarose gel (0.01 g/ml). The purity of DNA was determined by estimating the ratio of absorbance at 260 nm to that at 280 nm (A 260/A 280).

Extraction of total RNA with TRIZOL

RNA was extracted from the leaves of *Rhamnus frangula*. To 100 mg of grounded leaves with liquid nitrogen, 1 ml of TRIZOL (Invitrogen) was added and centrifuged for 15 min at 12000 rpm at 4°C. The RNAs were then precipitated with isopropanol. The pellet was washed with 70% ethanol, after incubation for 1 hour at -80°C and centrifugation for 10 min at 12,000 rpm and 4°C. Assessment of the RNA quality was performed by agarose gel electrophoresis.

2.3 Primer designing

Designing primers to amplify used NH2-terminal sequence (MKSTIFFIFL FCATTTSYLP SLIVDFVLDN NGN), and used NCBI Genebank database and downloaded the sequence of Kunitz trypsin inhibitor 3 *Glycine max* (soybean) . Were designed two forward (FPI, FPII) and two reverse (RPI, RPII) primers Tables 1.

Table 1:Primers used for amplification of *Rhamnus frangula* protease inhibitor gene

Primers	Sequence	Annealing Temperature (°C)
FPI	5' ATGAAGAGCACCATCTTCTTTCTCT3'	59,43
FPII	5' CCCCAAAAATGAAGAGCACCAT 3'	59,70
RPI	5' CTCACTCACTGCGAGAAAGG 3'	58,29
RPII	5' CTTGTGTCTCACTCACTGCG 3'	58.86

2.4 Amplification of DNA by PCR

The PCRs were carried out in the "BioMetra®" according to the following program: initial heating step (94 °C for 5 min), a DNA amplification step for 40 cycles of denaturation (94 °C, 30 s) annealing (55 °C for 30 s), extension (72 °C for 3 min) and a step of termination of the DNA synthesis (72°C, 10 min).

2.5 cDNA synthesis

A solution of total RNA (5 µg) was incubated at 65°C for 5 min, then added 1 µl of specific reverse primer and incubated at 25°C for 10 min, added 4 µl of 5X "first strand reaction buffer", 2 µl of DTT, 1 µl of the

MMLV enzyme and 1 µl dnTP and incubated at 37°C for 1 h Finally, to stop the reaction by inactivating the enzyme was incubated at 65°C for 5 min. The cDNA has been obtained and used as a template for PCR then separated on a 1% agarose gel.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Genomic DNA extraction from *Rhamnus frangula*

we extracted genomic DNA from a 300 mg plant. The concentration of genomic DNA obtained was estimated, by spectrophotometry at 260 nm, at 250 ng/µl, the ratio of readings at 260 nm and 280 nm. The quality of the extracted genomic DNA was checked on a gel of 1% agarose. A band of approximately 15,000 bp of genomic DNA remains at the top of the gel is observed **Error! Reference source not found.**. This band corresponds to the expected size since the total genomic DNA is large.

3.1.2 Extraction of total RNA from Rhamnus frangula

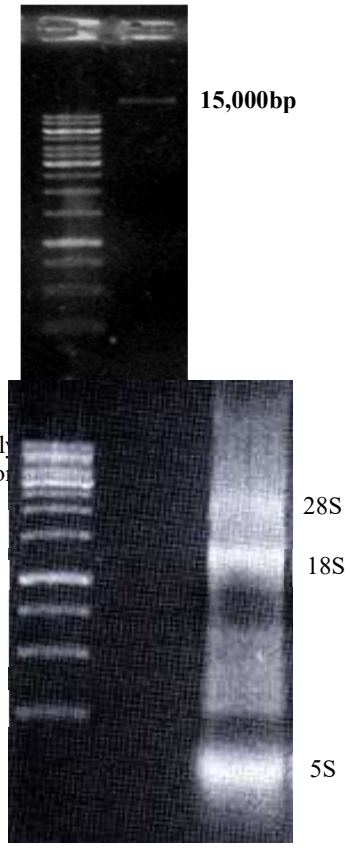

Fig 1:Anal
Geno

Fig. 2: Analysis of *Rhamnus frangula* RNAs quality

Total RNA must first be extracted and cDNA synthesized before the determined coding sequence of the *Rhamnus frangula* protease inhibitor RfIP1. By using specific primers, it was possible to amplify the coding sequences of this inhibitor by RT-PCR. In fact, total RNA extraction was performed from 100 mg of plant. This extraction, carried out with the TRIZOL reagent (Invitrogen), was followed by precipitation with isopropanol. The quality of this extraction was also visualized after electrophoresis on 1% agarose gel and

stained with BET. Analysis of this gel reveals three bands of ribosomal RNA, 28S rRNA 18S rRNA and 5S rRNA Figure 2.

3.1.3 Amplification of the genomic and coding sequences of Rhamnus frangula protease inhibitor gene

In order to isolate and sequencing the gene encoding *Rhamnus frangula* protease inhibitor, different pairs of primers were chosen and designed to amplify the entire gene **Error! Reference source not found.**.

By using the degenerate primer the PCR reaction made it possible to amplify the total genomic sequence of

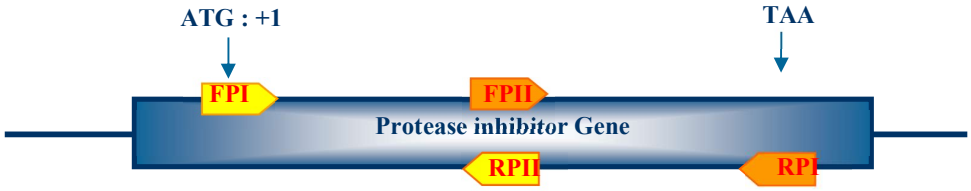

Fig. 3: Input sites pairs of primers to amplify the entire gene

Rhamnus frangula protease inhibitor. Amplification was performed under the following PCR conditions: 94°C for 60 s, 54°C for 60s, and 72°C for 60s (40 cycles). The result of amplification on an agarose gel showed a

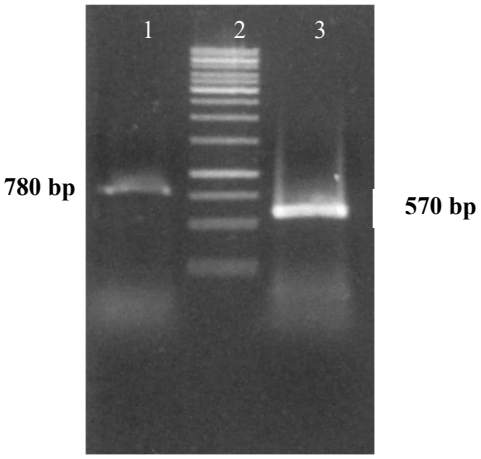

Fig.4: Electrophoresis on agarose gel (1%) of the PCR amplification products obtained.

Lane 1: PCR on genomic DNA of *Rhamnus frangu*la.
Lane 2: Concomitant migration of the 1kb Ladder Molecular weight marker.
Lane 3**:** PCR on the protease inhibitor cDNA.

band of approximately 780 bp Figure 4. From the total RNA, the cDNA is directly synthesized by a reverse transcription reaction using degenerate oligonucleotides FPI and RPI for the *Rhamnus frangula* protease inhibitor gene. Then amplification and isolation of a single product of about 570 bp Figure 4 covering the entire coding sequence of the protease inhibitor of *Rhamnus frangula*.

3.2 Discussion

The biggest and most significant subfamily of protease inhibitors is known as serine protease inhibitors, or serpins. By covalently attaching to their target protease, they function as suicide substrates and cause their

inactivation [22]. The ability of the widely distributed serine protease inhibitor to modify the proteolytic activities of various proteases revealed that are crucial regulators in a variety of biological processes in metazoan, plant, and some viral organisms that necessitate precise control of protein hydrolysis [12]. Plants produce a range of chemicals, both proteinaceous and non-proteinaceous, in reaction to insect attacks. Of these, Protease Inhibitors are the most researched family of plant defense proteins.

Proteases are necessary to survive and in biological processes proteolysis performs many such as DNA replication and transcription, cell proliferation and differentiation, wound repair, blood coagulation, inflammation, immunity, protein digestion, and apoptosis. Yet alterations in proteases activity result in multiple pathological conditions such as cancer, and inflammatory and cardiovascular diseases. So, their activities need to be kept strictly under control by protease inhibitors. It is found in numerous animal tissues and fluids, plants, and microorganisms [11]. The main function of endogenous proteases inhibitors is the prevention of unwanted proteolysis. Because their molecules represent an efficient way to control the activity of proteases, they are gaining attention in the biotechnological, food industry, and medicine [11,12,23]. In this study, we extracted serine protease inhibitors from *Rhamnus frangula* L and we got total genomic DNA and was size large 15.000 bp shown on a gel of 1% agarose Figure 1. In order to synthesis cDNA to get the determined coding sequence of the *Rhamnus frangula* protease inhibitor RfIP1 we extracted the total RNA with the TRIZOL reagent, where ribosomal RNA appeared in agarose gel Figure 2 as three bands 28S rRNA 18S rRNA and 5S rRNA. The 5S rRNA band was clearly visible without smearing. In addition, the 18S rRNA band was more intense than the 28S rRNA band.

Using degenerate primers, genomic amplification by PCR and an RT-PCR were done on genomic DNA and a cDNA, respectively. Results show amplicons on an agarose gel that with different size. A 780 bp with genomic PCR and 570 bp with RT-PCR Figure 4. This result shows that serine protease inhibitor gene contains a 220 bp intron unlike several other types of inhibitors of different sources which do not contain. In fact, A full-length Kunitz-type protease inhibitor gene from *Dolichos biflorus* has a frame of 669 bp, and don't contain intron sequence [24]. Same observation was seen in *Hevea brasiliensis* leaves that the cDNA of 210 bp encoded a 70 amino acid protein and don't contain introns [17]. Equally, the serine protease inhibitor codant gene from cotton bollworm of *Helicoverpa armigera* was 1176 bp long, with an open reading frame encoding 391 amino acids; there is one exon and no intron [25]. While alkaline serine protease gene amplified from genomic DNA and cDNA specific fragment of the antagonistic yeast-like fungus *Aureobasidium pullulans* PL5 were of 1351 bp and 1248 bp, respectively [26]. The gene encoding a novel serine protease inhibitor of *Schistosoma japonicum* (*SjSPI*) was sequenced and showed that *SjSPI* contained an open reading frame of 1,218 bp, which encoded 405 amino acid residues. Chromosomal structure analysis showed that *SjSPI* gene was comprised of six exons separated by five introns [27]. The *Rhamnus frangula* protease inhibitor Kunitz-type designated RflP1 was purified and biochemically characterized for the first time, followed by this study which allowed us a molecular characterization of this inhibitor followed by this study which allowed us a molecular characterization of this inhibitor through an amplification of the coding gene and determination of its size with a revelation of the existence of an intron sequence. This study will add the protease inhibitor to

others extracted from different source of plants, and will be useful for further study to find amino acid sequence the RflP1 protein.

REFERENCES

[1] Shamsi, T. N.; Romana, P.; and Sadaf, F. "Characterization, biomedical and agricultural applications of protease inhibitors: A review," Int. J. Biol. Macromol, 91 , pp.1120-1133, 2016.

[2] Figueiredo, A.; Filipa, M.; and Mónica, S. "Subtilisin-like proteases in plant–pathogen recognition and immune priming: a perspective," Front. Plant Sci, 5, pp. 739., 2014.

[3] Bond, J. S. "Proteases: History, discovery, and roles in health and disease," JBC, 294.5, pp. 1643-1651, 2019.

[4] Rani, K.; Rachita, R.; and Sanchi, D. "Review on latest overview of proteases," Int J Curr Life Sci 2.1, pp. 12-18.,2012 .

[5] Harish, B. S.; and Kiran, B. U. "Microbial serine protease inhibitors and their therapeutic applications," Int. J. Biol. Macromol, 107, pp. 1373-1387,2018 .

[6] Malefo, M. B.; Mathibela, E. O.; Crampton, B. G.; and Makgopa, M. E. "Investigating the role of Bowman-Birk serine protease inhibitor in Arabidopsis plants under drought stress," Plant Physiol. Biochem, 149, pp. 286-293, 2020.

[7] Turra, D.; Bellin, D.; Lorito, M.; and Gebhardt, C. "Genotype-dependent expression of specific members of potato protease inhibitor gene families in different tissues and in response to wounding and nematode infection," J. Plant Physiol, 166.7, pp. 762-774, 2009.

[8] Billinger, E.; Zuo, S.; Lundmark, K.; and Johansson, G. "Light scattering determination of the stoichiometry for protease-potato serine protease inhibitor complexes," Anal. Biochem, 582, pp. 113357, 2019.

[9] Volpicella, M.; De Leo, F.; Sciancalepore, M.; Sonnante, G.; Pignone, D.; Gallerani, R.; and Ceci, L. R. "Identification and characterization of protease inhibitors in Diplotaxis species," Plant Physiol. Biochem, 47.3, pp. 175-180, 2009.

[10] Srikanth, S.; and Chen, Z. " Plant protease inhibitors in therapeutics-focus on cancer therapy," Front. Pharmacol, 7, pp. 470, 2016.

[11] Cotabarren, J.; Lufrano, D.; Parisi, M. G.; and Obregón, W. D. "Biotechnological, biomedical, and agronomical applications of plant protease inhibitors with high stability: A systematic review," Plant Sci. 292, pp. 110398, 2020.

[12] Cid-Gallegos, M. S.; Corzo-Ríos, L. J.; Jiménez-Martínez, C.; and Sánchez-Chino, X. M. "Protease inhibitors from plants as therapeutic agents-a review," Plant Foods Hum. Nutr, 77.1, pp. 20-29, 2022.

[13] Katoch, R., Singh, S. K.; Thakur, N.; Dutt, S.; Yadav, S. K.; and Shukle, R. "Cloning, characterization, expression analysis and inhibition studies of a novel gene encoding Bowman–Birk type protease inhibitor from rice bean," Gene, 546.2, pp. 342-351, 2014.

[14] Pramanik, A.; Paik, D.; Pramanik, P. K.; and Chakraborti, T. "Serine protease inhibitors rich Coccinia grandis (L.) Voigt leaf extract induces protective immune responses in murine visceral leishmaniasis," Biomed. Pharmacother, 111, pp. 224-235, 2019.

[15] García-Gasca, T.; García-Cruz, M.; Hernandez-Rivera, E.; López-Matínez, J.; Castaneda-Cuevas, A. L.; Yllescas-Gasca, L.; ... and Blanco-Labra, A. "Effects of Tepary bean (Phaseolus acutifolius) protease inhibitor and semipure lectin fractions on cancer cells," Nutr. Cancer, 64.8, pp. 1269-1278, 2012.

[16] Yamaya, M.; Shimotai, Y.; Hatachi, Y.; Kalonji, N. L.; Tando, Y.; Kitajima, Y.; ... and Nishimura, H. "The serine protease inhibitor camostat inhibits influenza virus replication and cytokine production in primary cultures of human tracheal epithelial cells," Pulm. Pharmacol. Ther, 33, pp. 66-74, 2015.

[17] Chinnapun, D.; Palipoch, S.; and Hongphruk, H. "Cloning and characterization of a putative gene encoding serine protease inhibitor (251Hbpi) with antifungal activity against'Trichophyton rubrum'from'Hevea brasiliensis' leaves," Plant. Omics, 9.2, pp. 142-148, 2016.

[18] Williams, C. E.; and Krock, S. L. "Patchy invasion of riparian savannas by Rhamnus frangula L.(Rhamnaceae) in northwestern Pennsylvania: is soil drainage a factor?," Castanea, 77.4, pp. 318-326, 2012.

[19] Brkanac, S. R.; Gerić, M.; Gajski, G.; Vujčić, V.; Garaj-Vrhovac, V.; Kremer, D.; and Domijan, A. M. "Toxicity and antioxidant capacity of Frangula alnus Mill. bark and its active component emodin," Regul. Toxicol. Pharmacol, 73.3, pp. 923-929, 2015.

[20] Bacha, A. B.; Jemel, I.; Moubayed, N. M.; and Abdelmalek, I. B. "Purification and characterization of a newly serine protease inhibitor from Rhamnus frangula with potential for use as therapeutic drug," 3 Biotech 7, pp. 1-13, 2017.

[21] Sambrook, J.; Fritsch, EF.; And Maniatis, T. Molecular Cloning: A Laboratory Manual. No. Ed. 2. Cold Spring, Harbour, New York, USA, 1989.

[22] Gettins, P. "Serpin structure, mechanism, and function," Chem. Rev, 102.12, pp. 4751-4804, 2002.

[23] Rudzińska, M.; Daglioglu, C.; Savvateeva, L. V.; Kaci, F. N.; Antoine, R.; and Zamyatnin Jr, A. A. "Current status and perspectives of protease inhibitors and their combination with nanosized drug delivery systems for targeted cancer therapy," Drug Des Devel Ther. pp. 9-20, 2021.

[24] Kuhar, K.; Kansal, R.; Mishra, A.; Koundal, K. R.; and Gupta, V. K. "Cloning, characterization and expression analysis of a novel gene encoding Kunitz-type protease inhibitor from Dolichos biflorus," 3 Biotech, 2, pp. 199-209, 2012.

[25] Shakeel, M. "Molecular identification, characterization, and expression analysis of a serine protease inhibitor gene from cotton bollworm, Helicoverpa armigera (Lepidoptera: Noctuidae)," Braz. J. Biol, 81, pp. 516-525, 2020.

[26] Zhang, D.; Spadaro, D.; Valente, S.; Garibaldi, A.; and Gullino, M. L. "Cloning, characterization, expression and antifungal activity of an alkaline serine protease of Aureobasidium pullulans PL5 involved in the biological control of postharvest pathogens," Int. J. Food Microbiol, 153.3, pp. 453-464, 2012.

[27] Ying, Z.; Jin, G.; Li H.; Hong-Y, Z.; and Guo-B. C. "Isolation and characterization of a novel serine protease inhibitor, SjSPI, from Schistosoma japonicum," Parasitol. Int, 67.4, pp. 415-424, 2018.

الملخص باللغة العربية
تضخيم تسلسل جين مثبط سيرين بروتياز من نبات السدر

جنى صالح الخرافي
المملكة العربية السعودية ،القصيم ،بريدة، جامعة القصيم ،كلية العلوم، قسم الأحياء

ايمان بن عبدالملك
المملكة العربية السعودية،القصيم ،بريدة، جامعة القصيم ،كلية العلوم، قسم الأحياء

مثبطات الإنزيمات هي عبارة عن بروتينات موجودة في الإنسان ومعظم الكائنات الحية كالنباتات والحيوانات ووظيفتها تنظيم نشاط التحلل البروتيني، ومن أكثر المثبطات التي حظيت بالاهتمام لتطبيقاتها المتعددة في التكنولوجيا الحيوية والطب الحيوي هي مثبطات سيرين بروتياز والتي تعد من أبرز المثبطات في النبات. تم تصميم البادئات بالاستناد على الأحماض الأمينية لمثبط كونيتز التربسين ٣ الموجود في فول الصويا (Glycine max) واستخدامها لتضخيم تسلسل جين الـ(RfIP1) من (DNAو RNA) المستخرجة من أوراق (*Rhamnus frangula*) باستخدام (PCR وRT-PCR)، اظهر الفصل الكهربائي اختلافًا في هجرة امبليكون من DNAوcDNA مما يكشف وجود متوالية داخلية مكونة من ٢١٠ نكليوتيدة. أظهرت نتيجة RT-PCR أن تسلسل cDNA يحتوي على إطار مفتوح يبلغ ٥٧٠ زوج قاعدي والذي يشفر ١٩٠حمض أميني.في الختام، نجحنا في تصنيع cDNA لمثبط سيرين البروتياز نوع كونيتز من نبات (*Rhamnus frangula*) والذي يضاف إلى مثبطات الأنزيم البروتيني الأخرى المستخرجة من النباتات.

الكلمات المفتاحية: مثبطات سيرين بروتياز، *Rhamnus frangula* L ، تسلسل جين cDNA ، RT-PCR