



EXTRACTION AND PURIFICATION OF B-GALACTOSIDASE FROM (ZIZIPHUS SPINA-CHRISTI)

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Abstract: β -galactosidase enzyme EC (3.2.1.23), lactase, can be described as an enzyme of glycoside hydrolase which performs the catalyzing of β -galactosides hydrolysis to mono-saccharides by breaking glycosidic bond. The objective of this study was to extraction and purification β -galactosidase from (Ziziphus spina-christi.), ten Different types of extraction were investigated to selection of the best extraction of the enzyme, The Na. phosphate buffer (0.2M and pH6) had given a highest Specific activity of crude enzyme has been 101.36 U/mg. protein. The purification procedures were performed with the use of the precipitation of ammonium sulfate, ion-exchange and gel filtration chromatographic techniques. 70% ammonium sulfate saturation has been the best method for precipitation and partially purification of enzyme with a purification fold 1.34 and enzymatic yield 63.90%. This was followed by the use of ion exchange chromatography by DEAE Sephadex A50 column, the purification times of the enzymatic ex-tract were 2.66, with an enzymatic yield 31.52%. After the final purification step of gel filtration chromatography using Se-phadexG-100 column, the enzyme has been purified 4.12 fold with 21.91% of enzymatic yield. The optimum enzymatic activity was found at pH 6. The enzyme's molecular weight has been estimated to 77.53 KD by the gel filtration chromatography meth-od, and 75 kD on SDS-PAGE.

Keywords: Ziziphus Spina-Christi, β -Galactosidase, Extraction, Purification Methods.

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1. Introduction

β -galactosidase (β -D-galactohydrolase as well) which is referred to as the lactase can be described as a set of the enzymes that are capable of cleaving the β linked residues of the galactose from a variety of the compounds and is typically utilized for cleaving the lactose to glucose and galactose [1], enzyme β -galactosidase (EC3.2.1.23) performs the catalyzing of the bonds of β (1-3) hydrolysis and β (1-4) galactosyl in the oligo and disaccharides, however, it performs the catalyzing od reverse reaction of hydrolysis as well, typically referred to as the trans-glycosylation. It has a wide range of distributions, encompassing microorganisms, plants and animals [2]. [3]–[5]. The enzyme β -galactosidases have been described with their capability in hydrolyzing the terminal non-reducing residues of the β -D-galactosyl from the β -D-galactosides [4]. (Figure 1).

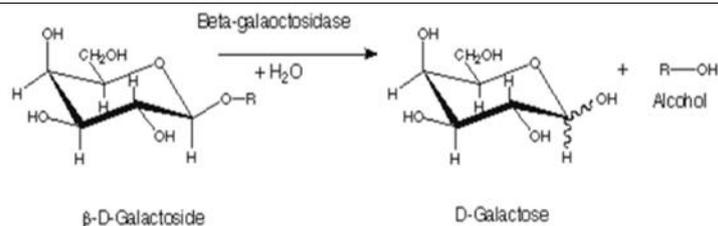


Figure 1. The reaction of β -galactosidase

Those enzymes were related to depleting the oligosaccharides and polysaccharides throughout the mobilization of the seed reserves [6], in addition to the fact that throughout the cell wall loosening process for the elongation of the cells [7]. In addition to that, they were associated with the processes of the fruit ripening [8], dietary lactose hydrolysis [9] lactose metabolism and other galactosides in the micro-organisms [10]. One of the significant properties of the galactosidases is their possibility of modifying the specificity of the blood group of the intact human erythrocytes through the removal of the residues of the galactose from the glycoproteins of the cell surface [11].

The majority of the plant β -galactosidases have specific optima of the pH in weak acidic solutions [12]–[14] that are close to the stomach and duodenal values of the pH. Which has raised the potential of the β -galactosidases (i.e. the residual in the ingested plant foods) which attack the gastric mucin. This type of attacks would result in the exposure of gastric wall to the acid and the proteolysis.

It has been a common knowledge that there are numerous isozymes of the β -galactosidase in quiescent seeds of a variety of the species of plants [15], [16] however, it's unknown whether those isozymes are species specific or they are different amongst the same species' cultivars.

It was purified from a variety of the sources of plants, such as mango [15], chick peas [17], almonds [13] *Vigna unguiculata* [18], apricot [19], tomato [2], watermelon [20], Peach [2] and cowpea [21]. Increasing the activity of the β -galactosidase throughout the germination of the seeds has been discovered as well in the plants like the soybeans and fenugreek [22]

The plant β -galactosidase is more suitable for the industrial applications due to its easy adaptability high availability, as well as cost effectiveness [23]. Due to those enzymatic properties, the β -galactosidase is capable of hydrolyzing the milk sugar, which is why, enzyme is often referred to as the lactase. As a result, those enzymes are of a high importance for dairy industries for the purpose of manufacturing the lactose-free and low-lactose products of milk [24]. In addition to that, the β -galactosidases are commonly utilized as well in the experimentations for the enzymatic synthesis of the galacto-oligosaccharides [25].

Lettuce is a greenish vegetable has an extremely decreased content of the calories and it is mainly made up of water, approximately 90%-95%. *Ziziphus spina-christi* as well includes fibers; substances such as the potassium, minerals, phosphorus, calcium, magnesium, iron and antioxidants like the β -carotene and vitamins C, E, and A, vitamin K, foliate in addition to a number of the vitamin B complexes [26]. The tissues of *Ziziphus spina-christi* contain small amounts of the phenolic compounds in the case where the plants have been grown under the non-stressful conditions [27]. Because of the unavailability of sources that indicate the production of lactase enzymes from the prickly lettuce plant that is found in a large and wide area in all regions of Iraq, therefore this study aimed to purify β -galactosidase from *Ziziphus spina-christi* and study some of its properties in order to invest this plant in the production of the enzyme and use it in some applications.

2. Materials and Methods

Materials: Samples of the fresh *Ziziphus spina-christi* have been obtained from local market (Baghdad) during February 2020. Characterizing β -galactosidase has been carried out at Biochemistry lab in Baghdad Univ. DEAE Sephadex A-50 was purchased from GE Healthcare, p-Nitrophenyl β -Dgalactopyranoside and sephadex G100 were purchased from Sigma-Aldrich, all other analytical grade materials have been obtained from Sigma-Aldrich and BDH.

Extraction of the enzyme: Crude plant extract has been made from the *Ziziphus spina-christi* and utilized as β galactosidase source. In order to determine the best solution for extraction of the enzyme, the following solutions have been used: extraction by distilled water, 0.5% sodium carbonate solution pH7.20, 0.2 M buffer sodium acetate solution pH 5, and 10% sodium chloride pH 5, 0.20 M buffer phosphate pH 7, 0.05 M ascorbic

acid pH 5, 0.01M citric acid pH5, 0.2% potassium chloride, 0.2% calcium chloride and glycerol 20%. 50gm Plant leaves were homogenized in 50ml from each solutions in a blender for four minutes. The homogenate has been filtered with the use of a cloth sheet and centrifuged afterwards for 20min (at 40C) at 10000rpm. Supernatants have been utilized for the β -galactosidase assay as a crude enzyme solution [27]

Estimation of the Protein: The protein content has been specified in a spectrophotometric way at 595nm using the Bradford method [28] with the use of the Bovine Serum Albumin (BSA) as standard.

Enzyme Assay: The activity of the Enzyme has been assayed through the measuring of rate at which it can hydrolyze the o-nitrophenyl- β -D-glucopyranoside (ONPG) [29]. With the existence of the β -D-galactosidase, the ONPG is hydrolyzed to the D-galactose (i.e. colorless) and o-nitrophenol (ONP) (i.e. yellow). The mix of the reaction of the β -galactosidase included 0.40 ml of 0.10 M acetate buffer (pH equal to 4), 0.50ml of 2mM of the substrate and 0.10 ml of the solution of the enzyme. Following a 15 min incubation at a temperature of 37°C, the reaction has been stopped by adding of 1ml of 0.10mM Na₂CO₃ and observed at 420 nm. The amount of o-nitrophenol released was quantified using the ONP standard (Figure 2). A single enzyme activity unit can be described as the enzyme amount liberating 1 μ mol of the o-nitrophenol per minute under the conditions of the assay.

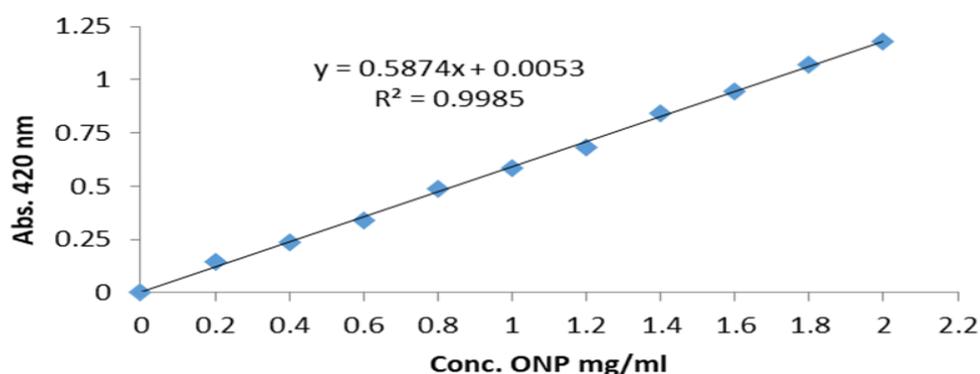


Figure 2. standard curve of ONP using to quantified o-nitrophenol released in the presence of β -D-Galactosidase.

Optimization of pH extraction: To determine the optimum pH of extraction β -galactosidase, a variety of the values of the pH, which range between 4 and 8, with the use of 0.10M concentration values of the following systems of the buffer: sodium acetate (pH 4 - 5.5), sodium phosphate (pH 6 - 7.5) and Tris-HCl (pH 8). Optimum pH for extraction was determined using the specific activity (enzyme activities/conc. of protein) for the crude extracts at different pH [30].

Purification of β -glucosidase: All of the steps of the purification have been carried out at temperature values that don't exceed 4-8°C: **Precipitation by Ammonium sulfate:** Determining the optimal concentration of the ammonium sulfate for the purification has been by treatment of ammonium sulfate 40%, 50%, 60%, 70%, 80% and 90% additions [30]. After weighing the ammonium sulfate concentrations, slowly mixing the enzyme with the stirrer overnight at 4°C to the point where it is homogeneous. After that centrifuging by a 3500rpm speed for 30min at 4 °C, following the completion of the process of the centrifugation, the precipitate has been dissolved in a 0.1M of Na-phosphate buffer (pH 7) and dialyzed against the poly ethylene glycol (M wt. 20000). The enzyme activity and protein concentration were estimated for solutions.

Anion exchange chromatography Sephadex A50: The DEAE sephadex A-50 column was prepared according to the supplier method (GE Healthcare) The column (2X20 cm) was filled by ion exchange material and equilibrated using phosphate buffer (10 mM, pH 7). Aliquot of 3ml of the enzyme solution which has been produced from precipitatin step has been loaded on the column of the ion exchange. The separated fractions have been gathered at a 0.5 ml/min flow rate with 5ml for every one of the fractions. The washing process has been done with the use of the same buffer that was utilized in equilibration, and elution has been accomplished by same buffer with linear gradient concentration of 0-1.0 M sodium chloride. The proteins have been observed at 280 nm and enzymatic activity was measured in each fraction. The fractions of active β -glucosidase were pooled and dialyzed for 24h by poly ethylene glycol, volume and protein concentration were measured.

Gel filtration by Sephadex G-100 column: The Sepadex G-100 gel was prepared according to the instructions of the processed company, and 5ml of purified enzyme obtained from The dialysed samples from anion exchange were loaded on Sephadex G100 column (1.5cm X 65 cm) equilibrated earlier with a 0.10M sodium phosphate buffer (pH = 7) that contains 0.10M Sodium Chloride to eluted by same buffer at a 0.5 ml/min flow rate.

The fractions have been obtained (5ml for each fraction) [31]. In every one of the steps, β -galactosidase activity and protein content have been specified. A fractions containing enzyme activity were collected and dialyzed against poly ethylene glycol. The protein concentration, total volume and enzymatic activity have been measured. The solution was kept under freezing at $-20\text{ }^{\circ}\text{C}$ until use in subsequent experiments.

Estimating the molecular weight with the gel filtration chromatography: The native enzyme's (β -galactosidase) molecular weight has been calculated with the chromatography of the gel filtration. Trypsin (23 kDa), pepsin (35 kDa), ovaalpomin (43 kDa), serum albumin (67 kDa) and collagen (105kDa) have been utilized as a reference. The blue dextran (2000kDa) has been utilized for the determination of void volume (V_0). The buffer of the elution has been 0.1 M phosphate buffer (pH=7) and flow rate has been 0.50mL/min. The elute's absorbance has been maintained at 280nm. The calculated β -galactosidase molecular weight has been found from plotting the molecular weights of the standard proteins against the retention times (V_e) [32].

Molecular weight estimation by SDS Electrophoresis: Molecular weight of β -galactosidase obtained from purification step by Sephadex G-100 column was determined using SDS Poly-acrylamide Gel Electrophoresis according to the method described by [33]. The molecular weight was calculated using the analysis of the linear regression from data which has been obtained by denaturing PAGE with the use 12.5% gel and a set of clear sample of standard proteins with known molecular weights.

Statistical Analyses: Every experiment has been applied in triplicates and results have been represented as values of average \pm standard deviations (SD) with the use of the Microsoft excel 2007.

3. Results and Discussion

Ten solutions for enzyme extraction from (*Ziziphus spina-christi*) have been used. The results mentioned in table (1) [1], indicate that extraction by 0.2 M buffer phosphate pH 7 is more efficiently in comparison with other extraction solutions. The enzymatic activity and specific activities of enzyme which has been extracted via the above-mentioned solution were 7.66 U/ml and 101.36 U/mg. protein respectively. According to the results mentioned in table (1), the high level of specific activity along with the enzyme activity is attributed to the increase in the ionic power which is of great effect on dismantling the ionic linkages connecting the enzyme and other components, a factor that increases the enzyme dissolution.

Table 1. Extraction of β -galactosidase from (*Ziziphus spina-christi*) by various solutions.

No	Method of Extraction	Abs. of ONPG at 420 nm	Enzyme Activity U/ml	Protein Conc. mg/ml	Specific Activ- ity U/mg
1	Water	0.327	4.90	0.07	72.67
2	NaCl (10%, pH5)	0.365	5.47	0.07	77.52
3	Na ₂ CO ₃ (0.5%, pH7.2)	0.341	5.11	0.06	81.84
4	KCl 0.2%	0.311	4.66	0.06	73.18
5	CaCl ₂ 0.2%	0.432	6.4	0.07	92.57
6	Buffer acetate (0.2M, pH5)	0.331	4.96	0.06	83.62
7	Buffer phosphate (0.2M, pH7)	0.511	7.66	0.08	101.36
8	Glycerol 20%	0.319	4.78	0.10	47.26
9	Ascorbic acid (0.05M, pH5)	0.311	4.66	0.09	51.48
10	Citric acid (0.01M, pH5)	0.303	4.54	0.09	53.47

T: 5.817, P: 0.000, d.f: 78, S: Significant.

Effect of the pH on the Activity of the β -galactosidase: Every one of the enzymes has an optimal value of the pH under which it shows its optimal performance: Any change in the value of the pH results in altering the structure of the enzyme and impacting their activities. With the increase or the decrease of the pH, specific amino acids are protonated or deprotonated, as a result, altering the activity and the conformation of the proteins [19]. The activity of the β -galactosidase has been discovered to differ according to the values of the pH (4 to 8), the optimal value of the pH of enzyme activity has been equal to 6 in Na. phosphate buffer solution 0.2M with specific activity 116.74 U/mg. protein (Figure 3).

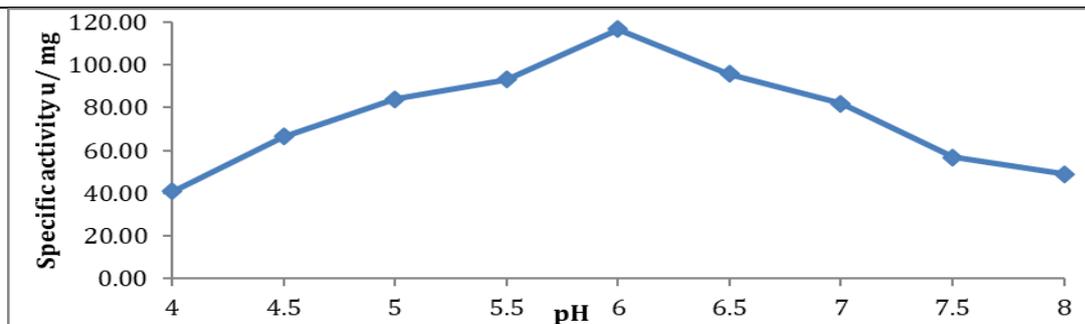


Figure 3. The Specific activity of the β -galactosidase in the crude extract of (*Ziziphus spina-christi*) at a variety of the values of the pH.

Those results have coincided with observations which have stated that optimal value of the pH of the plant β -galactosidase lie in the range of the weak acidics. It was discovered that extracting 3 iso-enzymes of the β galactosidase from the apricots had an optimal value of the pH from 4 to 6 [12], in almonds has been 5.50 and from black bean was 4.5 [14]. While the optimal value of the pH of the β -galactosidase was more acidic from chick pea seeds with 2.8 [17], and from peach was 3.0 [2].

Purification of β -galactosidase: For purifying β -galactosidase, the crude extract has been precipitated using ammonium sulfate in different saturation ratios (30-90%), and dialyzed against poly ethylene glycol (M wt. 20000) overnight to remove an extra salt. Results showed that β -galactosidase was precipitated efficiently with ammonium sulfate in saturation ratio of 70% (Fig. 4). After dissolving precipitate and dialyzed supernatant, Enzyme specific activity increase to 125.78 U/mg, with a purification fold 1.34, and yield of 63.74% compared with crude extraction (table 2). Ammonium sulfate is the most widely utilized reagent to salt out proteins as a result of the fact that its high solubility provides the ability to achieve a solution with a high ionic strength [34]. [13], Purified β -galactosidase from Almond with 15-60% precipitation, Purifying the crude extract of the Apricot seeds with the ammonium sulphate (30%-70%) has resulted in increasing specific activity and the purification fold to 29.07U/mg proteins and 2.92, respectively [18]. Dong [2], purified the β -galactosidase from Peach by using 85% ammonium sulfate saturation which increases Specific activity with yield 45% and 21 purification fold. [20] used 80% ammonium sulfate precipitation method for the partial purification of β -galactosidase from Watermelon, precipitated enzyme yielded 60% recovery and two folds purification of the enzyme. Such difference can be a result of the gradation of the salt which is utilized to purification or the concentration of the salt and the saturation duration for the purification.

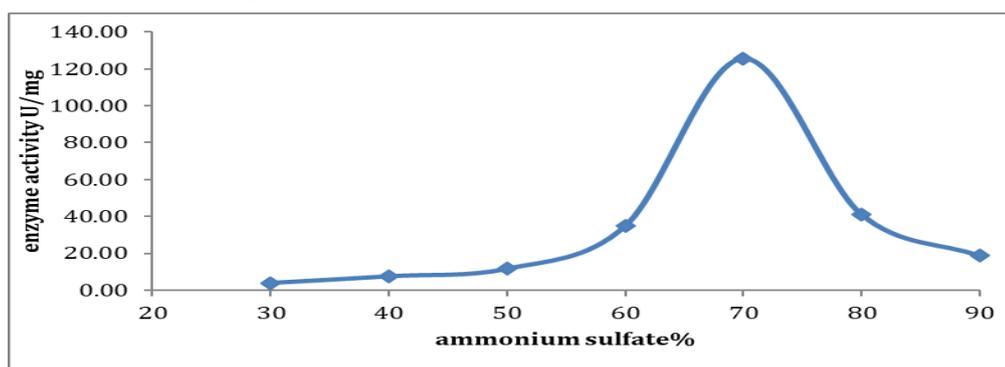


Figure 4. enzyme activity of β -galactosidase using precipitation by ammonium sulfate in different saturation ratios.

Dialyzed enzyme produced from ammonium sulfate precipitation step was more purified throughout DEAE Sephadex A-50 column. Results illustrated in figure (5) showed that one peak of proteins was appeared in elution step represents enzyme activity. This indicates that β -galactosidase carry negative charge differ from the column charge. Fractions of enzymatic activity were collected and dialyzed using poly ethylene glycol (M wt 20000). In this step of purification (ion exchange chromatography), enzyme specific activity was 238.31 U/mg. protein with purification fold 2.66 and yield reach 31.52% as can be seen in table2. In another study, [20] purify extracted from two Cowpea Cultivar, subjected to the ion exchange chromatography via the DEAE-sephadex has been fractionated in 2 separate peaks (DS-I & DS-II) both of which displays a β -galactosidase activity. Whereas the DS-I has been unbound to column DS-II has been bound and eluted afterwards with 0.70M NaCl.

[34] purified enzyme via the ion-exchange chromatography column using DEAE-Cellulose (Diethyl amino ethyl Cellulose).

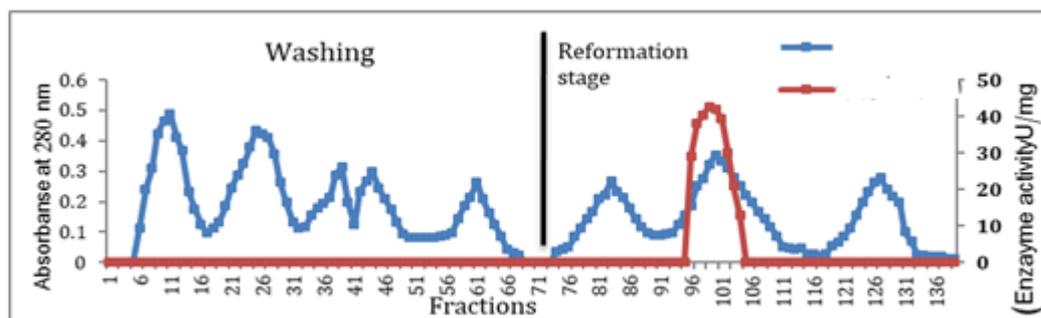


Figure 5. Ion exchange chromatography for purification of β -galactosidase extracted from (*Ziziphus spina-christi*) using DEAE-Sephadex A50 column.

Enzyme solution which has been obtained from the ion exchange chromatography purification step has been more purified by a technique of the Gel filtration chromatography throughout Sephadex G100. One protein peak represents enzyme activity was appeared figure (6). The specific activity has been reached to 368.62 U/mg. protein with a purification fold 4.12 and enzyme yield of 21.91% as shown in table (2). Columns of gel filtration used in the purification of β -galactosidase in scientific studies were varied, sephadex G100 column was used to purify β -galactosidase from peach, with 19 times purification fold and enzymatic yield 16.5% [2], such column used to purification enzyme from Apricot [13], the purification fold recorded 4.5 and 40.7% enzymatic yield. [2] used Sephacryl S200 when they purification enzyme from *Aspergillus niger*, the purification fold was 5.054 with 25.269% yield. Another study used Sephadex-75 to purification enzyme from Almond seeds at a yield of 136.4% and fold purification 2.9 [18].

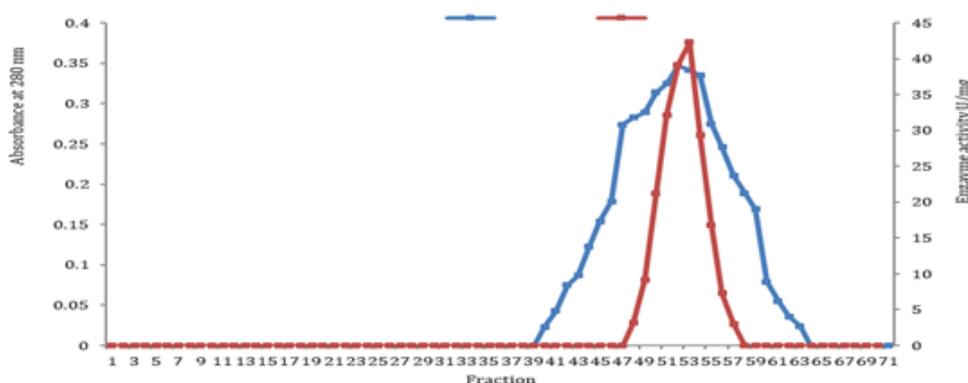


Figure 6. Gel filtration chromatography for purifying β -galactosidase extracted from (*Ziziphus spina-christi*) with the use of the Se-phadex G-100 column.

Table 2. Purification Steps of β -galactosidase extracted from (*Ziziphus spina-christi*).

Step	Volume (ml)	Activity U/ml	Specific Activity U/mg	Total Activity (Unit)	Purification fold	Yield 100%
Crude extract	100	7.38	89.45	738.00	1	100
precipitation by ammonium sulphate 70%	24	19.65	119.54	471.60	1.34	63.90
Dialysis	9	25.65	131.54	230.85	1.47	48.95
IE Chromatography DEAE-Sephadex A50	11	21.15	238.31	232.65	2.66	31.52
Gel filtration Sephadex G-100	6	26.96	368.62	161.73	4.12	21.91

Determination of the Molecular Weight: β -galactosidase molecular weight which has been purified from (*Ziziphus spina-christi*) has been found by two methods. The first method done with gel filtration chromatography throughout sephadex G100 in presence of five standard proteins as a size indicators and plotting the relationship between V_e/V_o (elution volume/ void volume) against log molecular weight. Results illustrated in figure (7) has shown that the enzyme's molecular weight has been 77.43 kD. In the second method, Electrophoresis with SDS-PAGE has been used to estimate the enzyme's molecular weight. Figure (8) shows the appearance of a single weak peak in the purified sample, as determined by relative mobility R_m (fig. 9), the single enzyme band in the sample give a molecular weight of 75 kD. The results from these two methods were close. The molecular weight determined with the gel filtration and SDS-PAGE confirms monomeric nature of the β -galactosidase from (*Ziziphus spina-christi*). This result was approximately similar to the result of [17], on chick pea with a molecular weight of 83 kD, and with study of [1]. on *Aspergillus niger* 76 kD, but it was lower or higher in other studies 42 kD [2], 52 kD [21], 165.98 kD [19]. The change in molecular weight due to the effect of several factors, such as the source of the extracted enzyme [22].

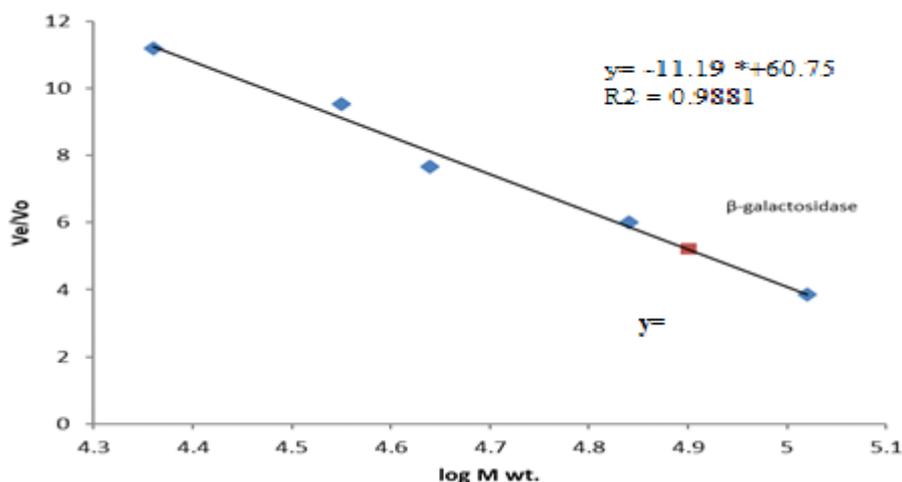


Figure 7. The determination of the β -galactosidase molecular weight by gel filtration using Sephadex G100 column purified from Lettuce.via.

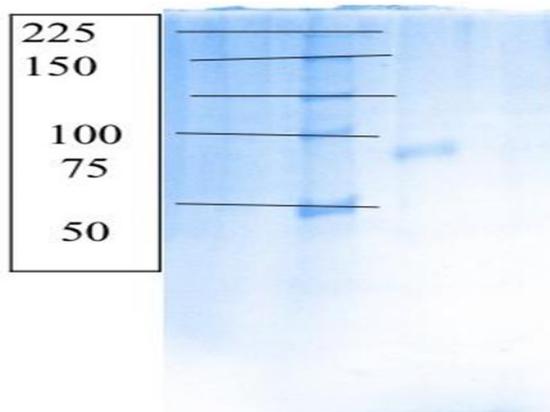


Figure 8. Determining the Molecular weight of the purified β -galactosidase enzyme on SDSPAGE analysis. a: marker of the molecular weight, b: purified β -galactosidase.

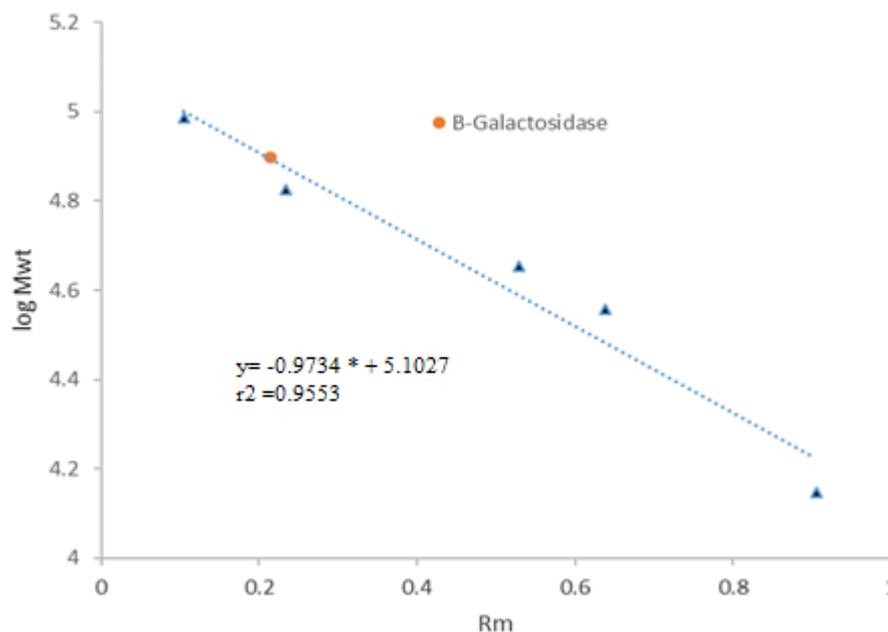


Figure 9. Determination of molecular weight for β -galactosidase from *Ziziphus spina-christi*) by SDS method.

4. Conclusion

The final purification step of gel filtration chromatography using Se-phadexG-100 column, the enzyme has been purified 4.12 fold with 21.91% of enzymatic yield. The optimum enzymatic activity was found at pH 6. The enzyme's molecular weight has been estimated to 77.53 KD by the gel filtration chromatography method, and 75 kD on SDS-PAGE. of the enzyme and used it in some applications.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

S. A. Hussien and K. S. Doosh; methodology, writing—original draft preparation, S. A. Hussien; writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement:

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No Data Availability Statement.

Conflicts of Interest:

The authors declare no conflict of interest.

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