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# Purification and characterization of trehalase from seeds of chickpea (*Cicer arietinum* L.)

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**Abstract:** In the present study, trehalase was purified and characterized from the seeds of *Cicer arietinum* L. 'Giza 1'. Crude extract was prepared and purified for electrophoretic homogeneity using ammonium sulfate, chromatography on DEAE-cellulose, CM Sepharose, and Sephadex G-200. The final specific activity was 7 U/mg protein, with 232-fold purification. The purified enzyme exhibited its pH optimum at 5.5. The optimum temperature was 60 °C. The determined $K_m$ value was 3.64 mM trehalose. The enzyme activity was stimulated by 20 mM $Mn^{2+}$, $Ni^{2+}$, or $Co^{2+}$, while it was inhibited by 20 mM $Na^+$, $K^+$, $Li^+$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, or $Fe^{3+}$. $Zn^{2+}$ proved to be a noncompetitive inhibitor, while mannitol and validamycin A proved to be competitive inhibitors. The inhibition constants ($K_i$) of $Zn^{2+}$, mannitol, and validamycin A were 7 mM, 9 mM, and 4 nM, respectively. The molecular mass of the native enzyme was 223 kDa by gel filtration. SDS-PAGE indicated that the enzyme consisted of 6 identical subunits with a molecular mass of 38 kDa.

**Key words:** *Cicer arietinum*, trehalase, trehalose, purification, molecular mass, validamycin A

## 1. Introduction

Trehalose is a nonreducing disaccharide, formed of 2 α-D-glucose molecules linked by an α,α-1,1-glycosidic linkage. It is widely distributed through the biological world. This sugar plays multiple physiological roles in stabilizing and protecting proteins and membranes against environmental stresses. It also serves as a source of energy and a source of carbon, and as a sensing and regulator compound (Elbein et al., 2003; Fernandez et al., 2010). Trehalase (EC 3.2.1.28) is a glycosyl hydrolase that hydrolyzes trehalose. It is detected in many prokaryotic and eukaryotic cells including bacteria (Carroll et al., 2007), fungi (Murata et al., 2001), and higher plants (Frison et al., 2007), as well as insects (Kamei et al., 2011) and mammals (Kamiya et al., 2004). It is the only known pathway of utilization of trehalose (Silva et al., 2004; Reguera et al., 2012). This enzyme plays an important role in trehalose metabolism, as it is either directly involved in the assimilation of exogenous trehalose, or it controls the level of this osmolyte in the cell. In many organisms, changes in trehalase activity are closely linked to alteration in physiological conditions or development, indicating that this enzyme plays an important role in such biological functions as homeostasis and developmental events.

Trehalase has been purified and characterized from various organisms such as *Saccharomyces cerevisiae* (Alizadeh and Klionsky, 1996), *Lentinula edodes* (Murata et al., 2001), *Acidobacterium capsulatum* (Inagaki et al., 2001), and *Medicago sativa* (Wolska-Mitaszko et al., 2005), as well as root nodules of *Phaseolus vulgaris* (García et al., 2005) and soybean (Müller et al., 1992; Aeschbacher et al., 1999). Recently, it has also been purified from the seeds of *Triticum aestivum* (Kord et al., 2012).

So far, the purification and characterization of the trehalase enzyme from seeds has not been properly studied. The present study deals with the extraction, purification, and characterization of trehalase from the seeds of *Cicer arietinum* 'Giza 1'.

## 2. Materials and methods

### 2.1. Plant material

In this study, purification of trehalase was carried out using seeds of chickpea (*Cicer arietinum* L.) cultivar Giza 1. The seeds were purchased from the Agricultural Research Center of Giza, Egypt.

### 2.2. Preparation of a crude extract

First, 300 g of chickpea seeds were surface-sterilized by immersion in 20% sodium hypochloride (v/v) for 20 min and were rinsed with sterile distilled water. The sterile seeds were homogenized in a prechilled mortar with 900 mL of extraction buffer composed of 100 mM cold sodium citrate buffer (pH 5.5), 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, 10 µM 2-mercaptoethanol, 10% glycerol (v/v), and insoluble polyvinylpyrrolidone (10

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mg/g fresh weight). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C.

### 2.3. Assay of trehalase

Trehalase activity was measured by estimating the glucose produced by hydrolysis of trehalose with a glucose oxidase-peroxidase kit (Spainreact) according to the method described by Bergmeyer and Bernt (1974). The reaction mixture contained 100 mM trehalose, 50 mM sodium citrate buffer (pH 5.5), and 0.25 mL of crude extract in a final volume of 1.5 mL. After incubation at 55 °C for 30 min, the reaction mixture was boiled for 3 min and then centrifuged at 5000 × *g* for 10 min. From the supernatant, 10 µL was taken and mixed with 1 mL from the glucose oxidase-peroxidase kit. The mixture was then incubated at 37 °C for 15 min. The absorbance of the sample was measured at 470 nm. Enzyme and substrate blanks were subtracted. The activity of trehalase enzyme was calculated according to the following equation:

$$\Delta A_{sample} \times \text{standard conc.} \times 0.0555 / A470_{standard} \times 2,$$

where ΔA = difference between optical density of sample before and after addition of substrate. Concentration of standard = 100 mg d/L. Conversion factor = 0.0555 µmol/mL. Dividing by 2 = hydrolysis of 1 mole of trehalose produces 2 moles of glucose. One unit (nkat) of trehalase activity is defined as the amount of enzyme that hydrolyzes 1 nmol trehalose per second at pH 5.5.

### 2.4. Determination of soluble protein

Soluble protein was routinely determined spectrophotometrically at 280 nm as described by Warburg and Christian (1942). In the presence of interfering compounds, soluble protein was determined according to Lowry et al. (1951).

### 2.5. Purification of trehalase

Step 1: Solid ammonium sulfate was added to the crude extract of chickpea seeds to reach the final saturation of 80%. The precipitated proteins were collected by centrifugation at 10,000 × *g* for 15 min at 4 °C. The precipitant was dissolved in the least possible amount of 100 mM sodium citrate buffer (pH 5.5), 1 mM EDTA, 10 µM 2-mercaptoethanol, and 10% glycerol (v/v). After dialysis and centrifugation, trehalase activity and protein content were determined.

Step 2: A DEAE cellulose column (35 × 1.5 cm) was equilibrated with 100 mM sodium citrate buffer (pH 5.5). The dialyzed fraction from ammonium sulfate was applied to the column. The proteins were eluted with a discontinuous gradient of 0.0, 0.05, 0.1, 0.2, 0.3, and 0.5 M KCl. Fractions of 3 mL were collected at a flow rate 0.5 mL/min. In each fraction, trehalase activity as well as the protein content were determined.

Step 3: The enzymatically active fractions of Step 2 were pooled and applied to a CM Sepharose Cl-6B column (20 × 2 cm). Preparation, equilibration, and elution of proteins were again performed as in Step 2. Fractions of 3 mL were collected at a flow rate of 1 mL/min.

Step 4: The enzymatically active fractions of Step 3 were pooled, lyophilized, and applied to a Sephadex G-200 (30×1.5 cm) column. The proteins were eluted with the same buffer used in column equilibration containing 0.2 M KCl. Three milliliters was collected at a flow rate of 1.5 mL/min. Trehalase activity and protein content were determined for each fraction.

All purification steps were carried out at 4 °C.

### 2.6. Characterization of trehalase

#### 2.6.1. Effect of pH

The effect of pH was assayed using 100 mM sodium citrate buffer for pH 3.5–5.5, 100 mM sodium phosphate buffer for pH 6.0–7.5, and Tris/HCl buffer for pH 8.0–10.

#### 2.6.2. Optimum temperature

The assay of optimum temperature was carried out using 100 mM sodium citrate buffer at pH 5.5 and at temperatures from 20 to 70 °C. For consecutive tests, the temperature was increased by 5 °C.

#### 2.6.3. Thermal stability

Test samples were preincubated for 30 min at temperature from 40 to 70 °C. Next, trehalose was added and the routine assay was followed as previously mentioned.

#### 2.6.4. Influence of chemical compounds

The effect of 5, 10, and 20 mM solutions of chloride salts of $Na^+$, $K^+$, $Mn^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Fe^{3+}$, as well as sulfate of $Li^+$, $Zn^{2+}$, and $Cu^{2+}$, were studied. The effects of 10 and 20 mM solutions of mannitol, succinate, borate, malate; 2 and 5 nM validamycin A; and 2 and 10 mM EDTA and ATP were also studied.

#### 2.6.5. Substrate specificity

The actions of the enzyme on 100 mM solutions of the following sugars were studied: trehalose, sucrose, cellobiose, lactose, and maltose. The results are expressed as relative percentage of the activity referred to trehalose reaction.

#### 2.6.6. $K_m$ determination

The dependence between the reaction velocity and the substrate concentration Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) was determined for trehalose, applying the Lineweaver–Burk equation. The reaction mixture contained the following in 1.5 mL: 50 mM sodium citrate buffer (pH 5.5), 1 nkat of purified enzyme, and different concentrations of trehalose ranging from 2 to 10 mM at 55 °C.

#### 2.6.7. Determination of the inhibition constant

The inhibition constants ($K_i$) for $ZnSO_4$ (5, 10, 20 mM), mannitol (5, 10, 20 mM), and validamycin A (2, 5, 7 nM) were determined. The reaction mixture contained the following in 1.5 mL: 50 mM sodium citrate buffer (pH 5.5) and 1.163 U of purified enzyme.

### 2.7. Determination of molecular mass

The molecular mass of the purified enzyme was estimated by gel fractionation on the Sephadex G-200 (30 × 1.5 cm), which was equilibrated with 100 mM sodium citrate buffer (pH 5.5). The following proteins were run separately through the same conditions: cytochrome c (13 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), catalase (250 kDa) (Serva Co.). The void volume ($V_o$) was determined by dextran blue (2000 kDa).

The molecular mass of the denatured form of purified trehalase was estimated with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Walker (1994). A 10% gel (w/v) was prepared; the run was performed at 100 V. The following proteins were used as molecular mass standards: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), REase Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa) (Fermentas).

All data of trehalase characterization are the means of 3 replicates. Standard errors were calculated and are represented in the figures.

## 3. Results and discussion

### 3.1. Purification of trehalase

Although trehalase has been purified from different bacteria, yeast and other fungi, mammals, and insects, few reports describe the purification of this enzyme from seeds. In this study we purified trehalase from seeds of chickpea. The purification protocol is given in Table 1. The specific activity of the crude extract of trehalase was 0.03 nkat/mg protein.

In Step 1, the specific activity increased to 0.12 nkat/mg protein, which represented 4-fold purification with 88% recovery after ammonium sulfate precipitation. In this context, the specific activity of crude extract of trehalase isolated from different plants expressed in nkat/mg protein was as follows: 0.1 for *T. aestivum* seeds (Kord et al., 2012), 0.4 for *P. vulgaris* nodules (García et al., 2005), and 0.8 for soybean nodules (Müller et al., 1992).

In Step 2, the protein fractions exhibiting trehalase activity were eluted from the DEAE-cellulose column as a single peak with 0.05 M KCl (Figure 1a). This indicates that the protein of trehalase exhibits a low density of negative charges, as the column contained positively charged beads.

In Step 3, the protein fractions that exhibited trehalase activity were eluted with 0.05 M KCl from the CM Sepharose column (Figure 1b). The fold purification was 173-fold with 37% recovery.

In Step 4, the specific activity of chickpea seed trehalase was 7 nkat/mg protein, which represented 232-fold purification (Figure 1c; Table 1). Generally, loss in activity and hence in specific activity was mostly due to the exclusion of some fractions that showed low specific activity, particularly after gel filtration chromatography. The final specific activity depends on several factors including the source of the enzyme, the type of tissue, and the state of purification and assay conditions. In the literature, the final specific activity of purified trehalase from *T. aestivum* seeds was 7 nkat/mg protein (Kord et al., 2012), while that of *P. vulgaris* nodules was 175.7 nkat/mg protein (García et al., 2005). *M. sativa* stem trehalase had a specific activity of 50 μg glucose/mg protein (Wolska-Mitaszko et al., 2005).

### 3.2. Characterization of trehalase

In the present study, trehalase from chickpea seeds had an optimum pH from 5 to 5.5. Lower pH values showed a slight decrease in the enzyme activity, but higher pH values showed sharp decrease in the enzyme activity; the highest stability of chickpea seed trehalase was at pH 5.5 (Figure 2). In this context, trehalase of *T. aestivum* seeds shows a broad optimum pH of 5.0–6.5 (Kord et al., 2012); that of soybean nodules has an optimum pH between 3.5 and 5.5 (Schubert and Wyss, 1995). Microbial trehalases have a wide range of pH optima: 2.5 for *A. capsulatum* (Inagaki et al., 2001) and 6.5–7.0 for *Mycobacterium smegmatis* (Carroll et al., 2007). Some fungal trehalases have a pH ranging from 4.0 to 7.0 (Murata et al., 2001; Reguera et al., 2012).

Optimum temperature of purified protein was 60 °C, and the lowest activities were found at 20 °C and 70 (Figure

**Table 1.** Purification scheme of chickpea seed trehalase. Data in this table are the means of 3 complete purification processes

| Purification steps | Total activity (nkat) | Total protein (mg) | Specific activity (nkat/ mg protein) | Recovery (%) | Fold |
|---|---|---|---|---|---|
| Crude extract | 559 | 18808 | 0.03 | 100 | - |
| $(NH_4)_2SO_4$ | 492 | 4062 | 0.12 | 88 | 4 |
| DEAE-cellulose | 311 | 120 | 3 | 56 | 87 |
| CM Sepharose | 206 | 39 | 5 | 37 | 173 |
| Sephadex G-200 | 100 | 14 | 7 | 18 | 232 |

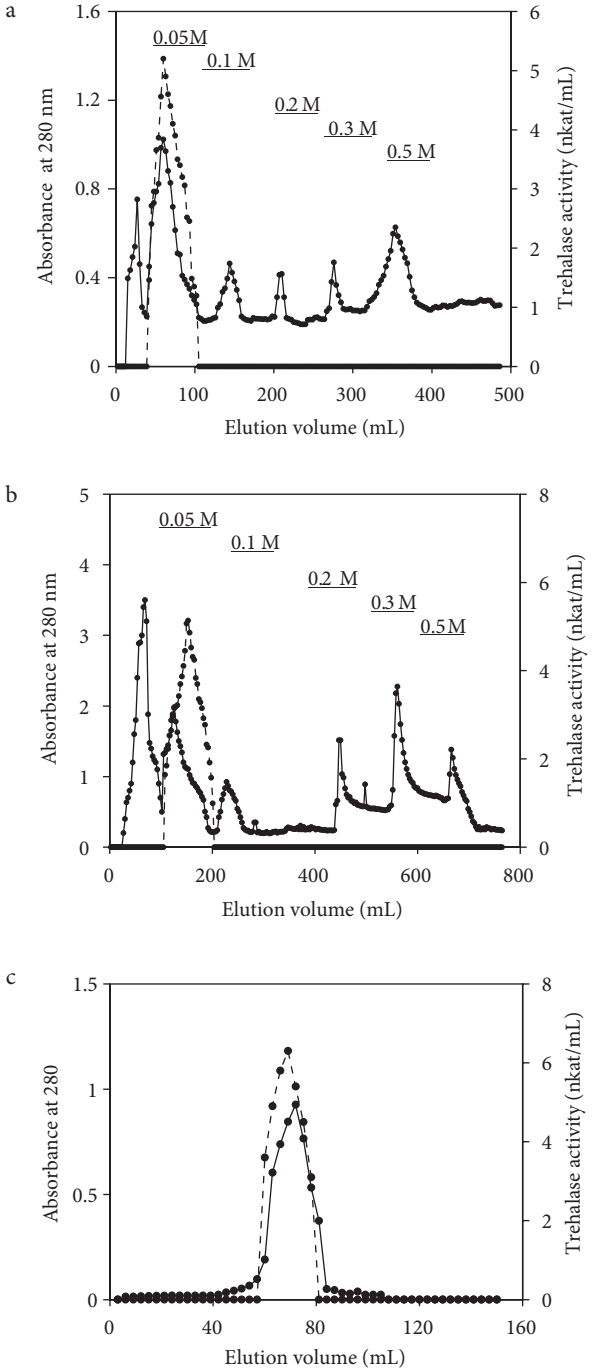


**Figure 1.** a. A typical elution profile for the behavior of trehalase of chickpea seeds on DEAE-cellulose column. The proteins were eluted with a discontinuous gradient of KCl as indicated. Solid line indicates the absorbance, dotted indicates activity. b. A typical elution profile for the behavior of trehalase of chickpea seeds on CM Sepharose column. The proteins were eluted with a discontinuous gradient of KCl as indicated. Solid line indicates the absorbance, dotted indicates activity. c. A typical elution profile for the behavior of trehalase of chickpea seeds on Sephadex G-200 column. Solid line indicates the absorbance, dotted indicates activity.

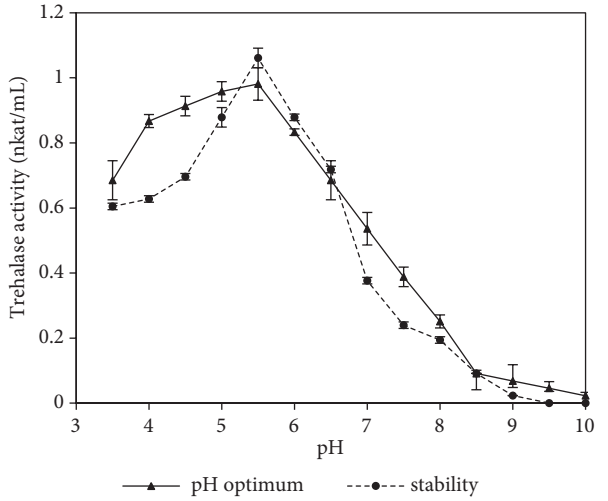


**Figure 2.** pH optimum and pH stability of chickpea seed trehalase. Standard assay conditions were used, except for buffer, which was varied as indicated. Bars indicate ±SE.

3). This corresponds with the information reported for trehalase isolated from *T. aestivum* seeds (Kord et al., 2012), *M. sativa* stem (Wolska-Mitaszko et al., 2005), and soybean nodules (Müller et al., 1992). Low optimum temperature was recorded for mealworm beetle trehalase (20–25 °C) (Yaginuma et al., 1996). A high optimum (88 °C) was reported for trehalase isolated from *Rhodothermus marinus* (Jorge et al., 2007). In this investigation, the highest thermostability of chickpea seed trehalase was reported between 45 and 60 °C (Figure 3). The same behavior was reported for trehalase isolated from *P. vulgaris* nodules (García et al., 2005) and soybean nodules (Müller et al., 1992).

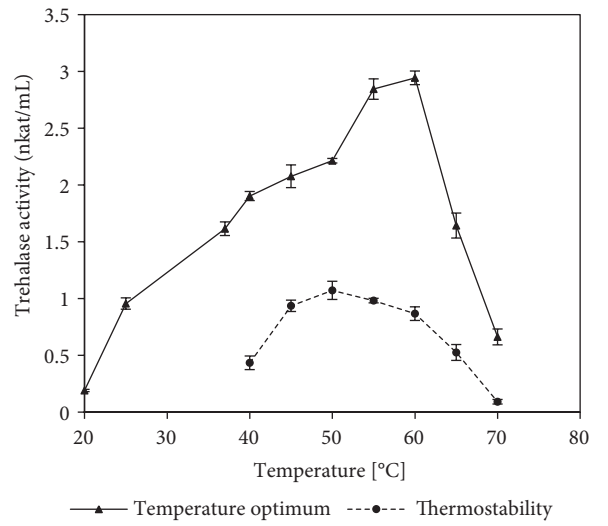


**Figure 3.** Temperature optimum and thermostability of chickpea seed trehalase. Standard assay conditions were used, except for temperature, which was varied as indicated. Bars indicate ±SE.

In this study, the relation between trehalose concentration [S] and the velocity [v] expressed as hydrolyzed trehalose (nkat/mL) showed a hyperbolic curve following Michaelis–Menten kinetics. The Lineweaver–Burk plot showing the linear relation between 1/[S] and 1/[v] is shown in Figure 4. The $K_m$ value for chickpea seed trehalase was 3.64 mM. This value was higher than that reported for trehalase isolated from other plants: 0.28 mM for soybean nodules (Müller et al., 1992) and 0.109 mM for *P. vulgaris* nodules (García et al., 2005). However, it is quite comparable to the values reported for trehalase isolated from *T. aestivum* seeds (2 mM) (Kord et al., 2012), *L. edodes* (2.14 mM) (Murata et al., 2001), and *Ascaris suum* (6.6 mM) (Dmitryjuk and Zółtowska, 2003).

The activity of chickpea seed trehalase as affected by the addition of different ions is represented in Table 2. It was observed that $Na^+$, $K^+$, $Ca^{2+}$, $Li^+$, $Zn^{2+}$, $Cu^{2+}$, and $Fe^{3+}$ inhibited enzyme activity, while $Mn^{2+}$, $Mg^{2+}$, $Ni^{2+}$, and $Co^{2+}$ increased enzyme activity.

In the literature, *T. aestivum* seeds' trehalase was inhibited with $Na^+$ and $K^+$ at 5, 10, and 20 mM concentrations (Kord et al., 2012). However, 10 mM concentrations of $K^+$ and $Na^+$ were activators for trehalase isolated from *P. vulgaris* (García et al., 2005). Many reports stated that $Ca^{2+}$ has a stimulatory effect on trehalase isolated from different sources (Dmitryjuk and Zółtowska, 2003; Wolska-Mitaszko et al., 2005). While 10 mM $Li^+$ showed no effect on *T. aestivum* seed trehalase (Kord et al., 2012), it was a stimulator for *P. vulgaris* nodule trehalase (García et al., 2005). $Zn^{2+}$, $Cu^{2+}$, and $Fe^{3+}$ had an inhibitory effect on *T. aestivum* seed trehalase (Kord et al., 2012). *T. aestivum* seed trehalase was stimulated by 5 mM $Ni^{2+}$; however, by increasing the concentration, this behavior was reversed. Inagaki et al. (2001) reported that 1 mM $Ni^{2+}$ inhibited trehalase from *A. capsulatum*, while 5 mM $Co^{2+}$ acted as a stimulator for *T. aestivum* seed trehalase (Kord et al., 2012)

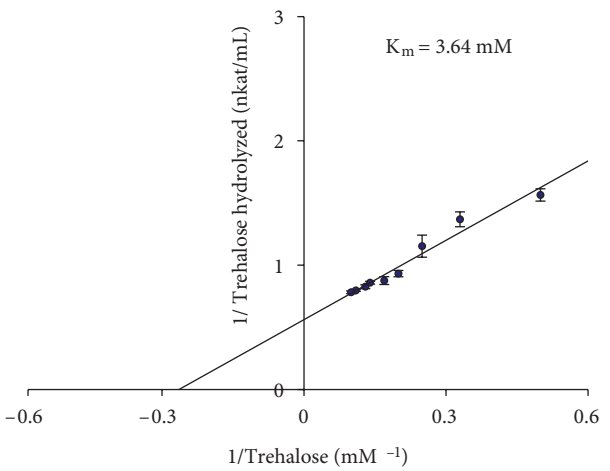


**Figure 4.** Lineweaver–Burk plot relating reaction velocity of trehalase of chickpea seeds to trehalose concentration. Bars indicate ±SE.

**Table 2.** Effect of different ions on chickpea seed trehalase activity. The values are the means of 3 samples.

| Ions | Relative percentage of activity at the indicated concentrations of ions | | |
|---|---|---|---|
| | 5 mM | 10 mM | 20 mM |
| None | 100 | 100 | 100 |
| $Na^+$ | 80 | 67 | 61 |
| $K^+$ | 84 | 74 | 69 |
| $Li^+$ | 88 | 47 | 12 |
| $Mn^{2+}$ | 99 | 108 | 210 |
| $Mg^{2+}$ | 133 | 120 | 98 |
| $Ca^{2+}$ | 95 | 94 | 67 |
| $Zn^{2+}$ | 76 | 39 | 22 |
| $Cu^{2+}$ | 67 | 20 | 14 |
| $Ni^{2+}$ | 114 | 114 | 122 |
| $Co^{2+}$ | 95 | 112 | 122 |
| $Fe^{3+}$ | 46 | 39 | 31 |

and 10 mM $Mg^{2+}$ increased the activity of trehalase from soybean (Müller et al., 1992) and *A. suum* (Dmitryjuk and Zółtowska, 2003).

Studying the inhibition constant ($K_i$) for $Zn^{2+}$ on the activity of chickpea seed trehalase revealed that $K_i$ was equal to 7 mM (Figure 5), which is lower than that recorded for trehalase purified from *T. aestivum* seeds (11 mM; Kord et al., 2012). Therefore, chickpea seed trehalase is more sensitive to $Zn^{2+}$ than *T. aestivum* seed trehalase.

The relative activities of chickpea seed trehalase in the presence of 20 mM mannitol, succinate, borate, and malate were 8%, 41%, 16%, and 37%, respectively (Table 3). The inhibition of trehalases by mannitol suggests that this sugar alcohol may be involved in the metabolism of trehalose in chickpea seeds. The same findings were reported for trehalase isolated from *Frankia* (Lopez and Torrey, 1985) and soybean nodules (Müller et al., 1992). In this study, chickpea seed trehalase was strongly inhibited by the addition of validamycin; 2 nM validamycin inhibited trehalase by 27% (Table 3). This result is consistent with that cited in many reports that dealt with the effect of validamycin on trehalases (Temesvari and Cotter, 1997; López et al., 2009). In the present work, an inhibition study of mannitol and validamycin revealed that both acted as competitive inhibitors with $K_i$ values of 9 mM and 4 nM, respectively. We represented only the inhibition study in the case of validamycin (Figure 6).

In this study, 10 mM EDTA and ATP showed strong inhibition of the enzyme activity, amounting to 84% and 90%, respectively (Table 3). Inhibition due to the addition of EDTA suggests that there are some metal requirements

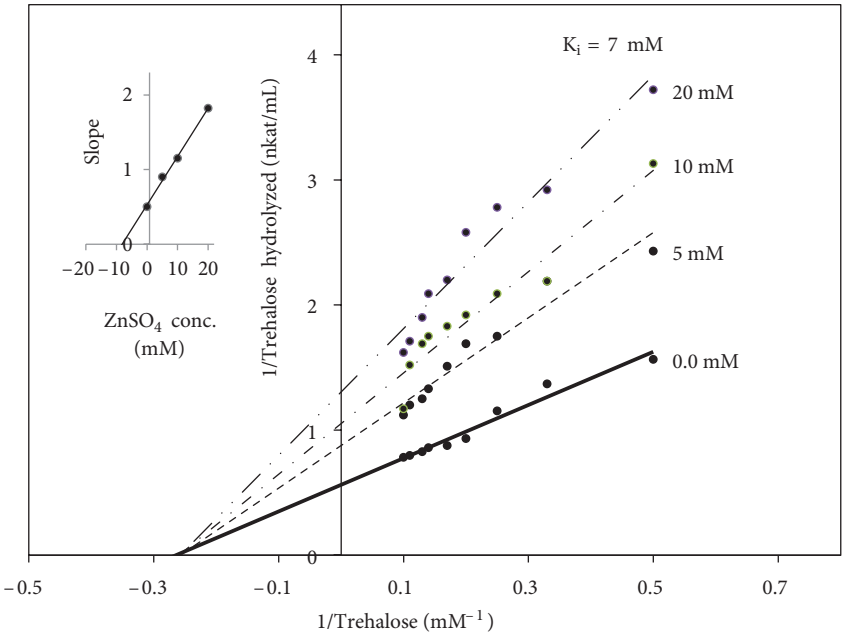


**Figure 5.** Inhibition study of chickpea seed trehalase with $ZnSO_4$. Plots of reciprocal of initial velocities versus reciprocal concentration of trehalose (mM). The inhibition constant ($K_i$) of $ZnSO_4$ was estimated from the replot of the slope.

for trehalase. It has been established that metal ions are subjected to noncompetitive inhibition by any agent that binds reversibly to the required ion. Among the noncompetitive inhibitors that function in this way is EDTA (a metal cation chelator), which binds to ions in the active sites of the enzyme, especially magnesium.

Effect of different substrate analogues (sucrose, cellobiose, lactose, maltose) on the activity of trehalase of chickpea has been studied. The results were expressed as relative percentage of the activity respective to trehalose reaction. The relative activities were 19%, 0.7%, 5%, and 0.2% for sucrose, cellobiose, lactose, and maltose, respectively. It was reported that *T. aestivum* seed trehalase showed a very high specificity for trehalose. Negligible activity was observed in the cases of sucrose and lactose; however, there was no effect on the other substrates (Kord et al., 2012).

**Table 3.** Effect of some additives on chickpea seed trehalase activity. The values are the means of 3 samples.

| Additives | Relative activity | |
|---|---|---|
| None | (%) | 100 |
| | (10 mM) | (20 mM) |
| Mannitol | 31 | 8 |
| Succinate | 51 | 41 |
| Borate | 16 | 16 |
| Malate | 45 | 37 |
| Validamycin A | (2 nM)<br>73 | (5 nM)<br>49 |
| | (2 mM) | (10 mM) |
| EDTA | 25 | 16 |
| ATP | 25 | 10 |

In the present study, the molecular mass for chickpea seed trehalase was 223 ± 4.2 kDa, as determined using a calibrated gel filtration column (Figure 7). This value was comparable to that reported for trehalase purified from *T. aestivium* seed (189 kDa) (Kord et al., 2012), *Pichia pastoris* (170 kDa) (Liu et al., 2007), and *L. edodes* (158 kDa) (Murata et al., 2001).

In this study, when the purified trehalase was electrophoresed on 10% SDS-polyacrylamide gel, 1 protein band with a molecular mass of 38 ± 2 kDa was observed (Figure 8). Since the native molecular mass determined by gel filtration was 223 ± 4.2 kDa, this indicated that chickpea seed trehalase was a hexamer of 6 identical subunits. The available data in the literature emphasize that trehalase is a monomer with a molecular mass ranging from 45–75 kDa (Inagaki et al., 2001; Jorge et al., 2007). However, some literature reported that it is a dimer with a molecular mass of 38 kDa for *A. suum* (Dmitryjuk and Zółtowska, 2003), or a trimer with a molecular mass of 63 kDa for *T. aestivium* (Kord et al., 2012). Carroll et al. (2007) reported that trehalase isolated from *M. smegmatis* consisted of a multimer of about 20 or more subunits, each with a molecular mass of 71 kDa.

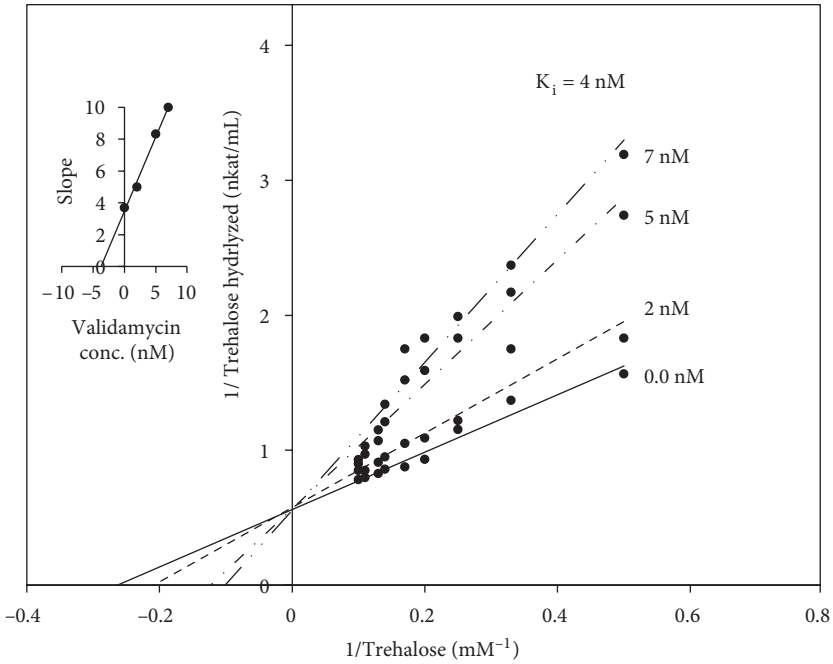


**Figure 6.** Inhibition study of chickpea seed trehalase with validamycin A. Plots of reciprocal of initial velocities versus reciprocal concentration of trehalose (mM). The inhibition constant ($K_i$) of validamycin A was estimated from the replot of the slope.

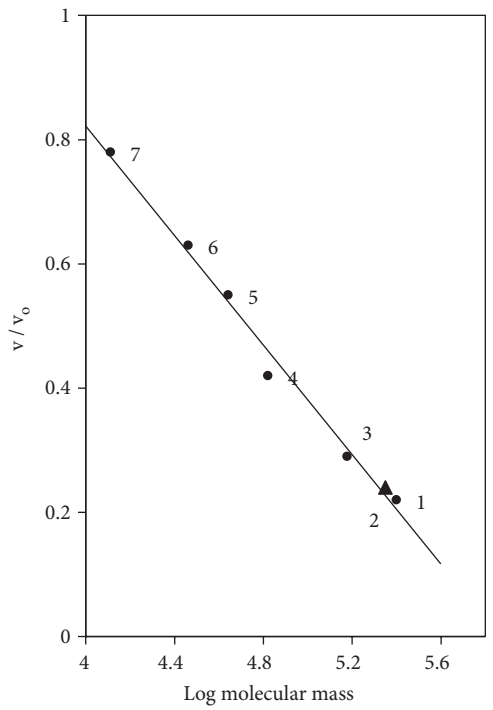


**Figure 7.** Molecular mass estimation of chickpea seed trehalase by gel filtration on Sephadex G-200 (30 × 1.5 cm). 1) Cytochrome C (13 kDa), 2) chickpea seed trehalase, 3) carbonic anhydrase (29 kDa), 4) ovalbumin (44 kDa), 5) serum albumin (66 kDa), 6) alcohol dehydrogenase (150 kDa), 7) catalase (250 kDa). The void volume ($V_o$) was determined with dextran blue (2000 kDa).

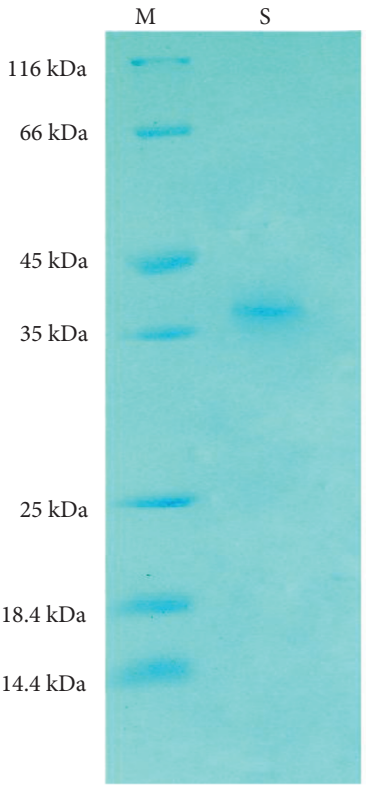


**Figure 8.** SDS-polyacrylamide gel electrophoresis of trehalase. Lane M contained 5-µL protein standards. Lane S contained 2 mg protein of purified chickpea seed trehalase. The 10% (w/v) SDS-PAGE was used. Protein bands for this gel were visualized with Coomassie blue.

## 4. Conclusion

Trehalase enzyme of chickpea seeds was purified until homogeneity with final specific activity of 7 U/mg protein and a molecular mass of 223 kDa. It consists of 6 identical subunits of 38 kDa each. The purified enzyme is relatively heat-stable with optimum pH 5.5 and optimum temperature 60 °C. It has a $K_m$ value of 3.64 mM trehalose. Validamycin A proved to be a competitive inhibitor, with $K_i$ = 4 nM. EDTA shows strong inhibition, suggesting that it is a metalloenzyme.

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