

# Isolation and Cloning of *sprA* Gene Alkaline Protease Activity from *Streptomyces flavogriseus* ADEM7 and Characterization of Its Antioxidant and Antitumor Activities

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Received March 22, 2023; Revised April 25, 2023; Accepted May 07, 2023

**Abstract** In the food industry, alkaline protease plays an important role in the formation of value-added products. Alkaline protease also plays a significant role in waste management. In this study, to construct a stable and high-yield alkaline protease producer, we investigated a combined strategy of gamma radiation as an enhancement factor and alkaline protease PGEM-T easy construct. The present study was focused on the isolation of the *sprA* gene-producing alkaline protease from *Streptomyces flavogriseus* ADEM7 (AB723783.1). Optimum growth media, fermentation conditions, and proteolytic activity assay were evaluated. The result showed that the free cells preparation produced alkaline protease with a specific activity of 550 U/mg after being exposed to gamma radiation at 10 KGy. The *sprA* gene was detected using polymerase chain reaction followed by cloning in PGEM-T easy vector, transformation in *E. coli* JM109, and direct construct sequencing. The *sprA* gene encoding streptogrisin a (alkaline protease-like activity) was isolated in this study and recorded in the gene bank with accession number AB827411.1. The extracellular protease enzyme was tested for antioxidant and anticancer activities. In future work, we will extract this alkaline protease from the same strain on a larger scale using a fermenter and will characterize its antioxidant activity under *in vivo* conditions.

**Keywords:** *Streptomyces flavogriseus*, alkaline protease, antioxidant and anticancer, gamma radiation

**Cite This Article:** Doaa E. El-Hadedy, Nesreen A. Safwat, Abir Pertila, and Rafat A. Siddiqui, "Isolation and Cloning of *sprA* Gene Alkaline Protease Activity from *Streptomyces flavogriseus* ADEM7 and Characterization of Its Antioxidant and Antitumor Activities." *Journal of Food and Nutrition Research*, vol. 11, no. 5 (2023): 345-352. doi: 10.12691/jfnr-11-5-2.

## 1. Introduction

For economic reasons, scientists found alkaline protease for broad applications, including cleansers, nourishment, pharmaceuticals, and calfskin [1,2]. They are characterized into different groups, such as serine protease, cysteine protease, aspartic protease, and metalloprotease [3].

Proteases represent no less than 60% of the enzyme market and around 66% of the proteases are derived from the microbial sources. Various studies have recently been focused on isolating protease from multiple microorganisms. Chemical factors, such as, media segments, carbon and nitrogen sources, and physical

factors such as temperature, pH, brooding time, and inoculum thickness [4] significantly affect the production of proteases by microorganisms. Important proteases produced by various microorganisms, particularly actinomycetes [5,6,7], have characterized for their activity at different pH, temperature, and dependability toward cleansers. The family *Streptomyces* constitute half of the population of soil actinomycetes.

Utilization of *Streptomyces* for protease generation has been explored due to their ability to emit the proteins into cell media, and generally recognized as safe (GRAS) by sustenance and medication organizations. Common *Streptomyces* sp. that produce proteases include *Str. clavuligerus*, *Str. griseus*, *Str. rimouses*, *Str. thermoviolaceus*, *Str. thermovulgaris* [8,9].

## 2. Material and Methods

### 2.1. Protease Production

#### 2.1.1. Growth Media and Fermentation Conditions

The inoculum was set up by growing the living *Streptomyces flavogriseus* ADEM7 (AB723783) on soybean for 9 days [10]. One ml of spore suspension containing  $3.5 \times 10^7$ , made in saline, was inoculated into 49 ml fluid (medium No. 5), which contains sucrose (20g/l), NaCl (0.5 g/l), KNO<sub>3</sub> (2g/l), K<sub>2</sub>HPO<sub>4</sub> (1 g/l), MgSO<sub>4</sub> (0.5 g/l), CaCO<sub>3</sub> (3 g/l), and FeSO<sub>4</sub>, ZnSO<sub>4</sub> and MnCl<sub>2</sub> (0.01 g/l each) in a 250 ml Erlenmeyer flask. The flasks were brooded for 6 days at 30°C in a shaking incubator (200 rpm). The medium was centrifuged at 12,000 xg for 10 min, and the supernatant was tested for protease activity and protein content.

### 2.2. Proteolytic Activity Assay

A quantitative assay of protease activity from the culture filtrate of *S. flavogriseus* was assayed by a modified method [11] with some modification by substituting casein as the substrate [10]. A 100 µl of enzyme solution was added to 900 µl of substrate solution [2 mg/ml of 1 w/v of casein in 10 mM Tris-HCl buffer (pH 8)] and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 1 ml of 10% w/v trichloroacetic acid. The reaction mixture was then allowed to stand on ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 12,000 x g for 10 min at 4°C and the acid-soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na<sub>2</sub>CO<sub>3</sub> solution. The color developed, after adding 0.5 ml of 3-fold diluted Folin Ciocalteu reagent and measured at 750 nm using a spectrophotometer. All assays were performed in triplicate. One protease unit is defined as the amount of enzyme that releases 1 µg of a tyrosine per ml per minute under the above assay conditions. The specific activity is expressed as the units of enzyme activity per milligram of protein.

### 2.3. Protein Concentration or Protein Assay

The protein concentration was determined by the Lowry's method [12] using tyrosine with concentration (0.1-0.7 µg/ml) as a standard.

### 2.4. Gamma Radiation

The *Streptomyces flavogriseus* ADEM7 was exposed to different doses of gamma radiation (2, 4, 6, 8, 10, 12, and 14 kGy) at room temperature, brooded at 30°C for 24h in separate 500 ml cone-shaped carafes. Results were reported as the mean ± S.E. of three autonomous culture arrangements performed in triplicate. The data were analyzed using one-way ANOVA.

### 2.5. SprA Gene Isolation and Cloning Genomic

Genomic DNA was extracted by a modification of the method as described previously [13]. The polymerase

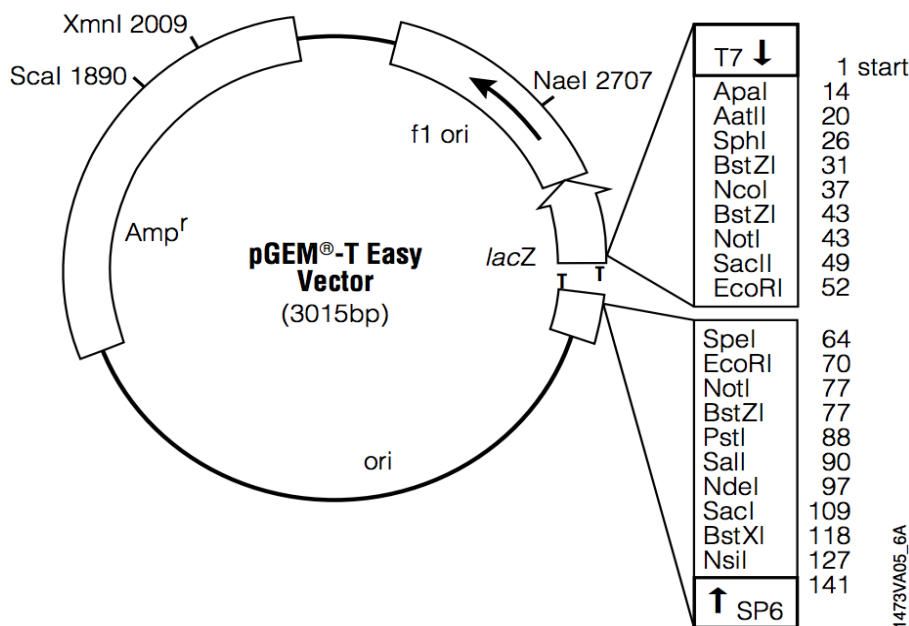
chain reaction (PCR) was performed after a few modifications of the method as described [14]. The PCR enhancement was performed in a 50 µl blend in a DNA thermocycler. The conditions comprised of 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72 h for 2 min. band detect at 1190 bp. The primers were designed from SprA genes multi alignments data from gene bank, the restriction sites EcoRI were added at 5' end for cloning purpose. Forward and reverse primers were as follows: primer F SprA 5'- ggtctacctgtacaacggct -3' TM 59 and R SprA 3' - gtctggatcatgccgtacac 5'- tm 58. The amplified PCR products were purified from low melting point agarose and the nucleotide sequences were determined by Promega lab, Biotechnology Company. SprA expressed alkaline protease PCR product was purified and ligated to PGEM®-T easy vector; ligated plasmids were transformed into E. coli JM109 and transformants were selected using the blue/white screening procedure. After growing on IPTG/X gal agar, plates were supplemented with 100 µg/ml ampicillin, and screening of recombinants was performed as described [15]. Purified plasmids were sequenced using a sequencer in Promega lab. The sequence alignment was prepared with DNA star software program.

**Competent cell solutions:** MgSO<sub>4</sub> (2M), Glucose (2M), and TSS solution (Transformation and Storage Solution) (1X) were prepared as follows: Solid PEG (M, 3350 or 8000) was added to L.B. broth to make 10% (w/v) solution. An aliquot of the 2 M Mg<sup>+</sup> stock solution was added to achieve a final concentration of 20-50 mM. The pH of the solution was adjusted to 6.5-6.8. The solution was sterilized by filtration through a disposable cellulose nitrate filter (0.25 µm pore size). Aliquots of DMSO were added to the filtrate solution to achieve a final concentration of 5% (v/v), and the solution was kept cooled and stored at 4°C. Solutions of E. coli competent cells were prepared fresh, filtered, and stored at 4°C.

Ampicillin was prepared as 100 (mg/ml) in distilled water, sterilized by filtration through a 0.25 µm disposable bacterial filter, and stored at -20°C. The working concentration for transformed E. coli cells was 100 (µg/µl). -X gal (0.4 g in 20 ml H<sub>2</sub>O). X-Gal (5-Bromo-4-chloro-3-indoyl-β degalactopyranoside) was prepared as 20 (mg/ml) in dimethyl formamide, sterilized by filtration, and stored in a dark bottle at -20°C. The working concentration of X-Gal was 40 (µg/µl). IPTG (1.19 g in 50 ml H<sub>2</sub>O) IPTG (Isopropyl- β-D-Thiogalactopyranoside) was prepared as stock solution 200 (mM) in water, stored at - 20°C; the working concentration of IPTG was (0.04 mM).

**Preparation of Plasmids:** A cloning vector is simply a DNA molecule possessing an origin of replication so that it can replicate in the host cell of choice. A PGEM-T easy vector was obtained from (Promega) (Figure 1) and used as a cloning vector.

**Restriction enzyme:** Restriction endonucleases are a group of DNA-cutting enzymes found in bacteria. They differ from other nucleases in only cutting a DNA chain at specific sequences called recognition sites, and the recognition sites may consist of 4 to 8 nucleotide pairs. EcoR1, Not 1, Pst1, Nde1, and Sac1 were obtained from Promega and Biolab Inc to performed these studies.



**Figure 1.** Restriction map and general specification of the cloning PGEM-T easy vector. The sequence of the multiple cloning sites is shown with a PCR product inserted by TAT cloning strategy

**Bioinformatics:** Plasmid sequence alignment was prepared with DNASTAR software programs (DNASTAR, INC., Madison, Wis.), manually edited with GeneDoc ([www.NCBI / blast.com](http://www.NCBI / blast.com)), and determined translation encoded regions from Web ([www.expasy.org/cgibin/dna\\_aa](http://www.expasy.org/cgibin/dna_aa)). Constructs contain sprA gene fragment was diagramed by NTI vector program.

**Antitumor activity:** The human carcinoma cell lines, including MCF-7 cells (human breast carcinoma), HepG-2 cells (human Hepatocellular carcinoma), and HCT-116 cells (human colon carcinoma) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/mL gentamycin (Lonza, Belgium). The cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were sub-cultured two to three times a week during the experiment. For antitumor assays, the tumor cell lines were suspended in a medium (cell density of 5x10<sup>4</sup> cells/well in a Corning® 96-well tissue culture plates) and incubated for 24 hours. The crude extract containing protease enzyme from *Streptomyces flavogriseus* ADEM7 were then added to the 96-well plates (six replicates) to achieve eight variable concentrations for each compound. Separate vehicle controls with only media were run for each 96 well plate as a control. After incubating for 48 h, the numbers of viable cells were determined by the MTT assay [16]. Briefly, the media was removed from the 96-well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red. Ten µL of the 12 mM MTT stock solution {5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (purchased from Sigma-Aldrich, St. Louis, MO) was added to each well including the untreated controls. The 96 well plates were then incubated in 5% CO<sub>2</sub> at 37°C for 4 hours. Afterward, an 85µl aliquot of the media was removed from the wells. Fifty µl of DMSO was added to each well, mixed thoroughly with the pipette, and incubated at 37 °C for 10 min. The optical density (O.D.)

was measured at 590 nm using a microplate reader (Sunrise, TECAN, Inc, USA) to determine the number of viable cells. The O.D. used to check the percentage of viability was calculated as [(ODt/ODc)]x100%, where ODt is the mean O.D. of wells treated with the tested sample and ODc is the mean O.D. of untreated cells. The 50% inhibitory concentration (IC<sub>50</sub>), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the Dose-response curve for each concentration using GraphPad Prism software (San Diego, CA. USA) [17].

**Antioxidant activity:** The antioxidant activity of the crude extract was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University by the DPPH free radical scavenging assay in triplicate, and average values were reported. Briefly, a fresh methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (0.004% w/v) was prepared and stored at 10°C in a dark environment. A methanol solution of the crude extracts was also prepared. A 40 ml aliquot of the methanol solution was added to 3 ml of DPPH solution and Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Spectronic 1201, Milton Roy, State, country). The decrease in absorbance at 515 nm was continuously determined, with data recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = [(AC- AT)/ AC] x 100 where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample + DPPH at t = 16 min [18]. The 50% inhibitory concentration (IC<sub>50</sub>), the concentration required for 50% radical scavenging activity, was estimated from graphic plots of the dose-response curve using Graphpad Prism software (San Diego, CA. USA).

### 3. Results

#### 3.1. Effect of Gamma Radiation on Alkaline Protease Activity

Table 1. Effect of gamma radiation doses on alkaline protease activity

Dose of gamma radiation (KGy)	The specific activity of alkaline protease (U/μg) (Mean ± SEM)
0	390±3
6	405±5
8	412±2
10	420±7
12	413±5
14	409±4

Results in Table 1 Shows the effect of different doses of gamma radiation (0, 6, 8, 10, 12, and 14 kGy) on alkaline protease activity from *Streptomyces flavogriseus* ADEM7. As reported previously [10], this strain was identified using 16sRNA. The sequence was submitted to gene bank with accession number AB723783.1. The data indicate

that the enzyme activity of alkaline protease was increased with increased radiation intensity exposure up 10 KGy, and thereafter further exposure inhibited the enzyme activity.

#### 3.2. Detection of Protease Gene Using PCR

The polymerase chain reaction (PCR) amplified the sprA gene sequence of alkaline protease of *Streptomyces flavogriseus* ADEM7. The DNA of the different *Streptomyces flavogriseus* was extracted directly from a single colony by boiling for 10 min in 100 μl of distilled water and was used to detect the sprA gene by using specific primers. One set of primers (forward and reverse) specific to the sprA gene, which was mentioned in the material and methods, was used and gave the expected PCR product of the sprA gene at 1190 bp (data not shown).

#### 3.3. Sequence Result

The fragment sequenced, and sequence edit was deposited at gene bank with accession number AB827411. The sequence of SprA 1190 bp of gene is given below:

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1   gtcgaccccc atctcattcc gggctcgcgg gcgcgaaatcc ggccttgctg cagggacggt
61  ccccgtaaac gattccagcg tgcaacttgg caggttcacg cccactccca ctgggtgaga
121 acctcgcgca ccaacggccc cacctcaccg gaccgggccc tccccccata cctcggagga
181 tctcgtgacc ttcaagcgct tctcgcgctc cagcagcacg tcaagatatg cacggctcct
241 cgccgtggcc tccggcctgg tggccgcgcg ggccttgccc acccctcctg ccgtcgcgcg
301 tcccagggcg gagtccaagg ccaccgtttc gcagctcgcc gacgccagct ccgccatcct
361 cgccgctgat gtggcgggca ccgcctggta cacggaggcg agcacgggca agatcgtcct
421 caccgcccgc agcaccgtgt cgaaggccga actggccaag gtcagcaacg cgctggcggg
481 ctccaaggcg aaactgacgg tcaagcgcgc cgagggcaag ttcaccocgc tgatcgcggg
541 cggcgaggcc atcaccaccg gtggcagcgc ctgttcgctc ggcttcaacg tgtcggtaaa
601 cggcgtcgcc cacgcgctca ccgccggcca ctgcaccaac atcagcgcca gctgggtccat
661 cggcacgcgc accggaacca gcttcccga aacagactac ggcacatcct gccactcgaa
721 cccggcggcg gccgacggcc ggggtctacct gtacaacggc tctaccaggc acatcacgac
781 ggcgggcaac gcctttgtgg ggcaggccgt ccagcgcgag ggcagcaca ccgggtgctg
841 cagcggctcg gtcaccggcc tcaacgccac ggtcaactac ggttccagcg ggatcgtgta
901 cggcatgata cagaccaacg tctgtgcca gcccggtagc agtgaggtag cgctcttgcg
961 gggcagcacc gctctgggtc tcacctccgg cggcagtgcc aactgccgga ccggcggcac
1021 cacgttctac cagcccgtca ccgaggcgct gagcgcctac ggggcaacgg tccctgtagcc
1081 ggtgccaccg gggcttcggg ctgaccgcgc accggccgcc cgaagccccc cgcgacgccc
1141 caccccggcg gaccgtgctc gcgcgcggtc cgccctcgcc gtgccaagaa

```

The predicted Amino acid sequence from the above DNA sequence is given below:

Amino acid sequence is

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5'3' _____ Frame _____ 2
STPISFRARGRESGLASGTVPVNDSSVQLGRFTPTPTG Stop EPRAPTAPP
HPTGSPHTSEDLVTFKRFSPLSSTRYARLLAVASGLVAAAALATPSAVA
APEAESKATVSQLADASSAILAADVAGTAWYTEASTGKIVLTADSTVSKA
ELAKVSNALAGSKAKLTVKRAEGKFTPLIAGGEAITTGGSRCSLGFNVSV
NGVAHALTAGHCTNISASWSIGTRTGTSFPNNDYGIIRHSNPAAADGRV
YLYNGSYQDITTAGNAFVGVQAVQRSGSTTGLRSGSVTGLNATVNYGSSG
IVYG Met IQTNVCAEPGDSGSLFAGSTALGLTSGGSGNCRRTGGTTFYQP
VTEALSAYGATVL Stop PVPPGLRADRRPAARSPARRPTPADRARARSALA
VPR

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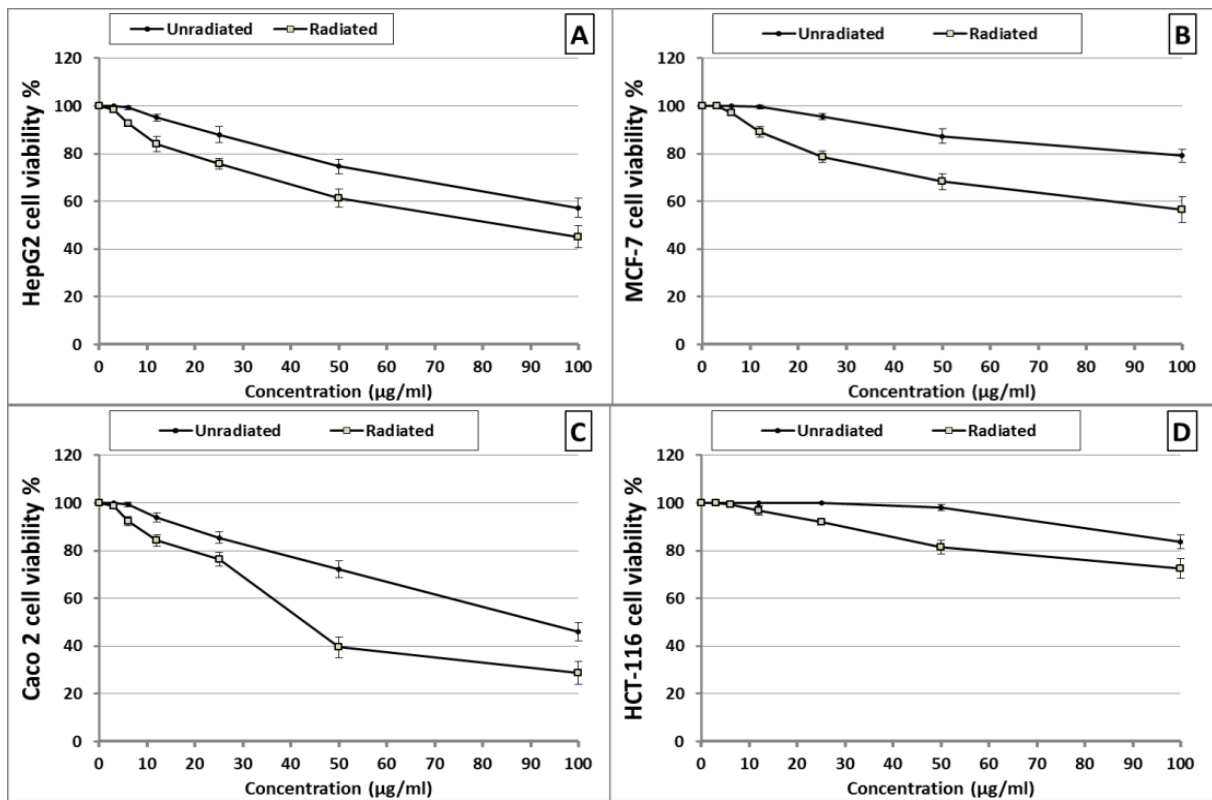
Amino acid with red color is open reading frame.

**Anticancer activity of Alkaline Protease.** The biological activities of the alkaline protease enzyme from the *Streptomyces flavogriseus* exposed to an optimum dose (10 KGy) was used to determine its anticancer activity and the results were compared to non-exposed strain. Cells were treated with protease enzyme at a concentration ranging from 3 to 100  $\mu\text{g/ml}$ , and then the percentage of cell viability was analyzed. Data were plotted for a dose-response curves to calculate IC<sub>50</sub>. Results are shown in Figure 2. Our data shows that HepG2 (liver cancer), MCF-7 (breast cancer) and HCT-117 (colon cancer cells exhibited an IC<sub>50</sub> over 100  $\mu\text{g/ml}$ . Irradiating the enzyme with gamma radiation increased the antitumor effects only by 20% in HepG2

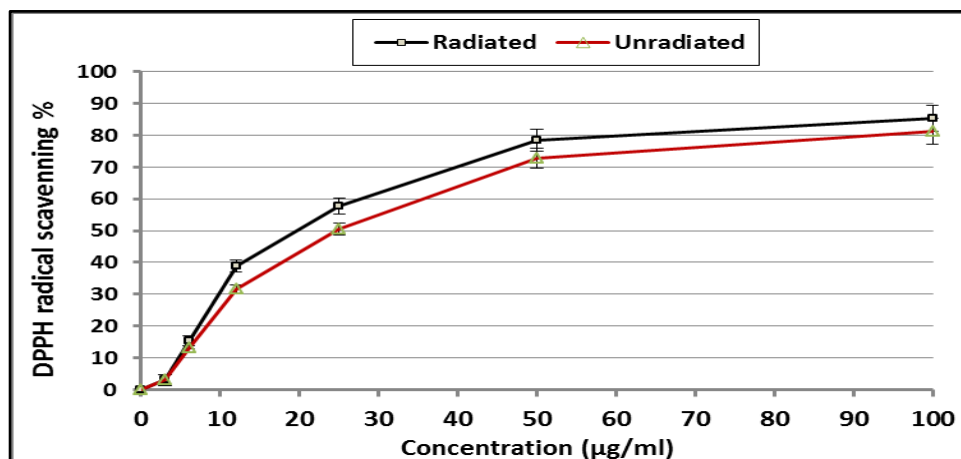
and MCF-7 cells whereas it only caused a 10% increase in HCT-117 cells. In CACO2 cells, alkaline protease exhibited an IC<sub>50</sub> of 45  $\mu\text{g/ml}$  on irradiation compared to 95  $\mu\text{g/ml}$  in the non-irradiated cells. However, the irradiation has also resulted in an increased antitumor activity by 20%.

### 3.4. Antioxidation Activity of Alkaline Protease

In the current study, the alkaline protease reduced the DPPH radical to a yellow-colored compound. The DPPH radical accepted an electron or hydrogen to become a stable diamagnetic molecule.



**Figure 2.** The dose-response curve showing the in vitro inhibitory activity of the alkaline protease enzyme from the *Streptomyces flavogriseus* (exposed to gamma radiation or non-exposed) against human carcinoma cells after 48 h treatment using MTT assay. Where: (A) liver (HepG2) cell line, (B) breast (MCF-7) cell line, (C) colorectal intestinal (CACO2) cell line, (D) colon carcinoma (HCT-116) cell line. Results were shown as the mean viable cells %  $\pm$  standard deviation of three independent experiments



**Figure 3.** DPPH radical scavenging activity of the alkaline protease enzyme from the *Streptomyces flavogriseus* (exposed to gamma radiation or non-exposed). Results were shown as the mean radical scavenging percentage  $\pm$  standard deviation of three independent experiments

The tested protease enzyme exhibited a dose-dependent increase in hydroxyl radical-scavenging activity up to 50 µg/ml, whereas increasing protease concentration over 50 µg/ml has very little increase in the antioxidant activity. Although the irradiated sample showed slightly more potent DPPH radical scavenging abilities than the non-irradiated sample (irradiated IC50 values of 19.87 and non-irradiated IC50 value is 24.85 µg/ml), the difference appears to be non-significant.

### 4. Discussion

In the food industry, alkaline protease plays an important role in the formation of value-added products.

Alkaline protease also plays a significant role in waste management. This study was performed to study the effect of gamma irradiation on *Streptomyces flavogriseus* ADEM7 alkaline protease activity. We performed the enzymatic assay of alkaline protease from *S. flavogriseus* using optimum medium conditions of 30°C, shaking at 200 rpm for diverse brooding periods (3 to 7 days) as described previously [10]. The alkaline protease activity increase was perhaps due to the penetration of gamma radiation to the cell and activation of alkaline protease enzyme up to 10 KGy gamma radiation dose. The gamma radiation over 10KGy dose may have caused denaturation or degradation to enzyme resulting in a reduction in protease enzyme activity over 10KGy dose.

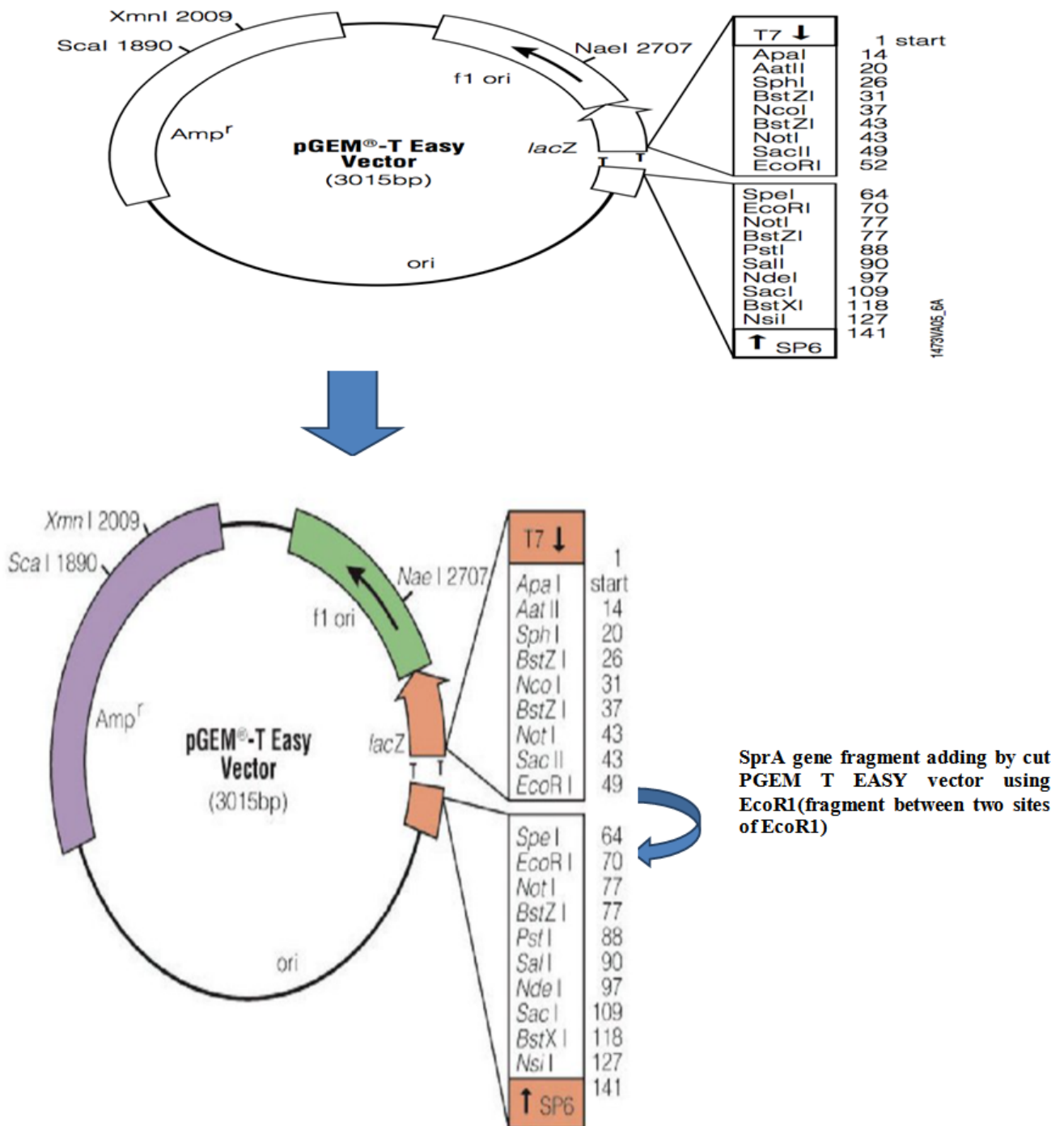


Figure 4. Diagrams illustrate Insertion SprA gene expressed extracellular protease alkaline fragment in PGEM vector by ligase enzyme

*Streptomyces flavogriseus* ADEM7 has *sprA* gene expressing streptogrisin A. *Streptomyces flavogriseus* ADEM7 was identified by 16 sRNA [10] and the sequence was submitted at gene bank with accession number AB723783.1. The PCR screening of recombinant clones from the *Streptomyces flavogriseus* ADEM7-based approach was used to identify specific recombinant clones containing the *sprA* gene. The primer was used specifically for *sprA* gene with several recombinant clones and gave the expected band 1190 bp (recombinant illustrated at Figure 4). Some were selected from these clones, and the amplified PCR products were electrophoresed on an agarose gel. This report describes the structure of *SprA* gene in *S. flavogriseus*, which is responsible for the expression of protease A. The DNA sequences suggest that each protease is initially secreted as a precursor, which is processed to remove an amino-terminal polypeptide (propeptide) from the mature protease. This property may be essential for the secretion of proteases in *Streptomyces* spp.

The predicted amino acid sequence from DNA sequence of *sprA* gene was used to determine homologues protein. For the predicted function of this gene, we tried to identify the homologous sequences. Homology depicts common evolutionary ancestry among different organisms and plays a crucial role in the prediction of protein function. Traditional homology-based methods can predict a function to an unknown protein based on the sequence similarity between the known and unknown protein. Homologous protein sequences are those with a common evolutionary origin. There are two types of protein homologs, depending on how they originated: paralogs, derived from a gene duplication event, and orthologs, originated from a speciation event. Our results identified Protease A and protease B, two of the serine proteases secreted by *S. griseus*, with a homologous sequences similarity of 61% based on amino acid identity [19]. These proteases also have similar tertiary structures, as determined by X-ray crystallography [20]. Although the structures of proteases A and B have been extensively studied, the genes encoding the proteases have not been characterized. Our data provides this essential information on the gene sequences of this group of proteases.

The cytotoxic activity suggests that the protease enzyme from the *Streptomyces flavogriseus* species is a good candidate for future therapeutic human trials. At 100 µg/ml concentration, the highest inhibitory activity was found against colorectal intestinal carcinoma (CACO2) cells (71.25% cell death) for radiated enzyme samples. Consistent with our data, in a previous study, using MTT assay, protease enzyme also exhibited a reduction in cell viability to 76.1% at 10 µg, 57.6% cell viability at 50 µg, and 45.3% cell viability at 100 µg concentration towards A 549 cell line [21]. Our data has shown much improved activity compared to this study, because we used crude and diluted enzyme preparation without precipitation or ion-exchange chromatography or lyophilization as it was used in the previous study. Furthermore, alkaline protease inhibited growth of CACO2 cells more potently than HepG2, MCF-7, and HCT-117 cells. It is possible that the enzyme binds to or enters easily in the CACO2 cells than the other cell lines. Our data suggest that this protease can be a good

candidate for colon cancer. Previous studies have also suggested that protease enzyme production from marine water bacteria is rich in pharmaceutical applications, such as digestive drugs, and anti-inflammatory drugs, anticancer agents [22].

The DPPH radical scavenging abilities of alkaline protease of un-irradiated and radiated alkaline protease produced from *S. flavogriseus* showed good antioxidant activity (ranging from 81-85.37%) in Figure 3. In another study, GMH hydrolysates showed relatively good antioxidant activity (72% at a concentration of 1.2 mg/ml); however, GMH effect was lower than BHA standard at the same concentrations [23].

**In conclusion**, alkaline protease produced by *Streptomyces flavogriseus* ADEM7 encoded by *sprA* gene exhibited improved activity on irradiation. The enzyme also exhibited antitumor and antioxidant activity under *in vitro* condition. In the future, we will be extracting this alkaline protease from the same strain on a larger scale using a fermenter and assaying the anticancer and antioxidant activity of alkaline protease under *in vivo* condition using appropriate mouse model.

## Acknowledgments

All authors very much appreciated their lab team for help and support.

## Author Contributions

Doaa E. El-Hadedy<sup>1\*</sup>, Rafat A. Siddiqui<sup>4</sup> performed experiments, nesreen and abir analyzed the data; doaa and rafat wrote and edited the manuscript. All authors reviewed and further edited the manuscript. All authors read and approved the final manuscript.

## Declarations of Competing Interests

The authors declare no competing interests.

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