Extraction And Purification Of Trans-Glutaminase Enzyme From Germinated and Non-Germinated Pea Seeds And Identification Of Active Compounds Using Infrared Technology.

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Abstract

The study was conducted in the laboratories of the University of Basrah, College of Agriculture, Department of Food Sciences in the laboratories of the Food and Consumer Protection Unit, where germinated and non-germinated pea seeds were selected from which the enzyme was extracted with various buffer solutions in order to know the bactericidal capacity of the enzyme.The use of distilled water, sodium acetate buffer solution pH 5, phosphate buffer solution pH 6, potassium phosphate buffer solution pH 7, and Tris-HCL buffer solution pH 8,Through the extraction process, a phosphate buffer solution with pH 7 was determined as the best solution for extracting the enzyme, as it was superior in the values of enzymatic activity and specificity than the rest of the extraction solutions. Then the enzyme was precipitated using ammonium sulfate to partially purify the enzyme and get rid of the impurities present in the enzyme extract, with a saturation percentage of 40-80%, as the precipitation was excelled to 80% with a specific and enzymatic efficiency of germinated and non-germinated pea and he used infrared technology to diagnose the active groups in germinated and non-germinated seeds and the effect of the germination process on the enzymatic extract, the appearance of wide bands of amide groups during the germinated period

Introduction

Legumes belong to the family Leguminosea Juss Fabaceae lindl and under the Papillinatea family and the genus Pisum whose English name is Pea and scientific name Mammoth melting. Pea occupies the fourth place among legume crops in terms of importance and basic crops for several reasons, where they are rich in nutrients and contain a lot of protein, Pea belong to the Fabaceae family and contain high levels of protein, so the study decided to extract the transglutaminase enzyme, which is one of the transporter and strengthening enzymes of the protein network and as a binding agent to contain the two amino acids glutamine and allicin (16), This makes it an ideal source of protein, especially in areas where meat and dairy products are not financially or economically accessible. Legumes are low in fat and rich in soluble fiber that can lower cholesterol . It helps in controlling blood sugar levels. Because of these properties, health organizations recommend it in order to combat non-communicable diseases, such as diabetes and heart disease. It has also been proven that pulses help to combat obesity (6) and (14).Legumes are an economic, industrial and nutritional source as they provide the human body with the necessary needs of energy, calories and protein, which made them the food of millions of peoples of the world, where the global grain production reached about 7.14 tons Because it contains mineral elements such as iron and calcium, in addition to vitamins B1, B2, B3, and a small amount of fats and carbohydrates (16) (17).Malt is a process known as fermentation or soaking, in which grains are soaked in water for their plants, and then the germinated process is stopped by drying them with hot air. Mono-glucose and dimaltose, trisaccharides raffinose and higher sugars called maltodextrines, It is also developing other enzymes, such as proteases, that break down grain proteins into models that can be used in yeast (4) and (12). Enzymes have witnessed a great development in the field of food industries as nutritional and industrial supplements due to their wide spread and can be used in a range of nutritional processes.

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The enzymes were extracted from microbial, bacterial and fungi sources, in addition to plant and animal sources, as the enzymes were introduced in general in the areas of food manufacturing in abundance, especially trans-glutaminase enzymes because of their importance in improving the quality of baked goods, and their health security and have no harm to human health (1).Transglutaminase is a group of transporter enzymes that form transferases (E.C. 2.3.2.13) called Protein-Glutamine-γ-Glutamyl transferase. It is a covalent bonding enzyme between a free amine group (for example, a protein or a peptide) and is a clidin complex. The acyl group is located at the end of a chain of protein sides of the reaction releasing ammonia.Transglutaminase is naturally present in most animal tissues and human body fluids, and is involved in biological processes (13 and 15). This enzyme acts only on proteins by catalyzing reactions in the formation of covalent bonds between the carboxyl amide group of the side chain on the glutamine residue (GLN) and the amino group of the lysine side chain. These groups can be formed between proteins of different types, such as: casein, soybean globulin , gluten, actins etc. These covalent bonds introduce new properties to the structure of the organisms, and increase their added value. The use of glutamine in meat and fish helps improve firmness, elasticity, and improve texture (3) and (18).The enzyme is present in the plant kingdom in green leaves and soybean leaves and is found in monocotyledonous grains such as wheat, barley and dicotyledonous plants such as pea and barley. This enzyme has the ability to bind primary amines to proteins in plant parts (13).Transglutaminase has a wide range of uses and applications that usually attract interest because it is used in food to make protein cross-linking. Transglutaminase is also used to produce protein membranes or edible compounds. Transglutaminase is an enzyme that catalyzes the formation of cross-links within a protein molecule and between them different protein molecules. This feature has an effect on changes in protein functions: solubility, emulsification, foaming and gel properties (8) and (14),Studies conducted The enzymatic cross-linking of β-casein was more resistant with pepsin than was the case with no β-casein cross-linking. Active groups were diagnosed using infrared rays technology. Infrared spectrum technology is one of the primary techniques in diagnosing active compounds, the composition of related materials and various functional groups, and giving an idea of the molecular structure present in the majority of chemical compounds (2).

The research aim

Using germinated and non-germinated legume seeds to know the enzymatic activity, purify it in part, and follow up the active groups during germinated by FTIR technique.

Materials and methods

The modus operandi of preparing the enzyme trans glutaminase

Preparation of extraction solutions Preparation of extraction solutions

it followed the method mentioned in Folk. and Finlayson. (1977) (7) with some modifications

1- Distilled water

2- Sodium acetate buffer solution with a solution concentration of 0.1 Mity and pH (5).

Prepared by mixing 5.4 ml of glacial acetic acid with 0.58 g of sodium chloride, the mixture was dissolved in distilled water, and the pH was measured to 5 complete to one liter by distilled water.

3- Phosphate buffer solution 0.05 mo, pH 6 =

It weighs 0.4 g of sodium hydroxide and 5.44 g of potassium dihydrogen phosphate.

4- A solution of phosphate buffer PH = 7 0.1 M was prepared with a weight of 0.255 gm of dihydrogen potassium phosphate and 1.088 g of potassium monohydrogen phosphate.

5- Potassium phosphate buffer solution 0.2 M, pH 8

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We prepared with a weight of 23.12 g of acid potassium dihydrogen phosphate and 5.22 g of potassium phosphate monohydrogen.

Extraction method

A quantity of germinated and non-germinated grains (for pea) was weighed with extraction solutions (1, 2, 3, 4 and 5) at a ratio of 5:1 (w/v) by means of a Magnetic Stirrer mixer for 10 minutes, then the mixture was centrifuged at a speed of 10,000 rpm at 5 C for 20 minutes, after which it was collected. The filtrate of each extraction solution and the activity of transglutaminase enzyme and protein concentration were estimated to calculate the specific activity of transglutaminase enzyme in the extraction solutions.The results showed that a solution of phosphate buffer pH = 7 0.1 M, which was prepared with a weight of 0.255 gm of dihydrogen potassium phosphate and 1.088 g of potassium phosphate monohydrogen, is the best solution for extracting enzyme from pea seeds.

To estimate the efficacy of the trans-glutaminase enzyme

The enzymatic activity was estimated using L-Glutamic Acid-γ-Mono-Hydroxamic Acid as a substrate by the colorimetric hydroxamic acid method used by Folk. and Finlayson (1977) (7).

Preparation of solutions

1- Tris-Acetate buffer solution 0.1 M and pH 6.

2- A hydroxylamine solution with a concentration of 2 M, which was prepared simultaneously from a solution of 4 M concentration and a solution of sodium hydroxide 5 M.

3- The solution of the Gly-Gln-L-CBZ substrate prepared by Sigma company is 0.1 M dissolved in 0.2 M sodium hydroxide solution.

4- Mix equal volumes of acetic acid trichloride 15% solution dissolved in 0.1 M hydrochloric acid and 5% ferric chloride solution dissolved in 2.5 M hydrochloric acid.

5- A stock standard solution of L-Glutamic Acid-γ-Mono Hydroxamic Acid prepared from Sigma company at a concentration of 0.002 M Materials and methods

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Preparation method

The reaction mixture was prepared in a test tube by adding 50 microliters of the enzymatic solution to 350 microliters of solution (1), then 25 microliters of solution (2) and 75 microliters of solution (3) and after good mixing by means of the tubes mixer.The mixture was incubated at 37 °C for 10 minutes, then the reaction was stopped by adding 500 µl of solution 4 and after good shaking, the mixture was left for 15 minutes. Then the centrifugation process was conducted at a speed of 3500 rpm for 10 minutes, the precipitate was neglected, the filtrate was taken, and the absorbance was read at a wavelength of 525 nm using a spectrophotometer. 37 AD .

As for the specific activity, it was calculated as follows:

Specific activity (enzyme unit / mg protein) = enzyme unit / protein concentration

Protein Estimation

To estimate the protein concentration during the separation and purification stages, the method of Lowry et al. (1951) (10) was followed. Materials :

1- A 2% sodium carbonate solution is dissolved in a 0.1 M sodium hydroxide solution.

2- Copper sulfate 1% solution.

3- Sodium tartrate 2% solution.

4- Mix solution No. (2) with solution (3) in equal proportions, and prepared immediately before the measurement.

5- Mix solution (1) with solution No. (4) in a ratio of 1:50 and prepared immediately before measurement.

6- Folin-Cioalteu solution prepared from the British company BDH.

7- A standard bovine serum albumin (BSA) solution of 125 µg/ml.

Enzyme precipitation

A 0.1 M sodium phosphate buffer solution was prepared with pH 7, following the method given in (14).

Precipitation with ammonium sulfate

Certain weights of ammonium sulfate were gradually added to the crude enzymatic

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extract at 5°C with continuous stirring using a magnetic stirrer to reach saturation rates ranging from 40-80%.The centrifugation process was carried out after each stage of addition at a speed of 12000 rpm for 25 minutes and at 4° C by means of a high-speed centrifuge and a cooler. The filtrate was separated and the precipitate dissolved in a small amount of buffer solution, then the enzyme activity and protein concentration were estimated. (14).

Infrared spectroscopy

The sample was prepared by mixing the samples under study with potassium bromide with KBr. It worked in the form of tablets and mixed well.It was placed in its designated place in the FT-IR infrared device model Shemadzu Affinty-1 FTIRSpectrophotometer of the College of Education for Pure Sciences/College of Education/University of Basra.

statistical analysis

Statistical analysis of the experiment was conducted for the data under study based on the Design Randomized Complete (DRC) design The results were analyzed within the ready statistical program Genstat and SPss based on the least significant difference between the averages R.L.S.D adjusted in the results at the level of probability (0.05).

Results and discussion

Extraction of trans-glutaminase enzyme

The results obtained in Table (1-1) indicated the qualitative and enzymatic activity of germinated and non-germinated pea using different extraction solutions to know the best solution to extract the enzyme, including distilled water and lead acetate buffer solution with pH 5 and phosphate buffer solution with pH 6 and a phosphate buffer solution with a pH of 7, and a Tris-Hcl buffer solution with a pH of 8, and this is the buffer capacity used for the purpose of knowing the extent of the enzyme's ability to withstand a range of pH and through the statistical analysis at the probability level $P<0.05$, there are significant differences between the extraction solutions used. The best extraction solution was the buffer solution with pH 7, with an enzymatic activity of 74.61 units/ml for germinated pea, while the efficiency of the non-germinated was 76.45 units/ml, while the lowest enzymatic activity was when extracting with distilled water for germinated and non-germinated pea 18.78 and 15.33 unit/ml, While when extracting with buffer 5 and buffer 6 solutions, the enzymatic activity showed significant differences between them, according to what was mentioned in Table (1-1) and these differences are in the enzymatic activity, It is due to the effect of the ranges of pH numbers used, which affect the exposed substance GBZ-GIU-GIY, and that this decrease and rise in the activity values leads to a change in the structure of the enzyme or the active sites that lead to a loss of enzymatic activity, The results in the same table (1-1) showed the values of the specific activity of germinated and non-germinated pea with significant differences between the averages, where it was noted that the highest specific activity of germinated pea reached 747.61 units/mg compared to the non-germinated with a specific activity of 246.09 units/mg.While {:gap {:kind :userinput}} noticed from the table that the specific effects of each of the harvested water, buffer 5 solutions, buffer 6 solutions and buffer 8 solutions for germinated pea is better than non-germinated pea as follows: 33.33, 117.11, 538.73 and 632.28 units/mg, respectively. While the non-germinated pea had the lowest specific effectiveness values, due to the effect of the germinated process and the increase and activity of the enzyme action, the results were in agreement with what was reached (9).

 ISSN 2072-3875

Table (1-1) Enzymatic and specific activity of transglutaminase enzyme extraction solutions Table

extraction solutions	germinated **pea**			non-germinated pea		
	specific efficacy Unit/mg	**protein concentration**	**Enzymatic activity unit/ml**	**specific efficacy Unit/mg**	**protein concentration**	**Enzymatic activity unit/ml**
distilled water	**33.33±0115**	**0.5633**	**18.78±0100**	**30.23±0100**	**0.507**	**15.33 ±0300**
Sodium acetate buffer solution 5	**117.11±0100**	**0.366**	**42.91±0100**	**47.57±0100**	**0.256**	**12.18±0100**
Phosphate buffer solution 6	**538.73±0100**	**0.1326**	**70.45±0100**	**98.23±0100**	**0.164±**	**16.11±0100**
Phosphate buffer solution 7	**747.61±0100**	**0.1024**	**74.61±0100**	**246.09±0100**	**0.290**	**76.45±0100**
Phosphate buffer solution 8	**632.28±**	**0.069**	**43.63±0100**	**98.55±0100**	**0.347**	**31.5±0100**
R.L.S.D . values	**0.0077**	**0.0173**	**0.0102**	**0.0122**	**0.0102**	**0.0161**

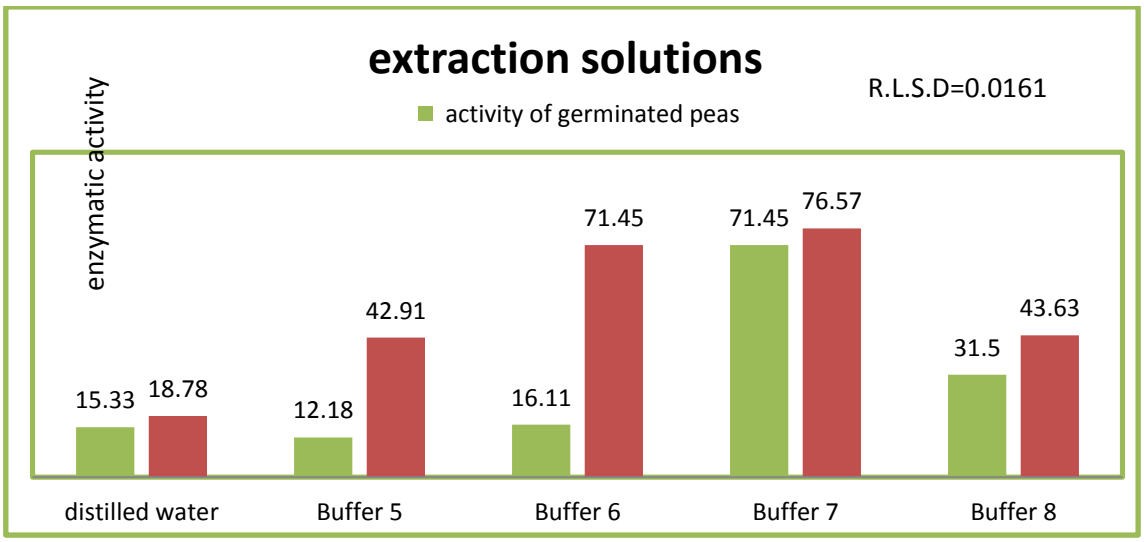

Figure (1-1) shows the enzymatic activity of the extraction solutions

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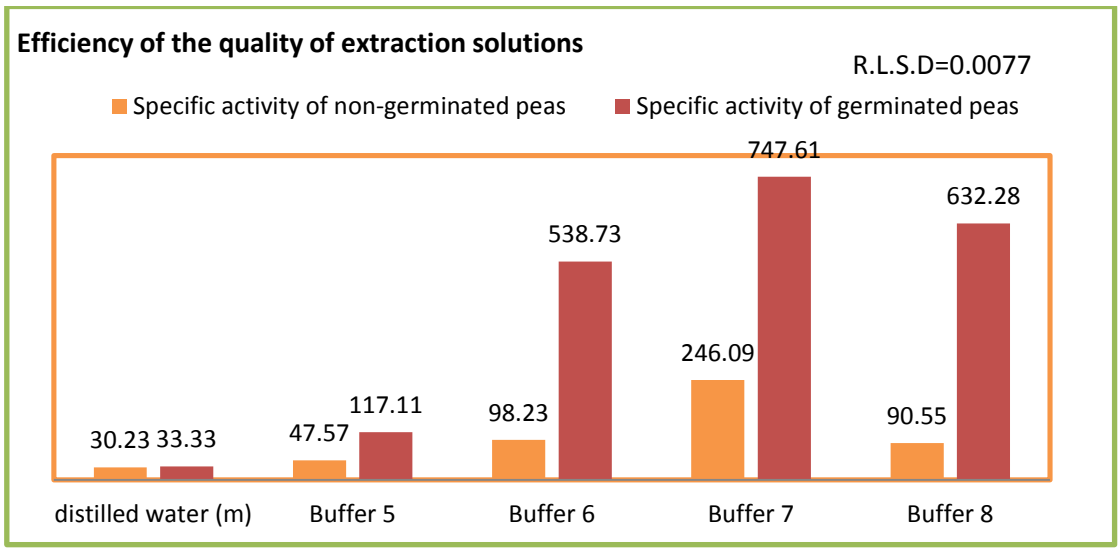

Figure (1-2) shows the specific effectiveness of the extraction solutions

Transglutaminase purification

The purification process is one of the important things to know the purity of the enzyme, and there are several steps to purify the enzyme, according to the study, including complete purification and partial purification.

The enzyme will be partially purified using ammonium sulfate (11).

Ammonium sulfate precipitation

The results of the statistical analysis at a significant level P<0.05 showed that there were significant differences between the sedimentation rates used in the study, where the highest qualitative effectiveness of germinated pea with a saturation rate of 80%, qualitative efficiency of 2088.68 units/mg, while in non-germinated pea it amounted to 56.11 units/mg, according to the figures shown in the figures.It was noted through the results that the values of the specific and enzymatic activity of germinated pea excelled on non-germinated pea with a saturation rate of 80%.While the results showed at 40% saturation of germinated and non-germinated pea the lowest values in terms of enzymatic activity and specificity with a significant difference at the level of 0.05, and that this discrepancy in the values of enzymatic activity and specificity of the most appropriate saturation is due to the efficiency of ammonium sulfate by extracting the largest possible amount of proteins and impurities, and that the use of. Different concentrations of saturation affect the enzymatic activity, which was added with gradual concentrations to get rid of the inactive proteins present in the crude extract before using the appropriate concentration to precipitate the largest amount of the enzyme with the highest qualitative effectiveness, and the saturation rates determine the enzyme's activity by its low and high, and the results were close to what was reached (5)).When the enzyme was isolated from Rosemary plant (*Rosmarinus officinalis* L) with ammonium sulfate with a saturation rate of 40-85%, an increase in the enzymatic activity and protein concentration was observed at 85% saturations, higher than the seven saturations at 40 and 50%, and the specific effectiveness was superior at 85% saturations. 280.66 units/mg

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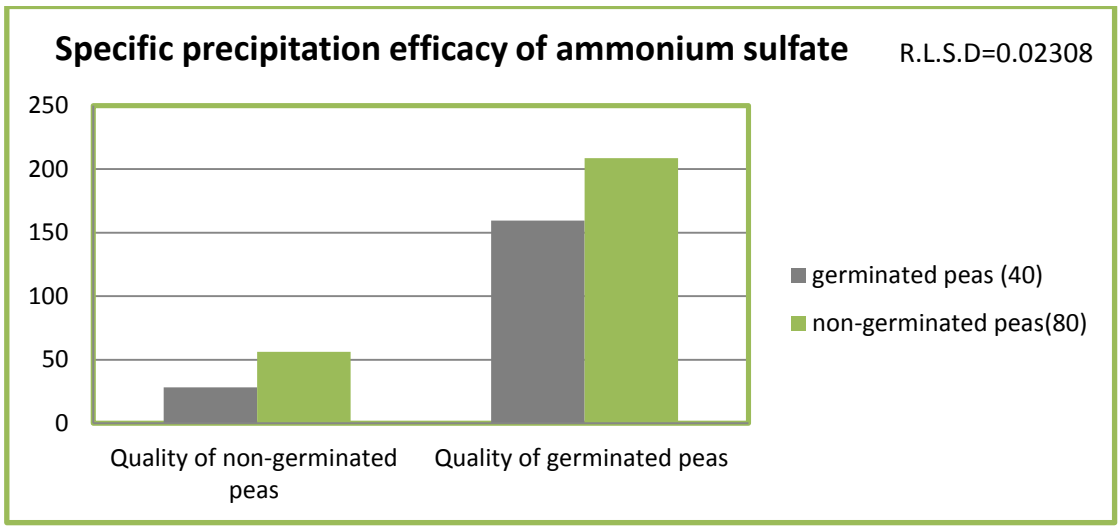

Figure (1-3) shows the specific activity of precipitation of ammonium sulfate

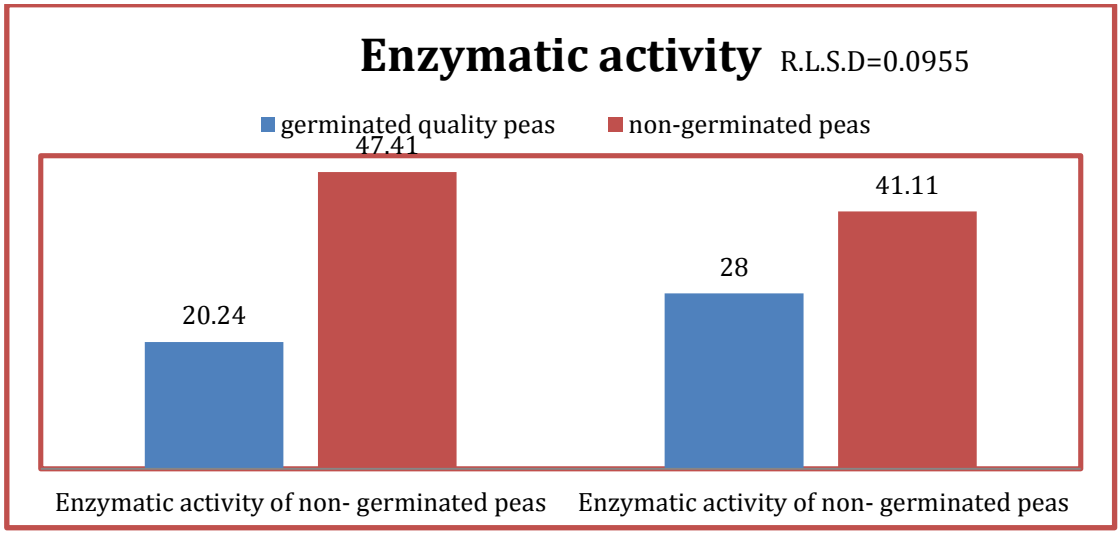

Figure (1-4) shows the enzymatic activity of the saturation ratios used for the transglutaminase enzyme

Infrared diagnostics

Infrared spectroscopy is one of the diagnostic methods used to identify molecules in their natural state, as well as when changes occur to molecules as a result of their interaction and the formation of new molecules. Organic compounds that contain reactive groups such as carbonyl groups, ester and methyl groups and other effective groups are diagnosed . Through the analysis of the sample under study for germinated and non-germinated pea, it was observed that the active groups were present in the germinated pea with a wide amplitude vibration than in the non-germinated pea, due to the effect of the starch granules and the extent of its consumption during the germinated process.

As it was noted that germinated increases protein and increases the elements sodium, magnesium, iron and zinc, as well as the acidity of all types of legumes, The fat content decreased in lentils, chickpea and lupine, while the carbohydrate content decreased (2).It was noticed that wide bands of spectral absorption belonging to methyl CH2 groups and groups belonging to the amplitude oscillation of C=C, C-N, N-H groups appeared, and the appearance of cyclic aromatic compounds, which are amides.),The results agreed with 14) among the infrared spectrometers, as the region of Amide I (1700-1600 cm^{-1}) was chosen as the spectral region most sensitive to these changes during the germinated process and that the amplitude vibration of the Amide I band

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(1700-1600 cm^{-1}) It is most relevant to the analysis of protein secondary structures due to its sensitivity to hydrogen bonds, and the architecture of the protein backbone

Table (1-2) of the effective totals of germinated and non-germinated pea

	Amplitude vibration of effective aggregates Wave numbers cm-1								Chemical groups
C-O;C-C 1151,900	C-O-H,C-C-H,C-O-H 1400-1150	Amide IIN-H,C-N1538	Amide IC=O 1652	Carboxyl C=O 1709	Ester C=O 1745	Methl $-CH_3$ 2854	Methylene $-CH_2$ 2925	OH,N-H 3316.5	groups
1157.29 1080.14 1043.49 1024.20 933.55	1342.46 1246.02 1157.29	1539.20	1651.07		1745.58	2858.51	2927.94	3522.02 3412.08 3396.64	germinated pea
1157.29 1080.14 1024.20 931.62	1425.40 1244.09	1516.05	1658.78	-	1797.66	2856.58 2517.10	2926.01	3408.22 3373.50 3010.88	non-germinated pea

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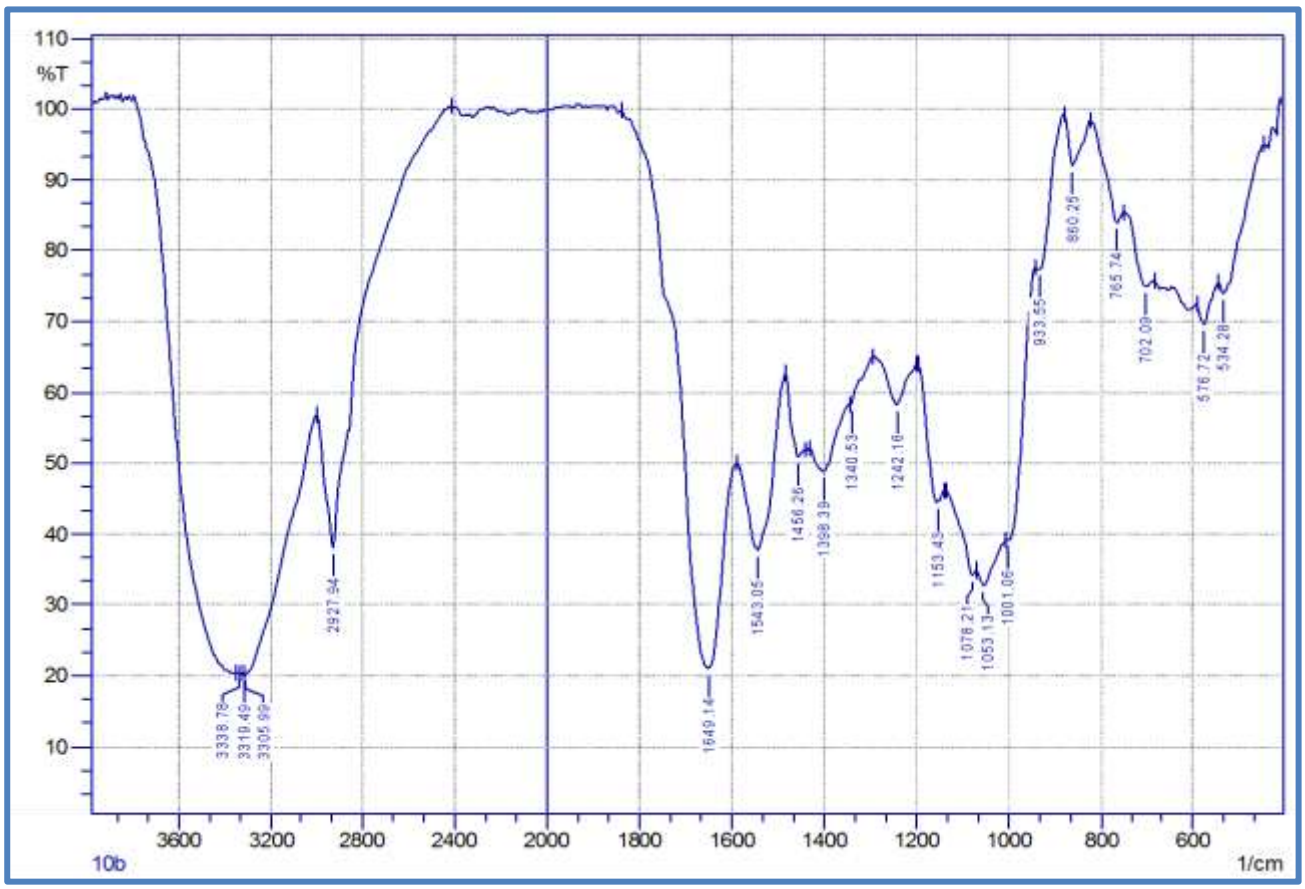
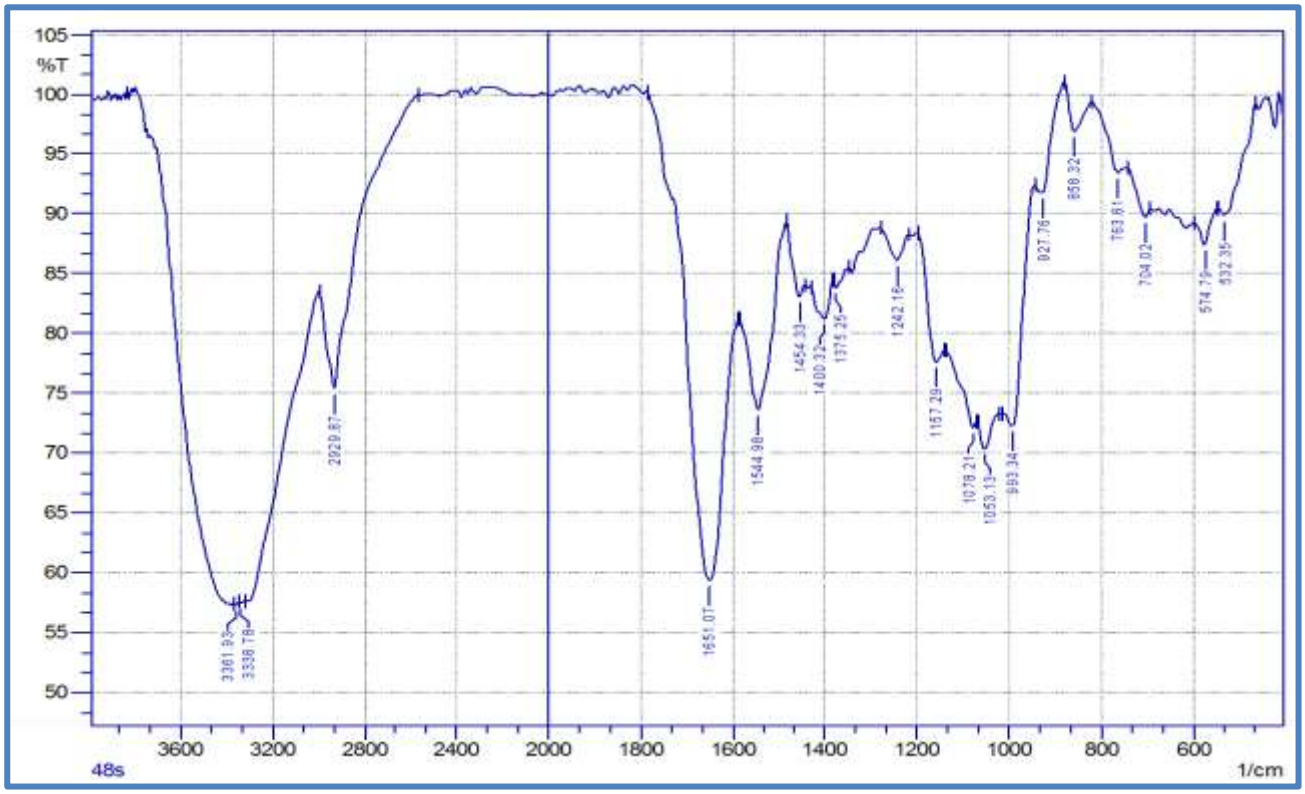


Pattern (1-5) infrared spectrum of ungermination peas



Pattern (1-6) infrared spectrum of germinated peas

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Conclusions

1- It was concluded that the enzyme can be extracted from plant sources, crops of grain and legumes with high protein content.

2- Various extraction solutions can be used to estimate the effectiveness

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