ISSN: 1343-4292
Volume 140, Issue 05, August, 2022

Partial Purification and Characterization of the Protease from Cress seeds as (*Lepidium sativum*) And Its Anti-bacterial Activity and Some Microorganisms

Huda Ahmed Fairooz[1*]

Medicine College, Al-Qadysia University, Iraq[1]

Corresponding author: 1*

ABSTRACT— Because the seeds of most plants, including the seeds of cress, contain a high level of proteases, therefore Purify the enzyme from the seeds of garden cress in several steps, including concentration with ammonium sulfate at a saturation rate of 50-60%. The specific activity of the enzyme reached 61.513 U / mg protein. Then the enzyme was passed through an ion exchange DEAE-cellulose column. The enzyme recorded a specific activity of 132,080 units/mg protein, with a purification fold 2.147, and yield 30.49%. Then gel filtration on a Sephacyl S-200 column. The enzyme recorded specific activity, purification fold and its yield, 660 U/mg protein, 4.1641, 27.72% respectively. The enzyme was characterized by setting the optimum pH and optimum temperature. The enzyme recorded the highest activity at pH 7 and pH stability from 7-8. The enzyme also showed the highest activity at a temperature of 35°C for a period of 15 minutes. The enzyme was also showed stable at a temperature 20-40°C for 15 minutes. The result of the effect of chemical compound on the activity of the enzyme showed that it was not affected by the present of sodium and potassium chloride except in a small percentage at concentration 5,10 mM, but it increase slightly when the enzyme was incubated with calcium chloride, it reached 104,110 and magnesium chloride 110,115 at concentration 5,10 mM, respectively. No effect was shown for thiol compounds such as cysteine at concentration 1,10 mM while it was affected by the presence of chelating agents EDTA. Also studied the effect of the activity of the enzyme purified from cress seeds against the types of positive and negative gram stain, as well as fungi and yeast, the result showed the activity of the enzyme at concentration 10,15 and 20 and that the highest response of the bacteria was at 20 concentration, while, no effect of fungi was shown except at concentration 20.

KEYWORDS: protease, seeds of cress, purification, characterization, bacteria, fungi, yeast

1. INTRODUCTION

Proteases or hydrolyzed peptides are a large group of enzymes that have the ability to hydrolyze the peptide bond [27], [28]. Protease are among the enzymes that have a major role in the metabolic processes of living cells due to their effect in biologically active proteins, as well as generating biologically active enzymes, hormones and peptides from inactive compounds [19]. These enzymes are divided into two group which are Exopeptidases (EC 3.4.11-19) and Endopeptideases (EC 3.4.21-24). The first group shows the ability to analyze amino acids near the amino end N-terminus and the carboxylic terminus C-terminus while the second group degrades the peptide bond in the middle of the peptide chain, so it is called Proteinases they have a special importance as they play important physiological roles and industrial applications. They are found in various living organisms such as plants, animals and microorganisms [29].

The use of plants as a source of proteases has become a pioneer at the present time when it is adopted by the

uses of this enzyme in the industrial and medical fields. One of the important plant proteases is papain, as this enzyme works in wide ranges of pH between 5-9 and the enzyme is thermally stable. Between a temperature of (80-90) °C (Schlereth et al., 2000). The seeds are the main store of protein in plants, and the hydrolysis enzymes (proteases) work to prepare the seed embryo with amino acids after analyzing the protein stored in the seed.

Cress seeds are considered scientifically known as (Lepidium sativum).it is one of the largest plant families, consisting of 300 genera, and many plants of this family are of medical importance [26]. However, few studies that have scientifically researched the properties of some of these plants and their biological effects, such as the crees plant and other [20], [21], [26] in recent year, interest has increased in using the enzyme and studying its effect in the treatment of many disease especially common bacteria such as intestinal and pulmonary infections, etc. and due to the lack of many studies in the region showing the effect of the protease purified from the seeds of cress, so the study aimed to purify the protease from seeds of cress seed and characterization it and studying the effect of some chemicals and metal ions on enzyme activity and inhibitory effect on pathogenic bacteria and fungi.

2. Material and Methods

2.1 Enzyme extraction from seeds of cress:

Followed the method that was described before [1] in extraction the protease enzyme from the seeds cress, weighing 5 g of ground seeds powder and adding to it 25ml of potassium phosphate buffer at a concentration of 0.25 M and pH 8. It was stirred on the magnetic stirrer for 15minutes and then left for 4 hours without stirring at $4C^{o}$. the mixture was discarded at a speed of 4000xg for 20 minutes at a temperature of $4C^{0}$ and the mixture was filtered through the gauze. the filtrate was used to estimate the enzymatic activity and protein concentration to calculate the specific activity.

2.2 Enzyme assay:

Followed the method mentioned before [18] in estimating the enzyme activity using 0.5% casein as a base material.

2.3 Estimation of protein:

The (Bradford, 1976) method was used to estimate the protein concentration using the standard curve for bovine serum albumin (BSA).

2.4 Protease purification:

The partial purification steps of the protease enzyme from cress seeds included concentration with ammonium sulfate at a saturation rate of (20-90)% and ion exchange chromatography using DEAE-cellulose. The column dimensions were (26×3) cm and used to wash the column as a sodium phosphate buffer (20mM, PH 7). The recovery was done by saline gradient of 20mM pH 7 NaCl, then gel filtration chromatography using sephacryl S-200, which was filled with a glass column with dimensions (85×1.5) cm the column was washed with sodium phosphate buffer (20mM, pH 7).

2.5 Optimum pH:

A 0.2M sodium acetate buffer (pH 4- 5.5), a 0.2 M sodium phosphate buffer (pH 6-7.5) and a Tris-HCl buffer (pH 7.5-9) was used as a reaction medium to determine the optimum pH for the enzyme's activity.

2.6 pH stability:

To determine the optimum pH for the stability of the enzyme. As for the estimation of the best pH for the stability of the enzyme, the activity of the purified enzyme was estimated at pH(4-9) at a temperature of 30 C^0 and for a period of 15 minutes using the casein substrate solution.

2.7 Optimum temperature

200µl of a phosphate buffer was incubated at a concentration of 20 mM pH 7 and 100 µl of the casein reaction solution at different temperatures ranging from 20-60 °C for 15 minutes, then 10 µl of the purified enzyme solution were added to it and left for another 15 minutes at the same temperatures, then the reaction was stopped and the enzymatic activity was estimated.

2.8 Temperature stability

The purified enzyme solution was incubated in a water bath at different temperatures ranging from(20-60)°C for a period of 15 minutes. Then the tubes containing the enzyme solution were transferred to an ice bath, then transferred to a water bath at 37 °C, and the reaction solution of casein and phosphate buffer was added at a concentration of 20 mM of the prepared and incubated for a period of time15 minutes, then the reaction was stopped and the enzyme activity was estimated.

2.9 The effect of metal ions:

The effect of metal ions. The solutions of the following metal chlorides were prepared at two concentrations of 5 and 10 mm separately for each of the used salts NaCl, KCl,CaCl2, MgCl2 The previous solutions were prepared by dissolving them with Tris-HCl solution at a concentration of 0.2M pH 7. The solutions of each of Cysteine were prepared at a concentration of 1 and 10 mM and EDTA at a concentration of 2 and 5 mM. 0.2 of the enzyme was added to each of the metal chlorides and the inhibitors separately and incubated at 35 °C for 30 minutes, then 0.2 ml of the enzyme solution was withdrawn and 0.5% casein was added to the reaction material solution and incubated at 37 °C for 30 minutes, then the reaction was stopped and the remaining enzyme activity was estimated as % The control factor was prepared by adding the untreated enzymatic solution to the reaction solution 0.5% casein dissolved in the gear solution at a concentration of 0.2 M and pH 7.

2.10 Lyophilization of enzyme:

The enzyme was dried from cress seeds using a special lyophilization device for the manufactured by Mini LyoTRAP Company purpose of conducting subsequent experiments.

2.11 Bacterial species and methods of isolate and grow them:

In this study, bacteria were used *Staphylococcus aureus* Gram-positive bacteria *Escherichia coli*, *Shigella. spp*, *Klebsiella sp* Gram –negative bacteria, As for the fungi, *Aspegellus niger*, As for the yeasts, it included *Candida sp*. Samples were obtained from Al-Diwaniyah Teaching Hospital and confirmatory tests were conducted for bacterial isolates based on what was mentioned in both Barnett and Hunter (1972), [9]. This has been diagnosed fungi depending on the morphological and color characteristics and the composition of the spores [5], [17], [16].

2.12 Revitalization of pure cultures of microorganisms:

The aforementioned tested isolates were activated using different culture media. For bacteria, they were activated 18-24 hours before the examine Brain Heart Infusion The nutrient medium was used Staphylococcus aurous, while Nutrient Broth was used for both *E.coli* and *Klebsiella Pneumonia*. Yeasts and fungi were also activated in the nutrient medium Malt Extract Agar At 25°C for four days for yeasts and five days for fungi.

2.13 Preparation of the bacterial vaccine:

method mentioned by [10] was used to prepare the bacterial inoculum by growing the activated bacteria on Nutrent Agar medium and incubated at 37 °C for 24 hours, then ten colonies of each type are transferred from The bacteria used in the experiment and under sterilization conditions were transferred to a test tube containing 5 ml of Nutrent Agar and then incubated at 37 °C for (4-6) An hour and then the appropriate dilutions are made for each type of bacteria so that the total number of cells is approximately $1*10^7$ per ml.

2.14 Enzyme antagonistic activity test on microorganisms:

2.14.1 Bacteria:

In the enzyme sensitivity test of bacteria, the dishes containing medium Nutrient Agar were inoculated By spreading a volume of 0.1 ml of the bacterial suspension then leaving the dishes on the medium at room temperature for 15 minutes for the purpose of absorbing the vaccine, then wells is made in the middle using a sterile cork drill with a diameter of 7 mm, Through a micropipette, 50 microliters are transferred From each concentration of the enzyme under study, it is placed inside the hole, and at the same time the work is done (Control) Control Dishes By placing 50 µl of sterile distilled water into the hole instead of enzyme , Then the dishes were incubated at a temperature of 37 °C for a period of (18-24) hour, after that you read the result By measuring the diameter of the inhibition zone, which represents the area of no bacterial growth surrounding the hole, using a ruler.

2.14.2 Fungi:

This experiment was carried out using 5 mm diameter discs from a 5-day-old activated colony of fungi, where one disc of each fungus was placed in the center of a Petri dish containing the Malt Extract Agar nutrient medium, Adding to it an appropriate amount of the enzyme under study, which gives the required concentration After that, the dishes were incubated at a temperature of 25 ° C for a period of one week, then the length of the colony's diameter was measured Also, control dishes were made containing the nutrient medium, on which the activated colony of fungi was implanted.

2.14.3 Yeasts:

The sensitivity of the yeasts to the purified enzyme from cress seeds was tested using the planning method, Where the filling of the loop was taken from the yeast suspension and plotted on the medium of Malt Extract Agar, to which different concentrations of were added enzyme In addition to the inoculation of control dishes containing only the nutrient medium, then the dishes were incubated At a temperature of 25 ° C for four days, after that the result is read based on the intensity of growth.

3. Result and dissection

3.1 Enzyme purification

Concentration of enzyme step is one of the steps of enzyme purification, one of the important steps in order to obtain an enzyme ready for the purification process by means of chromatography. where the enzymes are concentrated to get rid of the large proportion of water and to obtain a degree of purity. use salts such as sulfates or chlorides of ammonium or sodium or organic solvent such as Ethyl alcohol and acetone [12]. to find out the best way to concentrate the protein extracted from cress seeds, ammonium sulfate was used with different saturation rates of 20-90%. The results showed that the specific activity of the enzyme increased when using ammonium sulfate with a saturation rate of 50-60% as the specific activity reached 61.513 unit/mg protein compared to the crude extract 41.633 unit/mg protein. the precipitation of organic salts occurs due to the neutralization of the charges on the surface of the protein and this leads to a decrease in the solubility of the protein and its precipitation. this process is called external salting out [24]. Passage

ISSN: 1343-4292
Volume 140, Issue 05, August, 2022

of the enzymatic solution after concentration with ammonium sulfate with a saturation of 50-60% and dialysis through the ion exchange chromatography. where the specific activity reached 132.080 unit/mg protein. the purification fold is 2.147 and yield 30.49%. the use of the ion exchange DEAE- cellulous.it is one of the methods that depend on the principle of the charge carried by the protein and therefore the separation that occurs depends on the net charge, ionic strength and the distribution of the charge on the surface of the protein, as well as for its high separation strength and high capacity to bind proteins, as well as its versatility and ease implementation. The ion exchange step was followed by the gel filtration step using sephacryl -S200 as the specific activity reached 660 unit/mg protein the purification fold 4.1641 and yield 27.72.

Table (1): Purification of the protease enzyme extracted from cress seeds

Step	Volume ml	Activity U/ml	Protein mg/ml	Specific activity U/mg	Total activity U	Purification fold	Yield %
Crude enzyme	**100**	**125.02**	**3**	**41.633**	**12502**	**1**	**100%**
Ammonium sulfate concentration 50-60%	**30**	**260.2**	**4.23**	**61.513**	**7806**	**1.47**	**62.438**
Ion –exchange DEAE-Cellulose	**25**	**95.23**	**0.721**	**132.080**	**2380.75**	**2.147**	**30.49**
Gel Filtration Sephacryl-S200	**10**	**66**	**0.12**	**660**	**820**	**4.1641**	**27.72**

3.2 Characterization of protease:

3.2.1 pH of enzyme activity:

One of the important characteristics of the protease enzyme that has been studied is the optimum pH for the activity of the protease, and it was 7. Figure (1) this study was in agreement with [13]. Where it was found that the optimum pH for an enzyme is 7 for the enzyme extracted from *Euphorbia cotinifolia*, while other researchers found that the optimum pH of the protease enzyme was 8 when the enzyme was extracted from the sprouted bean seeds [11].

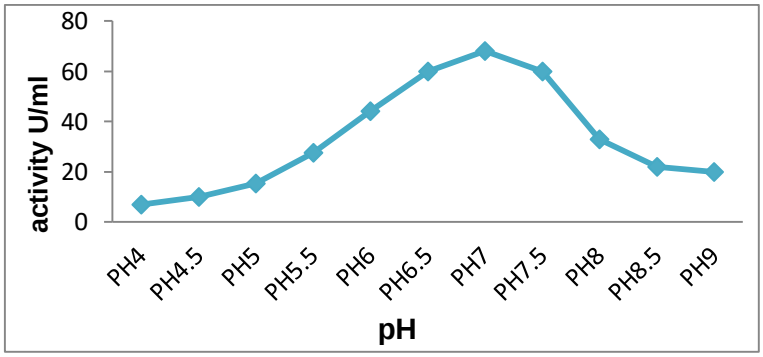

Figure (1): The optimum pH for the activity of protease from cress seeds

3.2.2 pH of enzyme stability:

The effect of the optimum pH for the stability of the protease extracted from cress seeds was studied, and it is considered one of the important characteristics, as it was shown from Figure (2). The enzyme is stable in the pH range (7-8). the decrease in the acidic and basic pH numbers can be attributed to the effect of the pH

number in the structure of the enzyme protein molecule, and irreversible denaturation of the enzyme may occur in highly acidic or basic solutions, which leads to a change in the active site and loss of enzyme activity [6]. This study was compatible with [13] that found the pH stability is (7-8).

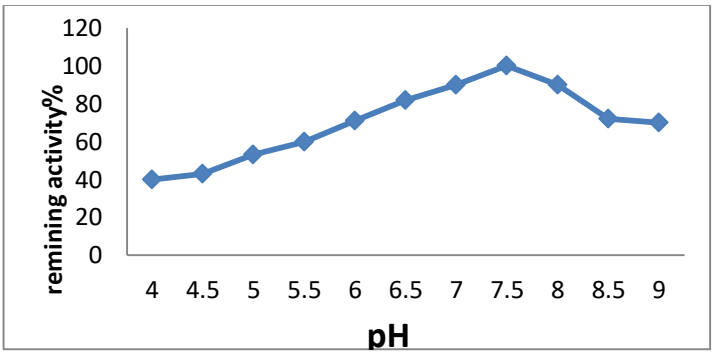

Figure (2): The optimum pH for the stability of the protease enzyme extracted from the seeds of cress

3.2.3 temperature of enzyme activity:

The rate of the enzymatic reaction increases with the increase in temperature within a certain range due to the increase in the kinetic energy of the molecules Until it reaches the point at which the reaction rate reaches its maximum value, but the temperature rises above the limits The optimization leads to a denaturation of the triple structure of the enzyme and thus a decrease in its effectiveness [23]. The results shown in Figure (3) show the effect of different temperatures on the activity of the protease, as it was noted that Increasing the enzymatic activity with higher temperatures, as the enzymatic activity reached its maximum at 35 °C This study was consistent with many studies that showed that the optimum temperature for protease is 35°C, [7]. While in another study, it was found that the optimum temperature of the purified protease enzyme from Euphorbia hirta was 50 °C [22].

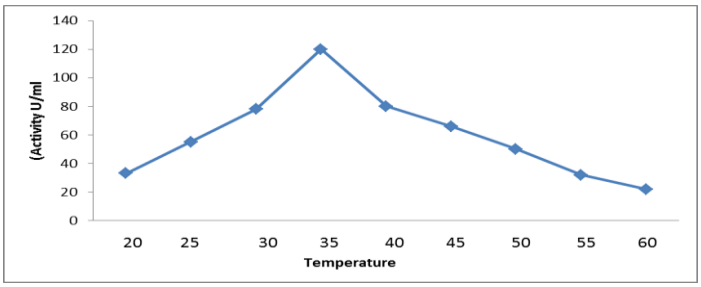

Figure (3) Optimum temperature for protease enzyme purified from cress seeds

3.2.4 temperature of enzyme stability:

The results of incubation of the enzyme at different temperatures (20-60)°C and a difference of 5°C for a period of 15 minutes showed that the purified protease enzyme from cress seeds has almost retained its full activity at temperatures of (20-40)°C, after which the enzyme activity gradually decreased to reach its lowest at a temperature of 60°C.Figure (4). And these results were in agreement with (Unchikoba 2001) where it was found that the stability of the purified protease enzyme from *Cucurbita metuliferus* is from (20-40) °C. While in another study, it was found that the best temperature stability of the purified protease enzyme, *Cucumis melo* var *agrestis*, is (40-60) °C [4].

ISSN: 1343-4292
Volume 140, Issue 05, August, 2022

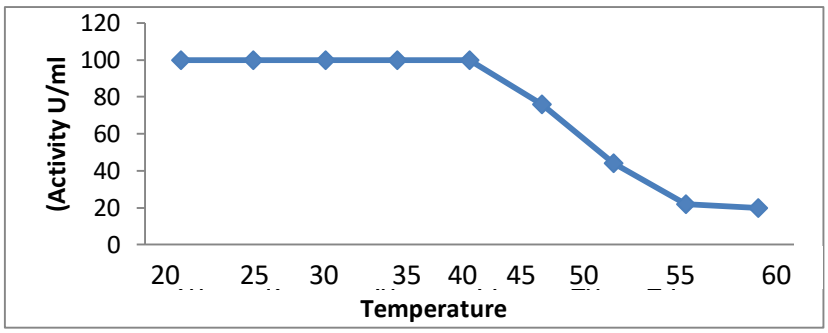

Figure (4): The optimum temperature for the stability of the protease enzyme extracted from the seeds of cress

3.3 Effect of some chemicals and mineral ions on protease activity:

The effect of metal ions on the effectiveness of the protease enzyme purified from cress seeds was studied by incubating the enzyme with metal chloride solutions at 35 °C for a period of 15 minutes. It was noted that the enzyme retained almost its full activity when treated with potassium ions 98%,95% It slightly increased when incubated with 110% and 115% magnesium ions at a concentration of 5 and 10 mM, table (2). The residual activity when incubating the enzyme with sodium ions was 97%, 93% and Calcium is 104% and 110%, respectively. Studies have indicated the role of calcium and magnesium ions in increasing the activity of the enzyme and have a role in stimulating the enzyme and its stability towards autolysis and thermal denaturation (Xiulan, 1984). It was also observed that reducing substances such as cysteine at a concentration of 1 and 10 mM did not affect the enzymatic activity, as it remained constant without change, which indicates that the enzyme does not have the s-type bonds necessary for its activity. Likewise with EDTA, the enzyme's activity was not inhibited when incubated with this substance, which is a chelating agent. The remaining activity of the enzyme when treated with a concentration of 2 and 5mM of EDTA reached 97% and 95%.)

Table (2): effect of metal ions and inhibitory and reducing substance on the activity of the purified enzyme from cress seeds

Subject	Concentration(m M)	remaining activity%
$CaCl_2$	5 10	104 110
NaCl	5 10	97 93
KCl	5 10	98 95
$MgCl_2$	5 10	110 115
Cystein	1 10	100 100
EDTA	2 5	97 95

3.4 Antibiotic sensitivity test for protease:

The enzyme purified from cress seeds showed a high inhibition efficiency when using the Wells method with different concentrations 5%, 10%, 15% and 20% due to the increase in the amount of the enzyme and its spread through the agar plate, which led to obtaining inhibitory areas in the table (3) the increase in the concentration of the enzyme increases its effect on inhibiting Microbial isolates, and this was evident when using concentrations (10, 15, and 20)% of the purified enzyme on the isolates used under study, and all results were positive by obtaining wide inhibitory areas.

Table (3): The diameters of the inhibition zones (mm) for the growth of bacteria treated with different concentrations of the enzyme

The diameter of the damping area (mm)					
20mg/ml Mean±SE	15mg/ml Mean±SE	10mg/ml Mean±SE	5mg/ml Mean±SE	Control Mean±SE	**Concentration g/ml bacteria**
14.33±0.66	11.33±0.2	0±0	0±0	0.0	*Staphylococcus aureus*
25.3±1	20.1±0.84	14.2±0.3	0±0	0.0	*Escherichia coli*
14.1±1.0	12.0±0.23	7.0±0.84	0±0	0.0	*Shigella .spp*
13.21±0.82	10.2±0.42	8.3±0.36	4.33±0.66	0.0	*Klebsiella sp*

The table (3) shows the results of the response between the bacterial strains towards the plant enzyme purified from the cress seed, which had an inhibitory effect on all the microorganisms used in the experiment with varying concentrations, which confirms the activity of the enzyme towards Gram-positive and negative bacteria as well as fungi and yeasts. The highest response of the bacterial strains to the pure enzyme was in *Klebsiella sp* bacteria at the inhibitory concentration 5 mg/ml. The results of the statistical analysis showed that there were significant differences $p<0.01$. There were significant differences between gram-positive and gram-negative bacteria, and positive and negative bacterial strains were not inhibited at the concentration 5 mg/ml) except for *Klebsiella sp*, Where the average diameter of the inhibition zone was 4.33 mm, While all strains showed an inhibitory response to concentrations 10, 15 and 20 mg/ml respectively. As for the analytical results of the response and inhibition of *A.niger* and *C.albicans*. We find that the fungus was inhibited at the concentration of 20 mg/ml, while the yeast was inhibited at the concentrations 15 and 20 mg/ml. Significant differences were clear compared to bacterial strains. We also note that the yeast C.*albicans* is more affected and preferable compared to the fungus *A.niger*. Where the yeast was inhibited with lower concentrations, which gave a higher response and effect, and this is due to the nature of the cellular structure of each of them, as the cell wall of *C. albicans* contains a high percentage of proteins, higher than that of mushrooms, which amounted to 10% In addition, the protein part is located in the outer part near the surface of the cell wall, which facilitates the process of attacking the proteins by the protease enzyme and thus analyzing them [8]. While the percentage of proteins in the cell wall of the fungus A.niger does not exceed 1.15% [15].

Table (4): response of fungal isolates to different concentration of protease enzyme purified cress seeds

)The diameter of the damping area (mm)					
20mg/ml Mean±SE	15mg/ml Mean±SE	10mg/ml Mean±SE	5mg/ml Mean±SE	Control Mean±SE	Concentration g/ml fungi
27.22±1.55	0±0	0±0	0±0	0.0	*Aspegellus niger*
30.33±2.4	20.33±1.03	0±0	0±0	0.0	*Candida sp*

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