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# Extraction, Purification and Characterization of Transglutaminase From Some Plants

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## Abstract

The present study aimed to Isolate trans-glutaminase EC:2.3.2.13 from some plants sources and Purified it and Studied it's Charactarestics as well as it's practical applications in the production of sausage. The enzyme was extracted from four types of plants (rosemary, chard, radish, arugula) using nine extraction solutions that included distilled water, Sodium chloride 3% solution, sodium chloride 5% solution, sodium phosphate solution 0.1 M and an pH 6.5, sodium phosphate 0.1 M and an pH of 7.5, Tris - Hcl solution 0.2 M and pH 7, Tris - Hcl solution 0.2 M and an pH 8, Tris - Hcl solution 0.1 M and an pH 7 and Tris - Hcl solution 0.1 M and an pH 8 in order to find out the best source of enzyme and the best extraction solution. chard was the best source of enzyme compared with other sources, Tirs -HCl 0.1M, pH 8 solution was the best extraction solution which gave the highest specific activity 8.104 unit / mg. Protein content for the crude enzyme extracts were concentrated using saturated ammonium sulfate in arrange 20-60%, Dialysis was done using distilled water. Then, the purification steps of the enzyme were completed using the gel filtration in the Sephadex G-100 Purification Folds 13.91 time and the yield was 20.04% %. Electrophoresis process using poly acryl amid gel in the absence of SDS observe the presence of one protein band which indicates the complete purification of transglutaminase. transglutaminase molecular weight was 42,660 Dalton when it was evaluated using poly acryl amide electrophoresis in the presence of SDS. the optimum pH for enzyme activity and enzyme stability was 7, while the optimum temperature for enzyme activity was 55°C, and the optimum temperature for enzyme stability was between 25-45°C.

Keywords: extraction, purification, transglutaminase, chard, cross-links

## 1. Introduction

Enzymes are biological catalysts that accelerate chemical reactions in living organisms, as they are extracted from cells and used to catalyze a wide range of processes of commercial interest [1]. Enzymes play an essential role in improving the physical, chemical and sensory properties of food products, so they can be used as alternatives to chemical additives which may be harmful especially if it is applied in the field of food manufacturing. One of these enzymes is the transglutaminase enzyme, which is often used to improve the functional properties of proteins in food [2,3]. Transglutaminase (EC:2.3.2.13) is an enzyme found in the plant and animal kingdoms that works to form internal and external cross-links in proteins between lysine and glutamine [4]. Transglutaminase is a transglutaminase enzyme capable of catalyzing acyl group transfer reactions by forming covalent bonds between proteins, peptides and various amines. The formation of bonds between proteins is stimulated by an acyl transfer reaction from a carboxamide group ( $\gamma$ -carboxamide group) of the amino acid terminal glutamine (an acyl group donor) to the amine group of the amino acid lysine (the acceptor of an acyl group) located at the other end [5,6]. The covalent bonds formed by this enzyme between proteins improve the functional properties of food products such as dairy products, grain products and meat products. These functional properties include texture, flavor, water-carrying capacity, viscosity, thermal stability, increasing the storage period and reducing people's allergy to certain foods, and these are among the most important characteristics of food quality [7-9]. Manufacture of meat products such as sauces, meatballs and steaks (4) as the enzyme works to strengthen the protein network, which leads to its durability and flexibility [10]. Transglutaminase has also been used as an improving agent in the manufacture of bread dough [11]. Transglutaminase improves the rheological properties and physical and chemical properties of the dough [12]. Transglutaminase has also been used in the dairy industry to increase the stability of the consistency of products such as milk, whipped cream and soft cheese [13]. This study objectives to discover new source of Transglutaminase for potential use to meet the growing demands for enzymes in commercial applications.



## 2. Materials and Methods

### 2.1. Preparation of material

Fresh plant was obtained from the local market of Basra city and the samples were cleaned, peeled and cut into small pieces.

### 2.2. Enzyme extraction

The trans-glutaminase enzyme was extracted from plants using nine extraction solutions, which are distilled water, sodium chloride solution NaCl at a concentration of 3%, sodium chloride solution of NaCl at a concentration of 5%, Sodium phosphate buffer solution with a concentration of 0.2 M and a pH of 6.5, Sodium phosphate buffer solution with a concentration of 0.1 M and pH 7. Tris - acetic acid solution at a concentration of 0.2 M and pH 7, Tris - acetic acid solution with a concentration of 0.2 M and pH 8, Tris - acetic acid solution with a concentration of 1.0 M and a pH of 7, Tris - acetic acid solution with a concentration of 1.0 M and pH 8. The enzyme was extracted according to the method [14], by mixing the previously mentioned plants with the extraction solutions in a ratio of 1:1 (weight: volume), where 30 gm of fresh samples (rosemary, chard, radish, arugula) were mixed, each separately, with 30 ml from extraction solutions. Then were left soaked in solutions for 24 hours in the refrigerator, then were mixed with an electric mixer (Blender) for two minutes until a homogeneous suspension was obtained. The mixture was filtered by a Cheese cloth prepared in several layers and then centrifugation process was performed at 10000 x g for 20 minutes and 4°C, the sediment was neglected and the filtrate was taken and used to estimate the enzymatic activity according to method [15], and protein concentration according to Lowry method [16].

### 2.3. Enzyme purification

#### 2.3.1. Ammonium sulfate

Concentrate the enzyme using ammonium sulfate at a saturated ratio of (20-60%), then centrifugation was carried out at a speed of 1000 x g for half an hour, and then the enzyme dialysis process was performed against water for 24 hours, with distilled water replaced every 6 hours. Then the extract collected the prepared for the next step.

#### 2.3.2. Gel filtration

Gel filtration of the enzyme was carried out using Sephadex G-100. Where the gel was prepared according to the instructions of the Swedish Pharmacia company. Which was equipped by dissolving 20 g of Sephadex granules in 750 ml of distilled water and mixing the mixture softly, then heated at a temperature of 90 ° C for 5 hours for the purpose of drinking Cevadex granules swelled and then left to cool. 0.02% of sodium azide was added to it to prevent microbial growth, then the air was removed (degassing) and the column was filled directly and left for the next day for compaction to give a gel with dimensions (1.6 x 78), and then the enzymatic activity was estimated according to [17].

#### 2.3.3. Determination of Enzyme Purity

The method of Polyacrylamide gel electrophoresis under non denaturated condition was followed according to Method [18], and described by Method [19], in testing the purity of the enzyme.

### 2.4. Determination of the molecular weight

Determine the molecular weight by following method of electrophoresis in a polyacrylamide gel and in the presence of Sodium dodecyl sulfate (SDS) according to method [20], and described before [21], using the method of gel filtration and in another way based on the relative mobility (Rm) of the protein bonds Standard and for the enzyme and extract the molecular weight of the enzyme by plotting the relationship between the logarithm of the molecular weights of standard proteins against their relative movement in the gel.

### 2.5. Determination of the optimum pH for enzyme activity

Buffer solutions of 0.2 M ionic strength were prepared over the pH range 3-9, as follows:

1. Solutions with pH 3-4-5 using an acetate buffer.
2. Solutions with pH 6-7 using sodium phosphate buffer

### 3. Solutions with pH 8-9 using a Tris-HCl buffer.

Then the enzymatic activity was estimated according to the method [22], and the relationship between the degradative activity of the enzyme and the pH values was drawn to determine the optimal pH for the enzyme's activity.

#### *2.6. Determining the optimum pH for the stability of the enzyme's activity*

Mixing a specific volume of the enzyme with an equal volume of buffer solutions with graduated pH numbers as mentioned previously, and incubating at a temperature of 35°C for 30 minutes, then the enzyme activity was estimated and the relationship between the different pH values was drawn against the percentage of effectiveness remaining to determine the optimum pH for the stability of the enzyme.

#### *2.7. Determination of the optimum temperature and the thermal stability of the enzyme*

The enzyme activity was estimated according to [23], in a range of temperatures ranging from (25 - 85 °C) and the relationship between temperature and enzyme activity was drawn to determine the optimum temperature of the enzyme. A specific volume of the enzyme was incubated at different temperatures ranging from (25-85 °C) with a difference of 10 degrees for 30 minutes, then transferred to an ice bath and the enzymatic activity was estimated. Optimum stability of the enzyme.

#### *2.8. Studying the effect of metal ions on enzyme activity*

Some ionic solutions of salts with concentrations (1 and 5) mM were prepared by dissolving them in distilled water. The ionic solutions of salts of FeCl<sub>2</sub>, FeCl<sub>3</sub>, CoCl<sub>2</sub>, ZnCl<sub>2</sub>, CuCl<sub>2</sub>, NaCl<sub>2</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub> and Pb(CH<sub>3</sub>COO) were prepared 2) Then 1 ml of the molecularly purified enzyme was incubated with 1 ml of each concentration of the prepared solutions, at a temperature of 35° C for 60 minutes, then the tubes were cooled directly in an ice bath, and the enzyme activity was estimated based on the remaining activity %.

## **3. Results and Discussion**

### *3.1. Enzyme extraction*

The results shown in Figures (1, 2, 3, 4) show the enzymatic and specific activity of TG enzyme extracted from plants. Figure (1) shows that the best solution for extracting the enzyme from rosemary is Tris-Hcl solution with pH 8 and 0.2 molar) and the enzymatic activity reached (6.32 units/ml and specific activity 3.059 units/mg), while Figure (2) shows that the best solution for extracting the enzyme from chard is Hcl- Tris extraction solution with a concentration of 0.1 molar and a pH number 8 The enzymatic and specific activity was (9.02 units/ml - 8.104 units/mg), respectively. Figure (3) shows the best enzyme extraction solution from radish plant is Hcl-Tris solution at a concentration of 0.2 molarity and pH 8, and the enzymatic and specific activity reached (7.18 units /ml - 2.972 units/mg) respectively, while Figure (4) shows the best enzyme extraction solution from arugula is 0.1 molar sodium phosphate buffer solution with pH 8. The enzymatic and specific activity reached 5.08 units/ml - 3.618 units/mg) respectively, and after comparing the results shown in the previous figure shows that the best plant for extracting trans-glutaminase enzyme from plants is chard, and the best extraction solution is Tris-Hcl with a concentration of 0.1 molarity and a pH of 8, as it gave the highest specific effectiveness compared to other extraction solutions.

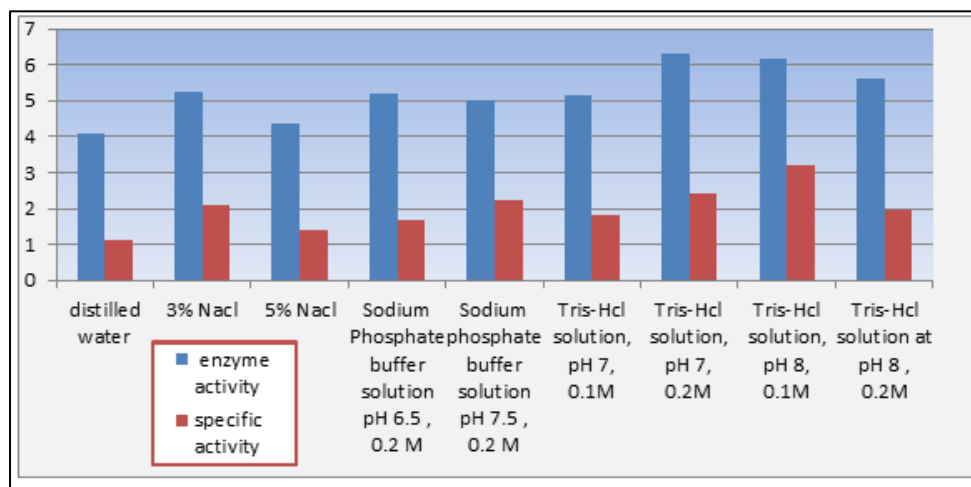
### *3.2. Enzyme purification*

#### *3.2.1. Enzyme concentration*

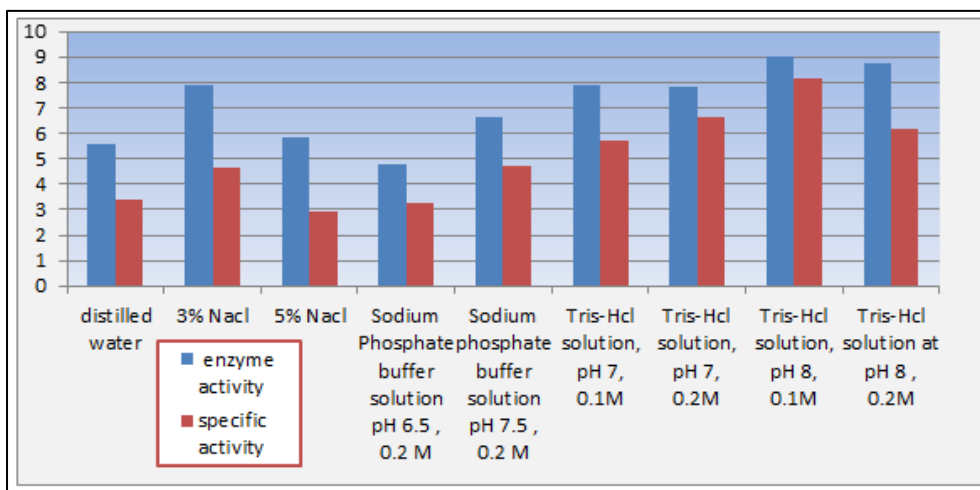
TGase extracted from chard was concentrated using ammonium sulfate salts with saturation rates ranging between 20-60%. This step gave a specific activity of 42.154 units/mg. This step achieved partial purification of the enzyme 5.3 times and an enzymatic yield of 59.59%, These results were in agreement with many studies that indicated the importance of the enzyme concentration with ammonium sulfate as a first step in purification, as it showed [24], that the concentration of TG extracted from rosemary with a 90% saturation rate gave a number of 1.68 purification times and a result of 32.34%, [25], also showed that the concentration of TG enzyme extracted from rosemary with a saturation percentage (80-40%) gave the number of purification times 2.12 and a result of 34.44%.

### 3.2.2. Gel filtration

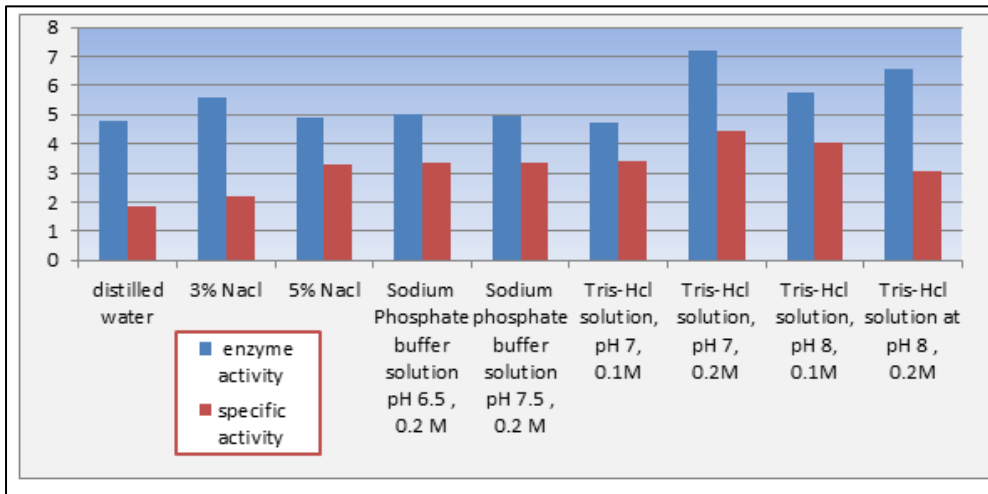
The gel filtration step is carried out as additional purification steps to complete the purification of the trans-glutaminase enzyme and to obtain highly purified enzyme extracts. Figure (5) shows the results obtained from the gel filtration method for the TG enzyme extracted from the chard plant. Where the presence of three peaks appeared when measuring the absorbance at a wavelength of 280 nm for the recovered parts, and when measuring the enzymatic activity of the parts of these peaks, it was found that the enzyme activity was limited to the tubes (68-80), which was represented by a peak identical to the second protein peak, which indicates a preliminary indication of the purity of the enzyme. Then the parts of this peak were collected and its size, protein concentration and activity were estimated. The enzymatic and specific activity reached 7.53 units/ml and 110.74 units/mg respectively. This step achieved an enzymatic yield of 20.04% and a number of purification times of 13.91 times. Sephadex G-100 gel was used in the gel filtration step to purify the TG enzyme by [26]. This gel was used to purify the TG enzyme from the rosemary plant and obtained a number of 7.31 times of purification and an enzymatic yield of 21.35%. [27], when using this gel to purify the enzyme, it was found that the number of purification times reached 6.3 times and an enzymatic yield of 26.51%.



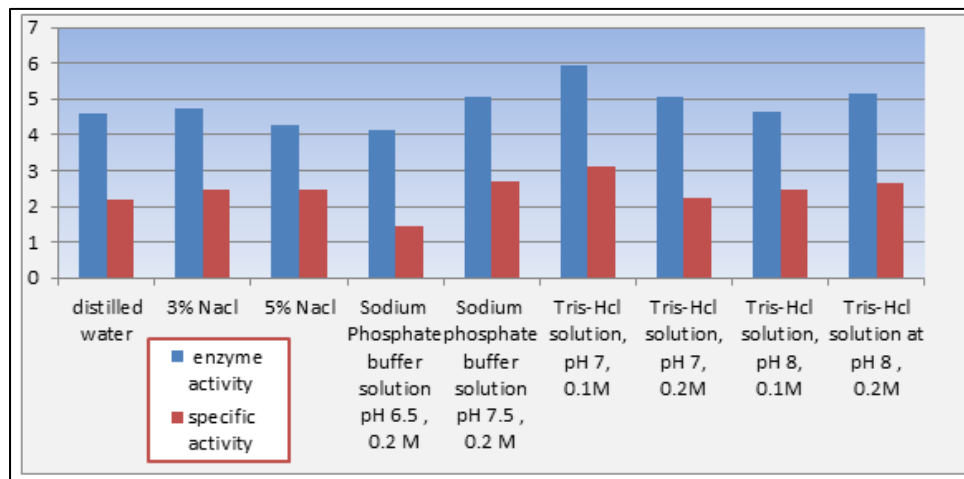
**Figure 1.** The enzyme activity and specific activity of TGase extracted from Rosemary using different solutions.



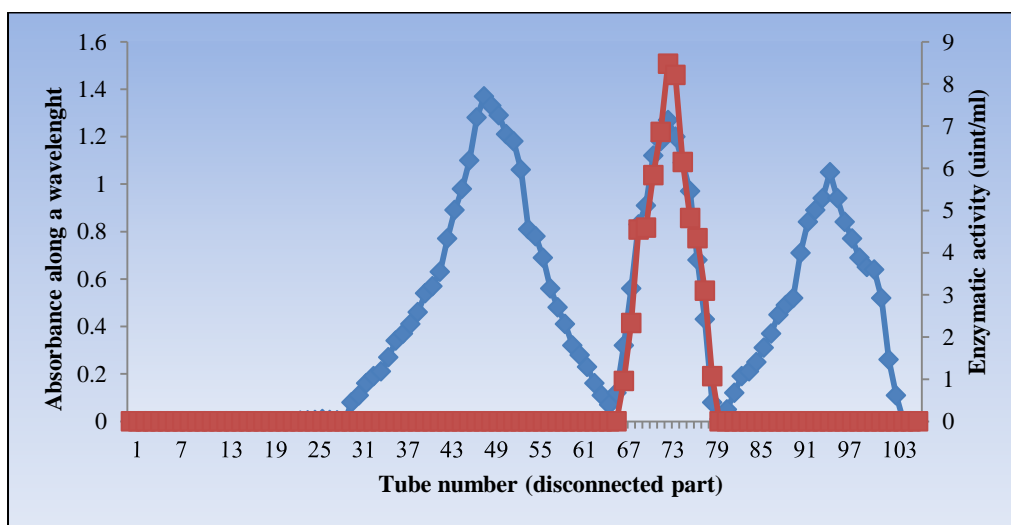
**Figure 2.** The enzyme activity and specific activity of TGase extracted from chard using different solutions.



**Figure 3.** The enzyme activity and specific activity of TGase extracted from radish using different solutions.



**Figure 4.** The enzyme activity and specific activity of TGase extracted from arugula using different solutions.



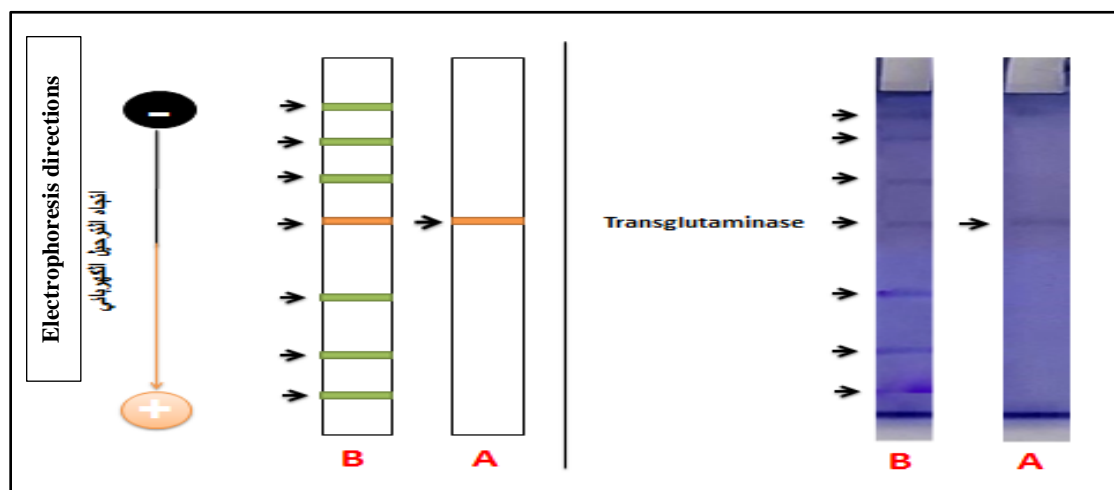
**Figure 5.** Gel filtration of Transglutaminase extracted from chard using a Sephadex G-100 column with dimensions (1.6 x 78) cm equilibrated with sodium phosphate buffer solution at a concentration of 0.02 molarity and pH 6.3 at a flow rate of 20 ml/hour at a rate of 3 ml/part.

**Table 1.** purification steps for TGase purified from chard.

step	purification steps	Volume (ml)	activity (unit/ml)	Protein (mg/ml)	Specific Activity Unit/(mg)	Total Activity (Unit)	Purification factor	Recovery %
1	Crude extract	150	9.02	1.133	8.104	1353	1	100
2	ammonium sulfate	21	38.36	0.91	42.154	805.56	5.3	59.59
3	Gel filtration	36	7.53	0.068	110.74	271	13.91	20.04

### 3.2.3. Determination of the purity of the enzyme

The method of electrophoresis is one of the important methods that determine the efficiency of the steps used in purifying the enzyme and ensuring its purity before starting to characterize the enzyme. Figure (6) shows the results of electrophoresis of TG enzyme purified from chard using ammonium sulfate and gel filtration with Sephadex G100, The electrophoresis was carried out in the absence of teratogens to ensure the purity of the enzyme and that it was free of any other proteins or enzymes. It is noted from the figure that one protein bond appears in the gel, which is one of the indications of the purity of the enzyme. This also indicates that the steps and conditions that were used to extract and purify the enzyme were efficient to the extent that one protein bundle was obtained for the TGase in the test to determine its purity.

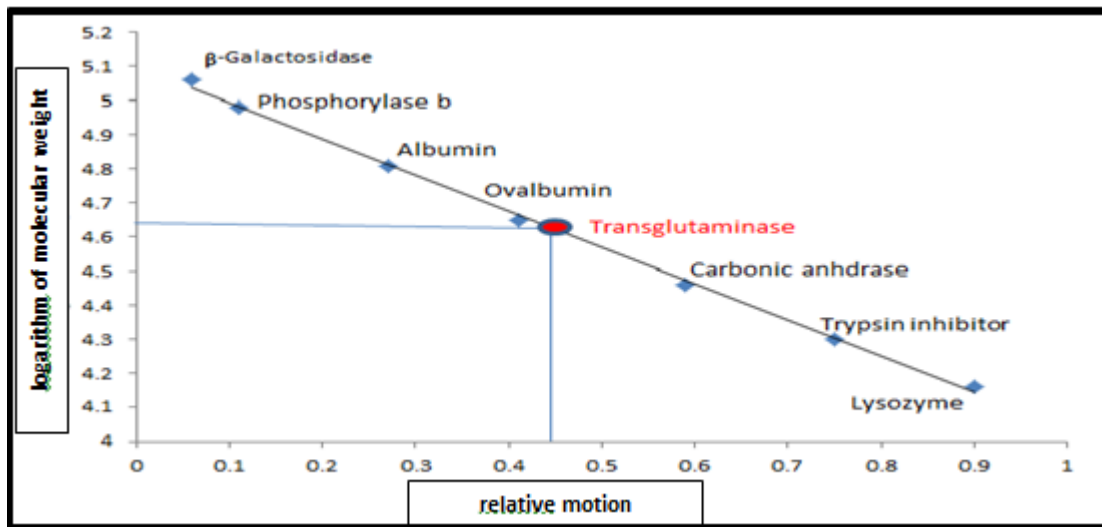


**Figure 6.** Electrophoresis in an acrylamide gel in the absence of SDS- PAGE. , (A) the enzyme purified by gel filtration (B) the crude enzymatic extract.

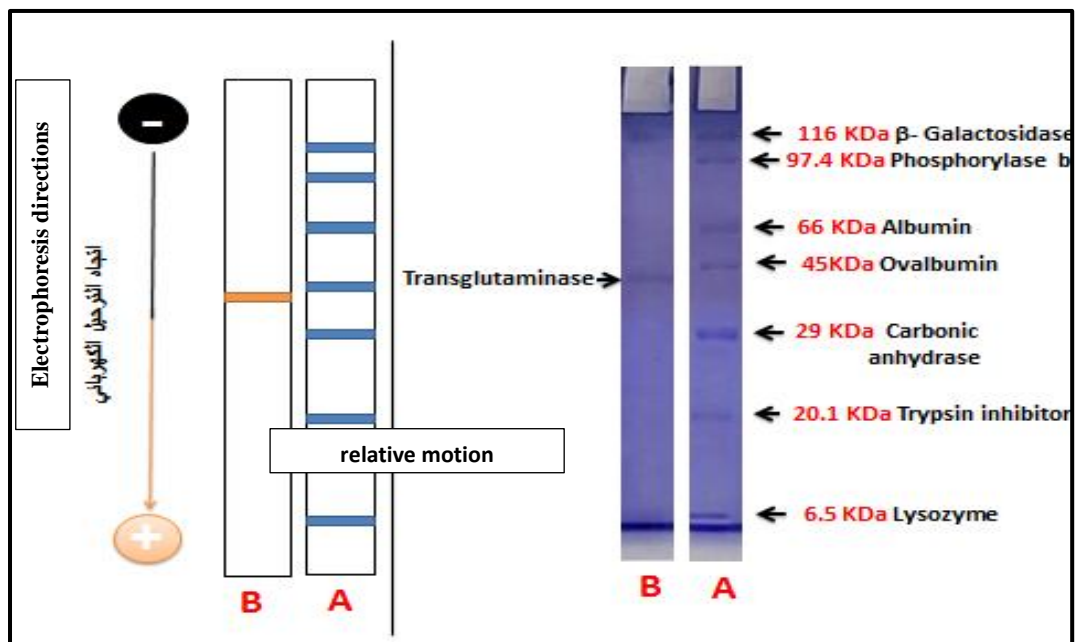
## 3.3. Enzyme characterization

### 3.3.1. Molecular weight Determination

Molecular weight of TG was determined by electrophoresis in a polyacrylamide gel in the presence of SDS- PAGE. Figure (7) shows the relationship between the logarithm of molecular weight and the relative mobility ( $R_m$ ) of standard proteins of known molecular weight. used to estimate the molecular weight of a pure enzyme. The relative mobility of the enzyme ( $R_m$ ) was measured and through it was possible to determine the molecular weight of the enzyme and it was found that it is equal to 42.66 kDa.



**Figure 7.** Standard curve for determination of the molecular weight of TGase purified from chard.



**Figure 8.** Electrophoresis in the presence of SDS- PAGE in a polyacrylamide gel (A) Standard proteins known molecular weight (B) purified TGase.

This result agreed with what was stated by [28], that the molecular weight of TG enzyme found in the chloroplast of plants ranges between 39-53 kDa, as it was close to what was obtained [29], when estimating the molecular weight of TGase from *Bacillus circulans* by electrophoresis method. The molecular weight was 45 kDa. And it was lower than what was obtained [30], when estimating the molecular weight of TG enzyme purified from Walleye Pollack Liver, which was 77 KDa.

### 3.3.2. Determination of the optimum pH for enzyme activity

Determine the optimum pH for TGase activity in a range of pH from 3-9 The results shown in Figure (9) showed that the optimum pH for the enzyme's activity was 7, as it gave the enzyme the maximum enzyme activity, which was 7.53 units/ml. The enzyme also worked with high efficiency with a range of pH ranging between 5-8, while the enzymatic activity was low at pH values 3 and 9 due to the effect of the pH of the reaction medium on the ionizable groups present in the active site, or because of changing the ionic state of the base material, or because Changing the ionic state of the enzyme complex - the substrate (ES) and the resulting enzyme complex (EP) [31,32]. These results were identical to what was found by [8], that the



optimum pH for the effectiveness of TGase purified from rosemary is 7. It is also in agreement with what was obtained by [33], when estimating the optimum pH for the activity of TGase purified from tilapia, which was 7.5, while [6], the number found that the optimum pH for the activity of TGase purified from rosemary is 6, and mentioned [9]. The highest activity of the enzyme purified from sunflower was at pH 8.

### 3.3.3. Determination of the optimum pH for the stability of the enzyme

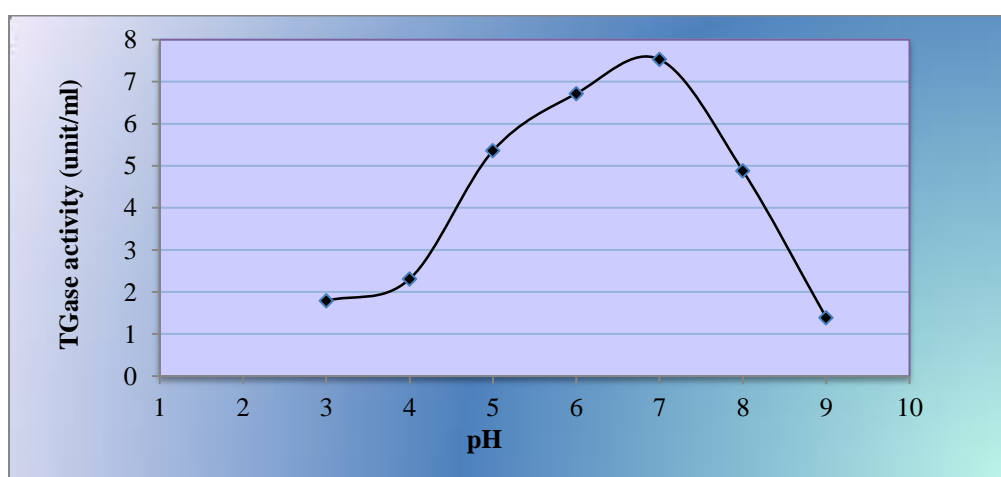
The results in Figure (10) indicate that the optimum pH for the stability of TGase purified from chard is 7, and it was noted that the enzyme retained its activity at pH 8,6,5 by more than 80%, while its stability decreased at pH 9 4,3. This decrease may be attributed to the effect of pH in changing the secondary and triple structure of the enzyme, changing the shape of the active site, and thus deforming the protein molecule and losing enzymatic activity [27]. These results agreed with what was found by [25], that the optimum pH for the stability of the activity of TGase purified from beef liver was 7.5. And a similar approach came to what was found (15 and 20) in that the optimum pH for the stability of the TGase enzyme purified from *Streptovorticillium mobaraense* and *Streptovorticillium ladakanu* bacteria, respectively, ranged between 5-7.

### 3.3.4. Determining the optimum temperature for the activity of the enzyme

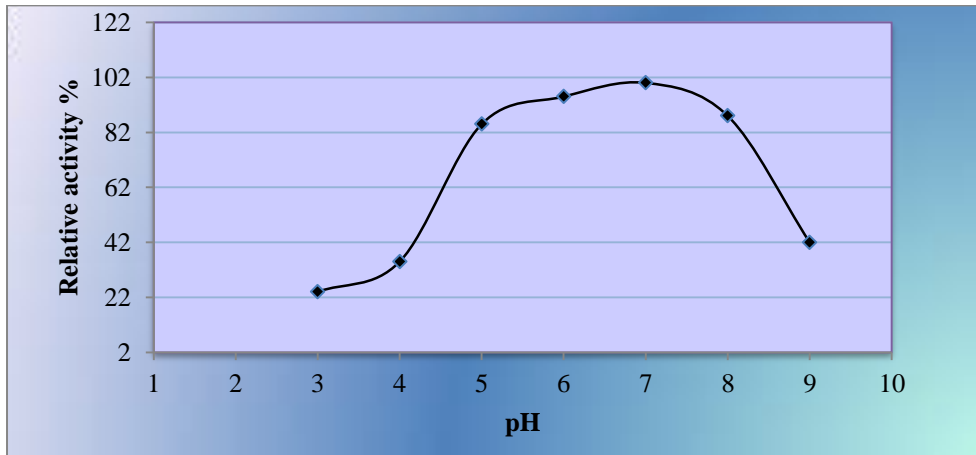
Figure (11) shows the effect of temperature on the activity of TGase, in which it is noted that the enzyme activity increased by increasing the temperature to reach its maximum at a temperature of 55 °C and then began to decrease gradually at a temperature of 65 and 75 °C until the enzyme completely lost its effectiveness at the temperature. The reason for the increase in the speed of enzymatic reactions with an increase in temperature to a certain limit is due to the increase in the collisions between the enzyme molecules and the base material as a result of the increase in the kinetic energy of the molecules due to the effect of increasing the temperature. It leads to changing the triple structure of the enzyme and then mutating it and losing part of its effectiveness [27]. These results were in agreement with what was reached (8 and 33) when studying the optimum temperature for the activity of TGase purified from sea bream and rosemary, respectively, which was 55 °C, and similar to what was found [6], when studying the optimum temperature for the activity of TGase purified from rosemary, which reached 60 m.

### 3.3.5. Determination of the optimum temperature for enzyme stability

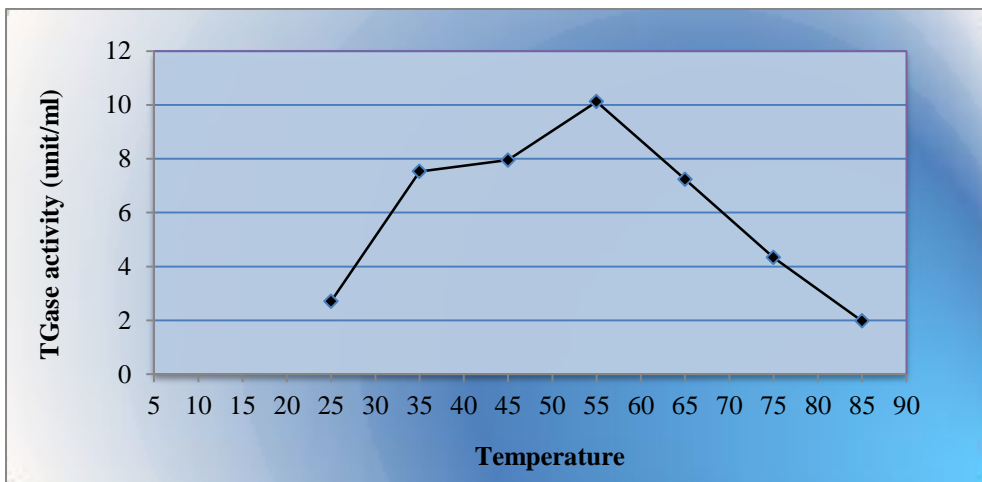
Figure (12) indicates the effect of incubating TGase enzyme purified from chard at different temperatures ranging from 25-85 °C for 15 minutes on the enzyme's activity. It can be seen that the enzyme retained its full activity in a temperature range between (25-45) °C. After that, the activity decreased with high temperatures until it was completely lost at 85 °C. These results came close to what was found by [2], when estimating the thermal stability of TGase purified from *Streptovorticillium*, which was 40 °C. It also agreed with what was reached [25], that the optimum temperature for the stability of TGase purified from cow liver ranged between 5-55 °C.



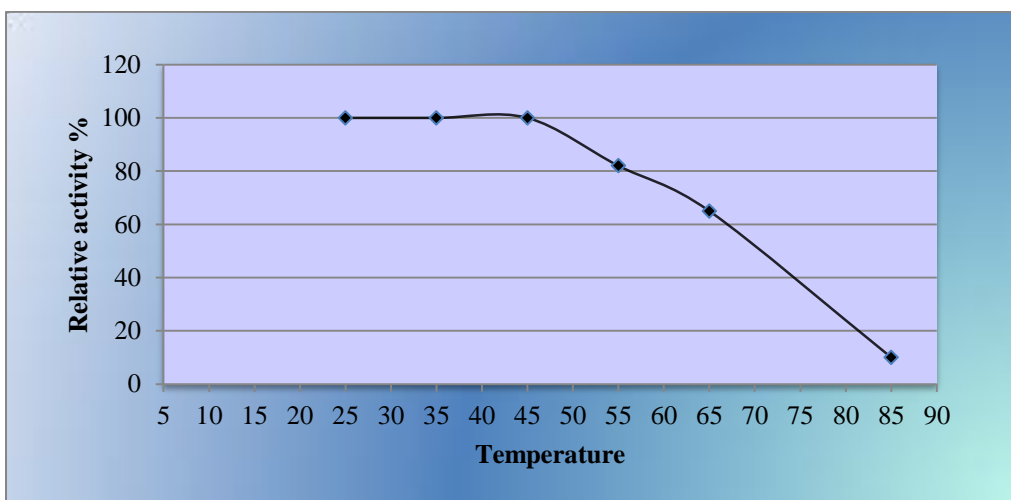
**Figure 9.** The optimum pH for the activity of the TGase purified from the chard.



**Figure 10.** The pH of the stability of the activity of the TGase purified from the chard.



**Figure 11.** The optimum temperature for the activity of TGase purified from the chard.



**Figure 12.** The optimum temperature for the stability of TGase purified from the chard.

### 3.4. Effect of some minerals on the activity of the TGase enzyme

The results in Table (2) indicate the effect of a number of metal ions on the activity of TGase purified from chard, it is noted that there is no effect of  $\text{Ca}^{+2}$  ion on the activity of the enzyme and this indicates that the enzyme does not depend on calcium ions in its effectiveness is similar to TGase found in bacterial sources [5], and different from TGase found in animal sources, which is characterized by its need for calcium ions [25]. The results also shows that the enzyme is greatly affected by heavy metal ions such as  $\text{Co}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Cu}^{+2}$  and  $\text{Pb}^{+2}$ , where these metals inhibit the activity of the enzyme and this is due to the fact that these ions have the ability to bind the thiols of the single cysteine unit It is found in the active site of the enzyme and thus reduces the activity of the enzyme significantly because the cysteine unit represents a part of the active site of the enzyme [20]. The results also showed that some minerals did not affect the enzyme's activity or had a slight effect on the enzyme's activity when treated with  $\text{Fe}^{+3}$ ,  $\text{Fe}^{+2}$ ,  $\text{Mn}^{+2}$  and  $\text{Na}^{+1}$  ions.

**Table 2.** Effect of metal ions on the activity of TGase purified from chard.

	Metal ions	Concentration (mM)	(%)Remaining efficacy
	Unprocessed enzyme	0	100
1	FeCl <sub>2</sub>	1	94
		5	90
2	FeCl <sub>3</sub>	1	103.5
		5	95
3	CoCl <sub>2</sub>	1	8.22
		5	6.33
4	ZnCl <sub>2</sub>	1	12.2
		5	9.8
5	CuCl <sub>2</sub>	1	38.76
		5	30
7	NaCl	1	92.47
		5	87.3
8	CaCl <sub>2</sub>	1	100.4
		5	95.4
9	Pb(CH <sub>3</sub> COO) <sub>2</sub>	1	12.77
		5	10
10	MnCl <sub>2</sub>	1	87.90
		5	82

### Acknowledgements

We acknowledge the authors gratitude to all staff of the department of food sciences in Agriculture Collage-Basrah University.

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