Isolation of protease enzyme from Aspergillus niger and its effect on tenderizing steaks

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Abstract

In this study, the protease enzyme from Aspergillus niger was extracted and purified, and its effect on the tenderness of steak was studied in different concentrations such as 0.2%, 0.4%, 0.6%, and 1% at the periods of 2, 4, and 6 hours. Those results were compared with the control sample, which was not treated with the enzyme.

The most important results were the chemical composition showed a significant increase in protein and moisture with a decrease in fat percentage compared to the control treatment. The E1 treatment was 1% for 6 hours; the pH values were 8.7, 35.90% protein, 8.22% fat, 76.22% moisture, and 7.98% ash.

The best treatment qualitative test was the E1 treatment (1%, 6 hours); the peroxide value was 3.51 Meq/kg less than the control treatment 5.01 Meq/kg, indicating reduced oxidation. The value of free fatty acids was 0.36%, and the lower value than the control treatment was 0.74%, indicating reduced fat deterioration. In addition, the protein tyrosine/tryptophan coefficient was 5.77, indicating an improvement in meat tenderness, while the non-protein tyrosine/tryptophan coefficient was 4.09, which refers to an additional improvement in tenderness.

The best treatment for sensory properties was also evaluated in E1 (1%, 6 hours). It obtained the best sensory evaluation in tenderness, juiciness, general acceptance, and color.

Conclusion: E1 treatment with protease enzyme extracted from Aspergillus niger at a concentration of 1% for 6 hours achieved the best results in terms of improving tenderness, nutritional value, reducing oxidation, and improving the sensory properties of meat, making it the ideal choice for improving meat quality in natural and safe ways, which enhances its use in food industries as an alternative to chemicals.

Keywords: Protease enzyme, Aspergillus niger fungus, amino acids, peroxide value

1-Introduction

Meat is an essential food source for humans, as it provides high-quality proteins, vitamins, and minerals necessary for the health of the body. Proteins contribute to building and repairing tissues, enhancing the functions of the immune system, and supporting the production of enzymes and hormones. [16], the tenderness and quality of meat are considered important factors that affect consumer acceptance of meat products [24] but there are still challenges and difficulties facing the process of improving this property by both the consumer and the producer, so many different methods have been used to improve tenderness [20] (Enzymes play a pivotal role in improving meat quality, as they are used as cofactors in softening muscle tissue and breaking down complex proteins, which leads to increased tenderness of meat. Among these enzymes is the protease enzyme

ISSN 2072-3857

extracted from the fungus Aspergillus niger, which has proven effective in improving meat properties. A study showed that the use of an enzyme extracted from the fungus Trichoderma hamatum contributed to softening beef meat and increasing its tenderness. [17]the meat industry faces multiple challenges related to the quality and preservation of meat products [7] One of the most prominent challenges is maintaining the tenderness of meat and preventing it from hardening during storage, which negatively affects the safety of the product and its suitability for consumption [27]. In this context, there is a need to develop natural and safe technologies to improve the tenderness of meat, away from the use of chemicals that may have side effects on the health of the consumer. The use of enzymes extracted from natural sources such as fungi is one of the promising solutions that combine effectiveness and safety. Fungi are considered rich sources of proteolytic enzymes, which can be used to improve the properties of meat in natural and safe ways. This research aims to extract and purify the protease enzyme from the fungus Aspergillus niger, conduct qualitative tests of the enzyme, and study its effect on the tenderness of steak.

2- Materials and methods:

2-1 Collection and activation of isolates:

The isolates were obtained from the Scientific Research Authority at the Environment, Water and Renewable Energy Center, Food Pollution Department. Aspergillus niger isolate was activated on PDB medium for 3 days, after which it was planted on PDA agar medium to obtain a pure and sexually identified culture using the polymerase chain reaction (PCR) technique. The glass slides containing 90% lactophenol blue dye were observed using a microscope with a magnification of 100X. The Aspergillus niger isolate was identified by the shape of the colonies, their growth, and the shape of the spores, using the taxonomic keys [11].

2-2 Preparation of the spore suspension:

The spore suspension was prepared by following the method of [13] and the spores were counted under the maximum power of 40X for the light microscope, and the following equation was applied [14]

Spore concentration=(z10×4×106)/n

-3 Enzyme production

Protease enzyme was produced in liquid state fermentations using a medium consisting of 10 g casein NH4NO3, 0.2 g KH2PO4, 0.1 g NaCl and 0.1 g MgSO4 in 100 ml distilled water and trace elements (ZnSO4.7H2O and FeSO4.7H2O) 0.01 ml each. Casein was added and the production medium was sterilized at 121.5 °C for 15 min, then cooled and inoculated with 1 ml of Aspergillus niger suspension and incubated at 28 °C for 7 days [8].

2-4 Enzyme purification:

2-4-1 Enzyme concentration using ammonium sulfate:

The method described by [34] was followed in the process of enzyme precipitation with ammonium sulfate. The precipitate was collected and dissolved in sodium phosphate buffer solution with a concentration of 0.1 and a pH of 7 to estimate the enzyme activity.

2-4-2 Dialysis:

In this process, bags with a retention capacity of 8-14 thousand kilodaltons were used for the purpose of membrane osmosis of the enzyme extract precipitated with ammonium sulfate resulting from the paragraph against the buffer solution in which the dialysis bags were immersed. The process was repeated with the replacement of the buffer solution every 6 hours for 24 hours, at a temperature of 4.0°C.

ISSN 2072-3857

The final volume of the sample was measured and the enzymatic activity of the enzyme was estimated. And the estimation of the enzymatic activity [6]

2-5 Estimation of the activity of the protease enzyme

The proteolytic activity in the solution was measured according to the method described by [21] with slight modifications using casein. One milliliter of (1% w/v casein in 0.1 M sodium phosphate buffer (pH 7.0)) was mixed with 1 ml of the crude solution. The mixture was then incubated for 10 min at 30 °C and the reaction was terminated by the addition of (2 m) triacetic acid (TCA) solution. The solution was then left for 30 min at room temperature followed by centrifugation at 10,000 rpm for 10 min. Then 1 ml of the filtrate was mixed with 5 ml of 3 Na2CO3 (0.4 M) and after ten minutes 1 ml of Follen's reagent was added. The tubes were incubated for 30 min at 30 °C and the absorbance was measured at 0-60 nm. A similar approach was used to prepare the controls. Casein was added only after the reaction was stopped. A standard curve was constructed using 600 µg/ml tyrosine. One unit of proteinase activity was defined as the amount of enzyme required to liberate one (1) microgram/mL of tyrosine under the assay conditions described. All assays were performed in duplicate.

Enzyme activity (unit/mol) = absorbance((A 660 nm))/(0.1×10×0.01)

Enzyme activity (unit/mol) = absorbance((A 660 nm))/(0.1×10×0.01)

2-6 Practical application of purified protease enzyme:

200 g of steak pieces were immersed in 100 ml of protease enzyme prepared at a concentration of (0.2, 0.4, 0.6, 1) of the enzyme for periods of time extending (6, 4, 2) hours. The treatments were grilled immediately after the end of the soaking period in a grill for 20 minutes in order to inactivate the enzyme at a temperature of 120 C, then cooled and stored in the refrigerator at a temperature of +5 C until later tests were conducted.

2-7 Chemical tests of grilled beef steak samples

2-7-1 Estimation of protein content

The Keldahl method was used to estimate the protein content in the samples, based on the method mentioned by [32] and according to the following equation.

Protein % = (Volume of HCL consumed × Standard × 0.014 × 6.25)/(Sample weight × 100)

2-7-2 Estimation of fat

The fat was estimated based on the method [1] where a weight of (10 g) was taken from the dried samples, placed in a filter paper and placed inside the thimble of the fat extraction device (Soxholet) and the percentage of fat was extracted according to the following equation:

Fat percentage (%) = Weight of the flask before extraction - Weight of the sample after extraction / Weight of the sample x 100

2-7-3 Estimation of the Ash Percentage

The percentage of ash in the sample was estimated by incinerating the sample after placing it in a ceramic bowl of known weight in the incinerator (incinerator) at a temperature of about (500-600 C) for (16 hours). [1]

Ash percentage (%) = weight of the pelvis with the sample after burning - weight of the empty pelvis × 100

Sample weight

2-7-4 Moisture estimation

The percentage of moisture in the sample was estimated as a loss in the sample weight before drying, using a precise weight of samples of about (3 g) and placed in a pelvis of a

ISSN 2072-3857

previously known weight and dried in an electric oven at a temperature of (105 C) for 16 hours according to the method of [2]

Moisture percentage = weight of the sample before drying - weight of the sample after drying

Weight of the sample before drying × 100

2-7-5 Estimation of the pH

The pH was measured according to the method provided by the scientist [28] which states that 10 g of the sample is taken and 100 ml is added to it, homogenized for one minute, then the sample is filtered and its pH is measured Using a (PH meter) device.

2-8 Qualitative tests in processed roast beef steak

2-8-1 Estimation of the peroxide value (P.V)

The estimate was based on [11] based on the following equation:

Peroxide number (Meq) = number of milliliters of sodium thiosulfate × 0.01 × 1000 / weight of the sample

The normal limit for the P.V value = 10 Meq/kg

2-8-2 Estimation of amino acids (tyrosine and tryptophan): The method presented by [12] In estimating the total tyrosine/tryptophan coefficient, which stipulates adding 70 ml of distilled water to 10 g of enzyme-treated meat and homogenizing well for 10 minutes, after which the sample was filtered using filter paper and the sample was measured using a wavelength of 280 nm. Tyrosine/non-protein Tryptophan (T/P.T.) coefficient, a 15% solution of TCA was prepared with distilled water and mixed with 5 ml of the filtrate in the previous paragraph at a ratio of (1:1) and measured at a wavelength of 280 nm. 2-8-3 Estimating the concentration of free fatty acids

Free fatty acids were estimated according to the method of [11] and the percentage of free fatty acids was calculated on the basis of oleic acid. Free fatty acid concentration kg = base volume × 0.01 × 28.2 × 100

Sample mass

2-9 Sensory evaluation

The sensory evaluation of beef steak treatments was conducted in the Department of Home Economics / College of Education for Girls by professors specialized in nutrition, as the number of evaluators was 10 professors. The method mentioned by [3] was followed in estimating the sensory evaluation scores of the product after the grilling process, which included all of the characteristics (color, flavor, tenderness, juiciness, and general acceptance)

2-10 Statistical analysis

The statistical program (2018) SAS- Statistical Analysis System was used to analyze the data to study the effect of different treatments on the studied characteristics according to a completely randomized design (CRD), and the significant differences between the averages were compared using the Least Significant Difference (LSD) test (0.05 >P). 3- Results and Discussion:

3-1 Activation of Aspergillus niger isolate.

Aspergillus niger growth appeared on Potato Dextrose Agar (PDA) medium, black mold growth, and the fungus was stained and identified using lactophenol blue stain, and the result showed fine blue fungal threads and fruiting forms (spores) with a pale blue background of Aspergillus niger (El-Garhi, et al., 2020) Figure 1).

ISSN 2072-3857

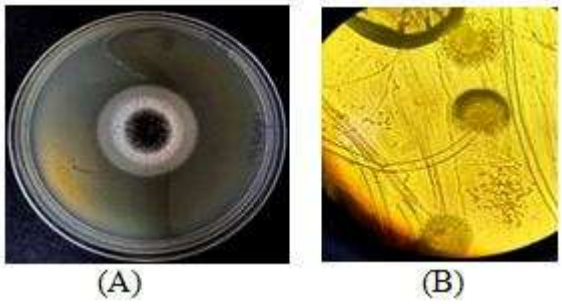

Figure (1) shows (A) the result of detecting fungal growth using lactophenol blue dye in staining Aspergillus niger fungus (B) A microscopic image showing the shape of the fungus (spore case, stalk carrying it and scattered spores.(

2-3Detection of protease enzyme production from A. niger fungus

The casein agar medium was inoculated with a stain from a colony of A. niger fungus and after incubating it for 48 hours, a halo appeared around the fungal growth measuring 42.7 mm, indicating the efficiency of the isolate in hydrolyzing casein as shown in Figure (2). It is consistent with what [19] concluded that Aspergillus protease acts as a good biocatalyst in protein hydrolysis, which increases the effectiveness as an antioxidant and its ability to hydrolyze soy protein, cow whey protein and egg white protein from 2.0 to ten times.

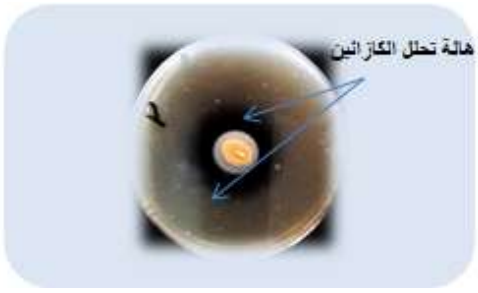

Figure (2) shows the ability of A. niger to grow and hydrolyze casein as a protein source and detect the production of protease enzyme.

3-3Purification of protease enzyme A. niger protease enzyme production

Table (1-1) shows the results of the precipitation concentration with ammonium sulfate used 80%. The enzyme gave a precipitation activity of 11.176 units/ml higher than the extract sample, which amounted to 8.546 units/ml. It can be concluded that the precipitation process of the enzyme protein occurs well at a concentration of 80% ammonium sulfate. While the purification process using dialysis tubes gave a high specific efficiency of 2.310 units/mg with significant differences ($P \leq 0.05$) compared to the rest of the treatments that gave 1.586 units/mg in the precipitation process and 0.955 units/mg for the crude extract. As for the efficiency %, the treatment using dialysis tubes amounted to 10.564 units/ml and gave a non-significant difference ($P \geq 0.05$) compared to the precipitation process, which amounted to 11.176 units/ml, and the differences in the yield % The efficiency of the purification process was shown between the treatments, which reached 9.88 using the dialysis purification process and 20.92 using the ammonium sulfate precipitation process 80%,

ISSN 2072-3857

so the differences were highly significant (P≤ 0.01) between the treatments. The concentration of the enzyme obtained from the partial purification process using dialysis tubes reached 10.564 units/ml with a higher specific efficiency than the rest of the treatments, which reached 2.418 units/mg, and gained a significant difference (P≤ 0.05), which showed that the purification process was efficient. The protease enzyme purification process begins by determining the appropriate ammonium sulfate concentration in order to precipitate the protease enzyme from the A. fungus extract. niger, and the importance of determining the concentration of ammonium sulfate lie in that it determines the level of activity and the amount of enzymes used in the subsequent purification step, and many studies have reported that the nature of the protease enzyme is excellent when precipitated at ammonium sulfate concentrations between 50-80%, and similar to the results of the research[29]reported that the concentration of ammonium sulfate of 80% is the best to use for precipitating proteins from enzymes.

Table (1-1) Purification of the protease enzyme from A. niger

Purification steps	Volume	Efficacy)unit/ml(	Protein)mg/ml(	Specific Efficacy)unit/mg(	Total effectiveness Unit	Number of purifications	Outcome %
Crude extract	25.02± 500 a	0.57± 8.55 b	0.56±8.14 A	0.11±0.955 c	0.43±4.273 c	0.1±0.25 c	0.00±100 a
Ammonium sulphate precipitation	6.40± 80 b	0.61±11.18 a	0.3±7.05 A	0.17±1.586 b	32.78± 899.08 a	0.28± 1.67 b	1.07± 20.9 b
Dialysis	2.75 ±40 c	0.58± 10.56 ab	0.19±4.57 b	0.24±2.310 a	18.67± 422.56 b	0.43±2.42 a	0.64± 9.88 c
L.S.D. value	22.95 *	1.984 *	1.668 *	0.531 *	51.755 *	0.519 *	8.961 *
Means with different letters within the same row are significantly different from each other. Means for three replicates (P≤0.05)*							

4-3Chemical tests of meat samples treated with protease enzyme and stored for different periods

1-4-3Acidic function:

The results showed in Tables (2-1), a significant difference (P ≤ 0.05) in the results of the acidic function when treated with an enzyme concentration of 0.2% and for a storage period of 6 hours (B3) amounted to 7 compared to B1 and A, which had a pH of 5.9 and 5.5 respectively, while the significant difference (P ≥ 0.05) was not reached when compared to the storage period of B2, which amounted to 6.3, as well as the results of the treatment with a concentration of 0.4% and 0.6%, each separately, Tables (2-2, 2-3, 2-4). The results were consistent with what was mentioned by [3,15] . In their results, a significant decrease in the pH rate was found as a result of treatment with raw protease

ISSN 2072-3857

enzyme at concentrations of 10 and 15% of the live weight and for different storage periods between (0-24) hours.

2-4-3Protein concentration:

The results showed in Tables (2-1), a significant difference ($P \leq 0.05$) in the results of protein concentration when treated with enzyme concentration of 0.4% and for a storage period of 6 hours (B3) amounted to 27.44% compared to B1 and A which amounted to 22.48 and 21.51 respectively, while the significant difference ($P \geq 0.05$) was not reached when compared to the storage period of B2 which amounted to 24.15%, as well as the results of treatment with concentrations of 0.4%, 0.6% and 1% each separately Tables (2-2, 2-3, 2-4.(

3-4-3Fat percentage: The results showed in Tables (2-1), a significant difference ($P \leq 0.05$) in the results of the fat percentage when treated with an enzyme concentration of 0.2% and for a storage period of 6 hours (B3) amounted to 5.89% compared to B1 and A, which amounted to 5.26 and 5.11 respectively, while the significant difference ($P \geq 0.05$) was not reached when compared to the storage period of B2, which amounted to 5.33%, and the same is the case with the results of the treatment with a concentration of 0.4%, 0.6% and 1%, each separately, Tables (2-2, 2-3, 2-4). 3-4-4 Moisture percentage: The results showed in Tables (2-1), a significant difference ($P \leq 0.05$) in the results of the moisture percentage when treated with an enzyme concentration of 0.2% and for a storage period of 6 hours (B3) amounted to 67.48% compared to B1 and A, which amounted to (64.25 and 62.65)% respectively, while the significant difference ($P \geq 0.05$) was not reached when compared with the storage period of B2, which amounted to 65.8%, as well as the results of the treatment with a concentration of 0.4%, 0.6% and 1% each separately Tables (2-2, 2-3, 2-4). It agreed with [33] that moisture increases in the treatments of the cake product to which mango peel powder is added with increasing replacement rates. The humidity reached (22.22 22.32 22.28) respectively compared to the control sample

5-4-3Ash percentage:

The results showed in Tables (2-1), with a significant difference ($P \leq 0.05$) in the results of the ash percentage when treated with an enzyme concentration of 0.2% and for a storage period of 6 hours (B3) reaching 6.89% compared to B1 and A, which reached (6.14 and 5.98)% respectively, while the significant difference did not reach ($P \geq 0.05$) when compared to the storage period of B2, which reached 6.54%, and the same is the case with the results of the treatment with a concentration of 0.4%, 0.6% and 1% each separately, Tables (2-2, 2-3, 2-4.(

The results of the tests of pH, protein, fat, moisture and ash showed that they increased as the storage period increased, and the treatment gained significant differences compared to the control model (untreated tender beef slices), and the models improved as the concentration of the enzyme treated with beef slices increased and gained significant differences. On the other hand, the treatment of beef slices with a concentration of purified protease enzyme 1% showed an improvement in the test results compared to the rest of the concentrations and for different storage periods, reaching 8.7, 35.9%, 8.22%, 76.22% and 7.98%, and gained significant differences with the rest of the concentrations. It agreed with the results of [5] as it indicated that the optimal pH is 7, and the concentration of the actinidin enzyme 1% recorded a significant difference in the chemical tests

ISSN 2072-3857

(pH, moisture content, ash content, fat content) and for a storage period of (2, 4 and 6) hours and at concentrations between 0.25 to 1%. On the other hand, it agreed with the results of [4] in the presence of a significant difference in the ash content of chicken meat samples treated with a mixture of kiwi and pineapple extracts at a rate of 5% for each of them and amounted to 1.63% with the control model not treated with the extract and amounted to 2.28%, while [23] found that the effectiveness of kiwi extract on chicken meat did not reach the significant difference, and this may be due to the experimental conditions and the increased rate of moisture loss, which leads to a difference in the percentage of mineral salts and thus to a difference in the ash content. One of the advantages of enzymes is their high activity, which makes them the most cost-effective choice for ingredients. Enzymes break down complex molecules into smaller units such as proteins into peptides and amino acids. They are natural substances involved in all biochemical reactions. Due to the enzyme specificity, each substrate has corresponding enzymes[22,31. [

Table (2-1) Testing some chemical tests for tender meat treated with protease enzyme at a concentration of 0.2% and heat treated

no.	sample / Storage time	pH	protein%	Fat%	Moisture%	Ash%
1	)(A control	c 0.32 ±5.5	c 0.85± 21.51	5.11 ±0.26 b	62.65 ±3.52 b	5.98 ±0.38 b
2	B1 2hours	bc 0.47 ± 5.9	bc 1.05± 22.48	5.26 ±0.34 ab	64.25 ±2.88 ab	6.14 ±0.42 ab
3	B2 4hours	ab 0.54 ±6.3	b 1.17± 24.15	5.33 ±0.28 ab	65.80 ±2.71 ab	6.54 ±0.39 ab
4	B3 6hours	a 0.58 ± 7.0	a 1.28± 27.44	5.89 ±0.35 a	67.48 ±2.57 a	6.89 ±0.55 a
L.S.D. value		0.761 *	2.705 *	0.693 *	4.025 *	0.892 *

Means with different letters within the same row are significantly different from each other.
Means for three replicates
)P≤0.05*(

Table (2-2) Testing some chemical tests for tender meat treated with 0.4% protease enzyme and heat treated

no.	sample / Storage time	pH	protein%	Fat%	Moisture%	Ash%
1	)(A control	0.32±5.5 c	0.85 ±21.51 c	0.26 ± 5.11 b	3.52 ±62.65 b	0.38 ±5.98 b
2	B1 2hours	0.47 ±5.9 bc	1.05 ±22.48 bc	0.34±5.26 ab	2.88±64.25 ab	0.42±6.14 ab

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3	B2 4hours	0.54 ±6.3 ab	1.17 ±24.15 b	0.28± 5.33 ab	2.71 ±65.80 ab	0.39 ±6.54 ab
4	B3 6hours	0.58±7.0 a	1.28 ±27.44 a	0.35± 5.89 a	2.57 ±67.48 a	0.55 ±6.89 a
		0.761 *	2.705 *	0.693 *	4.025 *	0.892 *
Means with different letters within the same row are significantly different from each other. Means for three replicates (P≤0.05)*						

Table (2-3) Testing some chemical tests for tender meat treated with 0.6% protease enzyme and heat treated

no.	sample Storage time	pH	protein%	Fat%	Moisture%	Ash%
1	)(A control	0.32±5.5 c	0.85 ±21.51 c	0.26 ±5.11 b	3.52 ±62.65 b	0.38 ±5.98
2	B1 2hours	0.38±7.6 b	0.88 ±29.45 b	0.39± 6.50 b	2.95± 70.14 b	0.37±7.18
3	B2 4hours	0.45± 8.0 ab	0.88 ±29.45 b	0.51± 7.44 ab	3.05 ±73.25 ab	0.45±7.57
4	B3 6hours	0.37 ±8.3 a	1.56 ±33.65 A	0.48 ±7.80 a	2.84 ±74.08 a	0.41±7.65
L.S.D. value		0.608 *	3.186 *	1.154 *	3.837 *	0.492 NS
Means with different letters within the same row are significantly different from each other. Means for three replicates)P≤0.05*(						

Table (2-4) Testing some chemical tests for tender meat treated with 1% protease enzyme and heat treated

no.	sample Storage time	pH	protein%	Fat%	Moisture%	Ash%
1	)(A control	0.32±5.5 c	0.85 ±21.51 c	0.26 ±5.11 b	3.52 ±62.65 b	0.38 ±5.98 b
2	B1 2hours	0.37±8.0 b	1.33± 31.25 b	0.42± 7.21 b	2.97± 72.65 b	0.36±7.40
3	B2 4hours	0.44± 8.5 ab	1.65± 34.59 A	0.39±8.05 ab	2.97 ±72.65 b	0.36±7.40
4	B3 6hours	0.47±8.7 a	1.08±35.90 a	0.37± 8.22 a	2.66 ±76.22 a	0.69±7.98
L.S.D. value		0.695 *	3.026 *	0.891 *	3.529 *	0.605 NS
Means with different letters within the same row are significantly different from each other. Means for three replicates)P≤0.05*(						

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5-3Qualitative tests Meat samples treated with protease enzyme and stored for different periods

1-5-3Peroxide value

The results of Table (3-1) showed that the rate (peroxide value) of the protease enzyme purified from A. niger fungus increases directly with the increase in the enzyme concentration, as the activity reached 3.51 Meq/kg fat at the enzyme concentration of 1% and with a highly significant difference ($P \leq 0.01$) compared to the control, which reached 5.01, while with a significant difference ($P \leq 0.05$) compared to the concentrations of 0.4 and 0.2, which reached 4.15 and 4.37 Meq/kg, respectively, while the peroxide number decreased with the increase in storage hours and the differences did not reach significance ($P \geq 0.05$). The results of the study [5] confirmed that the peroxide number is affected by two important factors, one of which is the concentration and the second is the storage period, as the enzyme concentration is inversely proportional to the peroxide value in chicken meat samples, and the peroxide value increases with the decrease in the storage period. The reason can be attributed to the good antioxidant activity of the enzyme.

Table (3-1): The effectiveness of the purified protease enzyme from A. niger fungus in reducing the peroxide value of meat samples stored for different periods.

Enzyme concentration % / Storage time (hour)	control	0.2	0.4	0.6	1	L.S.D. value
	Meq/kg					
2	5.01	0.25±4.37 a	0.19±4.15 ab	0.21 ±3.94 ab	0.23±3.51 b	0.622 *
4	5.01	0.31±4.50 a	0.28±4.48 a	0.25 ±4.17 ab	0.30 ±3.88 b	0.581 *
6	5.01	0.36±4.66	0.33±4.52	0.33±4.29	0.26± 4.16	0.507 NS
L.S.D. value		0.443 NS	0.473 NS	0.408 NS	0.612 *	

Means with different letters within the same row are significantly different from each other.
Means for three replicates
(P≤0.05*)

2-5-3

Percentage of free fatty acids in meat samples stored for different periods. The results of Table (3-2) showed that the rate of free fatty acids of the purified protease enzyme from A. niger fungus increases directly with the increase in the enzyme concentration, as the activity reached 0.36% at an enzyme concentration of 1% and a significant

ISSN 2072-3857

difference ($P \leq 0.05$) compared to the control, which reached 0.74% and a concentration of 0.4 and 0.2, which reached (0.56 and (0.60%), respectively, while the antioxidant activity decreased with the increase in storage hours.

Fat oxidation is one of the most important causes of meat and meat products deterioration, as it affects unsaturated fatty acids, especially polyunsaturated fatty acids, in membrane phospholipids as well as cholesterol, especially low-density cholesterol (LDL). The final products of this process reduce the color, smell, flavor and tenderness of meat and meat products, thus reducing the nutritional value. In addition to nutritional deterioration, oxidation generates compounds with cellular and genetic toxicity that harm human health [25[

The use of microorganisms To produce protease enzymes or to enhance food fermentation to obtain biologically active peptides offers many advantages due to the wide and diverse range of protease enzymes that can be obtained in large quantities, along with the possibility of genetic modification to generate enzymes with unique properties [27] the use of proteolytic enzymes that are soluble in an acidic environment confirms that the antioxidant activity can be attributed to soluble proteins and not other biologically active compounds [9.[

Table (3-2): (Free fatty acids) in meat samples stored for different periods.

Enzyme concentration% / Storage time) hour(	**control**	**0.2**	**0.4**	**0.6**	**1**	**L.S.D. value**
2	**0.74**	**0.07±0.73 a**	**0.05±0.56 ab**	**0.03±0.45 b**	**0.05±0.36 b**	**0.208 ***
4	**0.74**	**0.09±0.74 a**	**0.05±0.60 ab**	**0.05±0.48 b**	**0.07±0.45 b**	**0.197 ***
6	**0.74**	**0.09±0.74 a**	**0.07±0.67 ab**	**0. ±050.53 b**	**0.08 ± 0.51 b**	**0.189 ***
L.S.D. value	**------**	**0.117 NS**	**0.134 NS**	**0.109 NS**	**0.162 NS**	**-----**

Means with different letters within the same row are significantly different from each other. Means for three replicates
(P≤0.05)*

3-5-3Testing the ratio of protein and non-protein tyrosine/tryptophan at different storage periods. After treating tender beef slices with different concentrations (0.2, 0.4, 0.6 and 1)% each separately of purified protease enzymes from A. niger fungus and storing them for different time periods (2, 4 and 6) hours each separately, all treatments were heat treated and the results were compared with a control group (not treated with purified protease enzyme), the results showed that the efficiency of the treatment with the enzyme concentration of 1% was higher in maintaining the tenderness of the meat slices compared to other treatments with the protease enzyme, as the ratio of protein tyrosine/tryptophan reached 5.08, 5.3, 5.77 and the ratio of non-protein tyrosine/tryptophan reached 3.6, 3.74, 4.09 for storage periods (2, 4, 6) hours respectively with significant differences ($P \leq 0.05$) compared to the treatment with a concentration of (0.2, 0.4 and 0.6) % each

individually and for different storage periods, while it gained a highly significant difference ($p \leq 0.01$), especially at a storage period of 4 and 6 hours each individually between the 0.4% and 1% treatments, Table (3-3-1) (3-3-2). These results agreed with what was found by (Abu Ahmed et al. 2023) with an increase in the percentage of the two amino acids tyrosine and tryptophan for three treatments added to the partially purified actinin enzyme compared to the control model after two hours, but it did not reach significance. This increase continued between treatments with the continuation of the storage process, and a noticeable decrease was recorded for each of the two acids with the continuation of the storage process for the same treatments until it reached the lowest percentage upon reaching 6 hours of the storage process, and it was confirmed by [18] after treating aged beef with different concentrations of protease enzymes (papain and bromelain) and after a storage period of one and two days, as the percentage of non-protein nitrogen increased with increasing concentration and during the storage period.

Table (3-3-1) The effectiveness of the purified protease enzyme in meat tenderness (T/T.T) for different storage periods.

Enzyme concentration % / Storage time) hour	control	0.2	0.4	0.6	1	L.S.D. value
	Tyrosine/Total Tryptophan Ratio T/T.T					
2 hours	0.13 ±2.51 d	0.24±3.66 C	0.28±3.95 Bc	0.22±4.78 ab	0.19 ±5.08 a	1.037 *
4 hours	0.17 ±2.50 C	0.20 ±3.78 b	0.31±4.58 Ab	0.35 ±5.11 a	0.24±5.30 a	1.176 *
6 hours	0.22±2.49 C	0.26± 4.22 C	0.28±4.89 ab	0.28±5.21 ab	0.36±5.77 a	1.176 *
L.S.D. value	0.459 NS	0.608 NS	0.879 *	0.563 NS	0.723 NS	1.088 *

Means with different letters within the same row are significantly different from each other.
Means for three replicates
)P≤0.05*(

ISSN 2072-3857

Table (3-3-2) The effectiveness of purified protease enzyme in meat tenderness (T/NP.T) for different storage periods.

Enzyme concentration % / Storage time) hour	control	0.2	0.4	0.6	1	L.S.D. value
	Tyrosine/Total Tryptophan Ratio T/T.T					
2 hours	0.13±2.18 c	0.16 ±2.55 bc	0.21 ± 2.80 Abc	0.27±3.43 ab	0.32±3.60 a	0.894 *
4 hours	0.09 ±2.28 c	0.16± 2.65 bc	0.25± 3.32 Ab	0.33± 3.62 a	0.28±3.74 a	0.866 *
6hours	0.12±2.36 c	0.18 ± 2.98 bc	0.22 ±3.55 Ab	0.28±3.70 ab	0.36 ±4.09 a	0.902 *
L.S.D. value	0.387 NS	0.491 NS	0.764 NS	0.508 NS	0.517 NS	
Means with different letters within the same row are significantly different from each other. Means for three replicates)P≤0.05*(						

6-3Sensory evaluation of meat samples treated with protease enzyme and stored for different periods

These results were reflected in the results of the tenderness test when treating purified fungal protease enzyme with steak compared to the treatment without enzyme in Table No. (4). The results showed that the treatment with protease enzyme at a concentration of 1% (the tenderness of steak reached (6 and 5) for two storage periods (2 and 6) hours each separately and significant differences were found at a storage period of 6 hours with treatment at a concentration of 0.2% and for a storage period of 2 hours which reached 4 and treatment at a concentration of 0.4% for a storage period of 6 hours which reached 4 while compared to the control treatment (without treatment with enzyme) which reached 2 gained a highly significant difference (P≤ 0.01.(

The results of Table (4) showed that the juiciness test of steak for treatment E1 and C1 which reached 6 and found a significant difference compared to treatment A, B, B1, C, D and E which reached 2,2,3,3,4 and 4 respectively while it did not gain significance with treatment D1 which reached 5.

The study of the flavor of the treatment of the purified protease enzyme from the fungus A. niger showed a significant superiority over the treatment E1, D1, C1, B1 and B which reached 6 over what was shown by the results of the sensory evaluation (flavor) for treatment E, D and A which reached 3, 4 and 4 respectively and did not show significance with treatment C which reached 5.

The results of Table (4) showed that the sensory evaluation of the color of the steak after treatment with the purified protease enzyme was significantly superior to the treatment E1, D1, C1 and A which reached 6 over what was shown by the results of the sensory evaluation (color) for treatment B which reached 4 and did not show significance with treatment B1, C, D and E which reached 5.

These results above were reflected in the results of the general acceptance test when treating the enzyme Purified fungal protease with steak compared to the treatment without the enzyme, the results showed that the treatment with the protease enzyme at a concentration of 1% (E1) and the treatment at a concentration of 0.6% (D1) reached 6 for a storage period of (6) hours each separately and significant differences were found in treatment A (without treatment with the enzyme) and C which reached 3, 4 and gained a significant difference ($P \leq 0.05$) while a highly significant difference was found ($P \leq 0.01$) compared to treatment B which reached the general acceptance of 1 while we did not find a significant difference compared to treatments B1, C1, D and E which reached 5 Table (4). The results differed from what was found in the results of (Abu Ahmed et al. 2023) that the treatment of adding the enzyme actindin at a concentration of 0.75% and incubating for 4 hours, 6 hours and at a concentration of 1% for a storage period of 2 hours obtained its superiority compared to the other treatments without a significant difference ($P\geq 0.05$). This was confirmed by the results of Delshad and Hassoun (2016) when they used the extract of the enzyme protease (actindin) from pineapple to tenderize sheep meat, which showed high evaluation scores for the qualities of tenderness, juiciness and general acceptance of the meat. The results of Jalal and Hatem (2017) showed that after adding the enzyme actindin with green tea extract to the meat of old chicken, the highest evaluation scores were recorded for the quality of flavor and aroma, reaching 3.8 and 4.4 compared to the control treatment, which recorded a decrease of 2.4 and 2.8, respectively

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Table (4): Sensory evaluation results for steak treatments

Treatment	Color	Flavor	Juicy	Tenderness	Overall Acceptability
control-A	6 ±0.27 a	4 ±0.17 bc	2 ±0.07 c	2 ±0.07 c	3 ±0.14 c
B	4 ±0.15 b	6 ±0.25 a	2 ±0.07 c	4 ±0.16 b	1 ±0.05 d
B1	5 ±0.26 ab	6 ±0.25 a	3 ±0.11 bc	5 ±0.24 ab	5 ±0.22 ab
C	5 ±0.27 ab	5 ±0.19 ab	3 ±0.11 bc	4 ±0.16 b	4 ±0.16 bc
C1	6 ±0.31 a	6 ±0.17 a	6 ±0.27 a	5 ±0.24 ab	5 ±0.23 ab
D	5 ±0.26 ab	4 ±0.16 bc	4 ±0.16 b	5 ±0.24 ab	5 ±0.23 ab
D1	6 ±0.31 a	6 ±0.31 a	5 ±0.26 ba	6 ±0.31 a	6 ±0.31 a
E	5 ±0.22 ab	3 ±0.07 c	4 ±0.17 b	5 ±0.22 ab	5 ±0.23 ab
E1	6 ±0.27 a	6 ±0.26 a	6 ±0.25 a	6 ±0.31 a	6 ±0.25 a
L.S.D. value	1.269 *	1.317 *	1.298 *	1.181 *	1.419 *

Means with different letters within the same row are significantly different from each other.
Means for three replicates
)P≤0.05*(

ISSN 2072-3857

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ISSN 2072-3857