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# Improvement of Characteristics of Maltase from Marrow (*Cucurbita pepo* L.) Cotyledons by its Immobilization on Chitin

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## Abstract

Maltase is one of the key enzymes in our digestive processes, as it is the main salivary enzyme in the digestion of carbohydrates in the mouth. The present investigation aimed to evaluate the impact of some additive materials on maltase activity, and the role of immobilization of the enzyme on chitin in improving its characters. After different purification steps, the recorded specific activity of maltase from *Cucurbita pepo* L. was 385 units $mg^{-1}$ protein and the fold was 4.4. Addition of EDTA, 0.5% (w/v) bovine serum albumin (BSA), 10 mM nicotinic acid, 20 to 30 mM PEG, glycine, proline, and alanine stimulated the maltase activity. On the other hand, 0.5 % (V/V) of Triton–X100, Tween-80, 10 mM diphenylamine, and 5% (v/v) 2-mercaptoethanol inhibited maltase activity. Also, maltase activity was inhibited by succinic anhydride > sodium fluoride >phethalic anhydride in the order as arranged. After 5 days of storage at 25 ℃, the enzyme retained 43.5% of its activity. After immobilization on chitin, the enzyme retained 162.4 U from 240 U of its activity after 6 cycles of reusability. Also, the pH and temperature of optimum activity were shifted from 6.0 and 50 ℃ in case of free maltase to 9.0 and 60 $^{O}$C in case of immobilized maltase respectively. Also, $K^{+}$, $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$, and $Zn^{2+}$ activated immobilized maltase with a higher rate compared with the free maltase. On the other hand, $Hg^{2+}$ and $Cu^{2+}$ inhibited the immobilized maltase activity at a lower rate than free maltase. Moreover, immobilized maltase expressed higher response to activation with sorbitol and mannitol than free maltase.

**Key words:** Maltase, Chitin, Cucurbita pepo L., enzyme Immobilization

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## Introduction

The communications between the cotyledons and the embryos improve the enzyme activities in plant cotyledons to regulate the hydrolysis of storage materials based on their requirements (He *et al.*, 2015). Maltase is one of the hydrolase enzymes. Hydrolases are the third group in enzyme classification. They cause the addition of water to a variety of bonds and generally lead to the cleavage of the substrate molecule (Trincone, 2015).

The α-glucosidase enzymes convert carbohydrates into glucose (Yuniarto *et al.*, 2018). The α-Glucosidase forms a group of exo-acting glycoside hydrolases (Sarian *et al.*, 2017). A number of α-glucosidases from fungi and other sources also catalyze reactions that produce α-glucosylated compounds (Kobayash, *et al.*, 2003).

Plant α-glucosidases hydrolyze soluble starch efficiently and degrade the insoluble polysaccharides in plant seed such as starch granules (Yamasaki *et al.*, 2005). In addition to plant α-glucosidases, plant α-amylases degrade starch granules (Zhang *et al.*, 2013). α-amylase catalyzes the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen, and different oligosaccharides, and simplifies the availability of sugars for the intestinal absorption (Karthikeyan *et al.*, 2018). In contrast to the above information, four different forms of α-glucosidases, which are thought to be formed by post-translational modifications were obtained from spinach (Sugimoto *et al.*, 1997). The existence of various forms of plant α-glucosidases may be because of the presence of numerous gene loci. Most of the α-glucosidases of plant origin have smaller molecular sizes (such as barley 81 KDa) than those calculated from their deduced amino acid sequences (97 KDa) (Nakai *et al.*,

2007). Plant α-glucosidase has been purified from the homogenate of rice (Ito *et al.*, 1989), sugar beet (Matsui *et al.*, 1981), and barley (Frandsen *et al.*, 2000).

Rice α-glucosidase has been purified from dry seeds of several varieties including *Oryza sativa* L. var. Koshihikari (Awazuhara *et al.*, 2000) and *O. sativa* var. Onnemochi (Ito *et al.*, 1989), the ripening seeds of *O. sativa* var. Yashiromochi (Yamasaki and Suzuki, 1997), and the germinating seeds of *O. sativa* var. Indica (Eksittikul *et al.*, 1993). All of these seeds contained many types of α-glucosidases. These forms are different in their molecular weight, isoelectric point, and kinetic parameters. Barley seed α-glucosidase can initiate the attack of raw starch granules, independently of the presence of α-amylase (Sun and Henson, 1990).

Many important enzymes are present in the digestive tract. Maltase is one of these enzymes that, is a key enzyme in the digestion of carbohydrates in the mouth by salivary α-amylase and in the gut by pancreatic α-amylase (Wolfson *et al.*, 2006). This digestion process results in simpler sugers which will then be absorbed into the body (Aligita *et al.*, 2018). In this way, maltase helps the whole digestive system function smoothly (Wolfson *et al.*, 2006). Therefore, the aim of the present investigation was to investigate some of the kinetic properties of maltase, the effect of some additive materials on its activity and the role of its immobilization on chitin in improving its characters.

## Materials and Methods

*Plant materials*

The experimental plant utilized in the present investigation was *Cucurbita pepo* L. (marrow, family Cucurbitaceae). Pure strains of seeds were obtained from the Egyptian Ministry of Agriculture.

*Sterilization of seeds*

Seeds were surface-sterilized based on El-Shora and Ap Rees (1991).

*Seed germination and growth conditions*

Seeds were germinated based on El-Shora (2002). The cotyledons of 5-days old plants were excised with a razor blade and stored on ice to be utilized for enzyme extraction immediately.

*Enzyme extraction*

The enzyme was extracted from the tissues as described by Sharma *et al.* (2004). After extraction, the extract was used for purification of maltase and its biochemical assay.

*Purification of the enzyme*

*Fractionation by ammonium sulfate:*

The supernatant was precipitated by 80 % ammonium sulfate, and the precipitated protein was collected by centrifuging at 5,000 rpm for 20 min and 4 ℃.

*Dialysis*

The precipitated pellets were dialyzed against 0.001 M acetate buffer pH 6.0 to remove ammonium sulfate using a dialysis membrane and treated with polyethylene glycol to reduce the volume. Then, the catalytic activity of maltase was determined.

*Gel filtration:*

The partially purified enzyme from ammonium sulfate was added to the Sephadex G-50 column and eluted by 0.1 M acetate buffer (pH 6.0). The rate of elution was 2 ml/6 min.

*Enzyme assay*

The Enzyme assay was carried out according to the methods of Yamasaki *et al.* (2007).

*Estimation of total proteins*

The total protein in the extract was determined by the method of Lowry *et al.* (1951).

*Immobilization of maltase by chitin*

One gram of chitin was mixed with 5 ml of 2.5% (v/v) glutaraldehyde for 2 h at 30 ℃. The carriers were collected by filtration and washed with distilled water to eliminate the excess glutaraldehyde. Each of the wet carriers was shaken with 2.5 ml of the enzyme solution for 2 h at 30 ℃ and the unbound enzyme was eliminate by washing with distilled water (Abdel-Naby *et al.*, 1998).

## Results

In this study, three plants were tested for maltase activities, *Cicer arietinum* 78.6 **U**, *Pisum sativum* 92.9 **U,** and *Cucurbita pepo* 141.4 **U**. *Cucurbita pepo* cotyledons showed the highest level of maltase. Therefore, *C. pepo* was chosen as a source of maltase in the present study.

*Purification of maltase from marrow*

The obtained results in **Table 1** showed that the highest specific activity was 385 units $mg^{-1}$ protein and the fold of purification was 4.4.

**Table 1:** Steps of partial purification of maltase from marrows cotyledons.

| Step | Total volume (ml) | Total activity (U) | Total Protein (mg) | Specific activity (U/mg protein) | Fold of purification |
|---|---|---|---|---|---|
| **Crude preparation** | 250 | 35350 | 400 | 88.4 | 1 |
| **The supernatant of crude extract** | 202.5 | 29646 | 291.6 | 101.7 | 1.15 |
| **After precipitation with ammonium sulfate (80%)** | 25 | 6690 | 50 | 133.8 | 1.5 |
| **After dialysis** | 31 | 7998 | 46.5 | 151.8 | 1.7 |
| **Polyethylene glycol** | 5 | 1500 | 8.5 | 176.5 | 2 |
| **Sephadex G-50** | 2 | 250.25 | 0.65 | 385 | 4.4 |

*Effect of different buffers on maltase activity*

The results in **Fig. 1** indicated that the acetate buffer was the best buffer followed by phosphate and phospho-citrate buffers for enzyme activity.

*Effect of chelating agents on maltase activity*

The impact of several chelating agents on purified maltase activity was studied. The obtained results in **Fig. 2** revealed that 5 mM EDTA as a chelating agent stimulated the maltase activity, whereas *o*-phenanthroline did not have any effect on the enzyme activity.

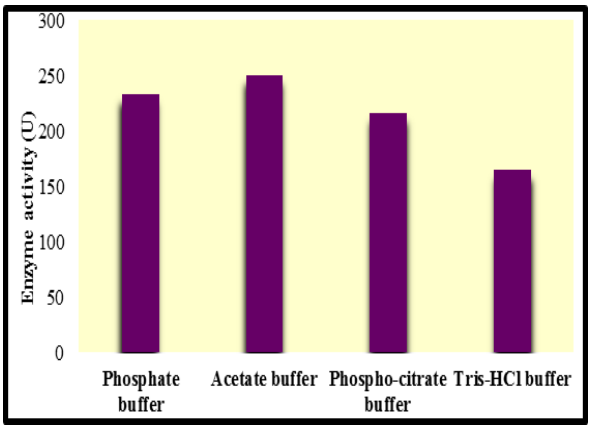


**Figure 1:** Effect of different buffers on maltase activity.

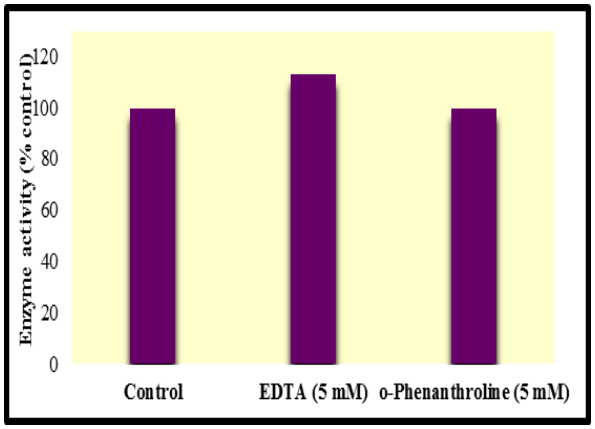


**Figure 2:** Effect of some chelating agents on maltase activity.

*Effect of some surfactants on maltase activity*

The impact of several surfactants on purified maltase activity was tested. The findings in **Table 2** revealed that 0.5 % (V/V) of Triton–X100 and Tween-80 decreased maltase activity.

**Table 2:** Effect of some surfactants on maltase activity.

| Substance | Enzyme activity (U) | Enzyme activity (% control) |
|---|---|---|
| Control | 249 | 100 |
| Triton X-100 (0.5%) | 238.1 | 95.6 |
| Tween 80 (0.5%) | 205.7 | 82.6 |

*Effect of some substances on maltase activity*

The impact of several substances on purified maltase activity was examined. The results in **Table 3** revealed that 0.5% (w/v) bovine serum albumin (BSA) and 10 mM nicotinic acid increased maltase activity when included in the reaction mixture.

**Table 3:** Effect of some substances on maltase activity.

| Substance | Enzyme activity (U) | Enzyme activity % (% control) |
|---|---|---|
| Control | 249 | 100 |
| BSA (0.5%) | 305.7 | 122.8 |
| Nicotinic acid (10 mM) | 277.2 | 111.3 |
| Sorbitol (1 M) | 273.2 | 110.6 |
| Glycerol (10% v/v) | 262 | 106.1 |
| Sucrose (1 mM) | 249 | 100 |
| Blue dextran (1mM) | 269.5 | 109.1 |

*Effect of polyethylene glycol (PEG-6000) on maltase activity*

The recorded results in **Fig. 3** revealed that the enzyme activity improved by increasing the concentration of PEG from 20 to 30 mM.

*Effect of some inhibitors on maltase activity*

The results in **Fig. 4** revealed that the three tested compounds inhibited maltase activity in the order arranged as succinic anhydride > sodium fluoride >phthalic anhydride.

*Effect of 2-mercaptoethanol and diphenylamine on maltase activity*

The results in **Table 4** showed that 5% (v/v) 2-mercaptoethanol and diphenylamine (10 mM) inhibited maltase activity, but diphenylamine was a stronger inhibitor than 2-mercaptoethanol.

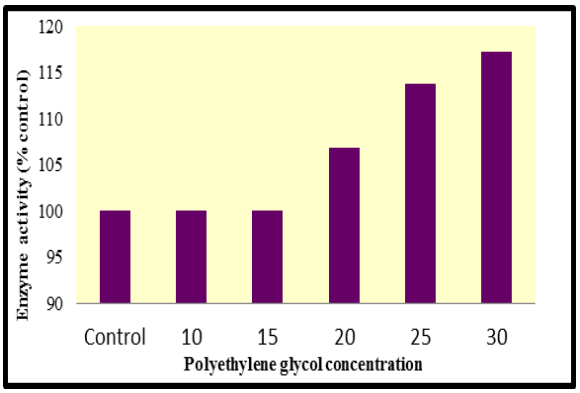


**Figure 3:** Effect of polyethylene glycol (6000) concentration on maltase activity.

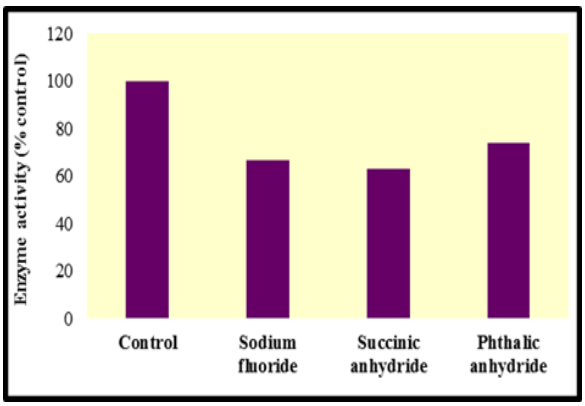


**Figure 4:** Effect of some inhibitors on maltase activity.

**Table 4:** Effect of 2-mercaptoethanol and diphenylamine on maltase activity.

| Inhibitor | Enzyme activity (U) | Enzyme activity (% control) |
|---|---|---|
| Control | 248 | 100 |
| 2-Mercaptoethanol (5%) | 33.8 | 13.6 |
| Diphenylamine (10 mM) | 214.2 | 86.4 |

*Storage stability of partially purified maltase at 25 ºC*

The results in **Fig. 5** revealed that the enzyme activity was declined gradually throughout the experimental period. After the 5$^{th}$ day of storage, the enzyme activity was 107.4 U, which represented 43.5% of the initial activity at zero time.

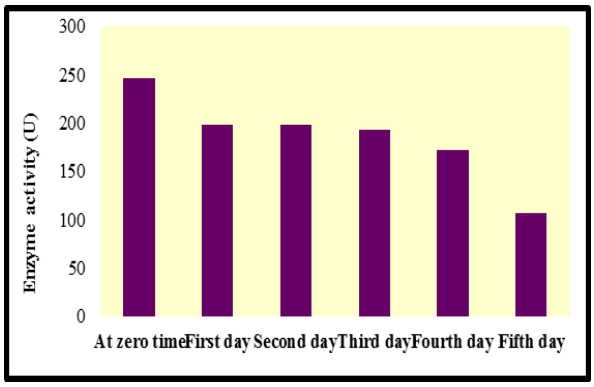


**Figure 5:** Storage stability of maltase from marrows cotyledons at 25 ºC.

*Effect of some amino acids on maltase activity at 60 $^{o}$C for 1 hr.*

The results in **Fig. 6** revealed that glycine, proline, and alanine induced the enzyme activity whereas methionine caused a slight increase in maltase activity.

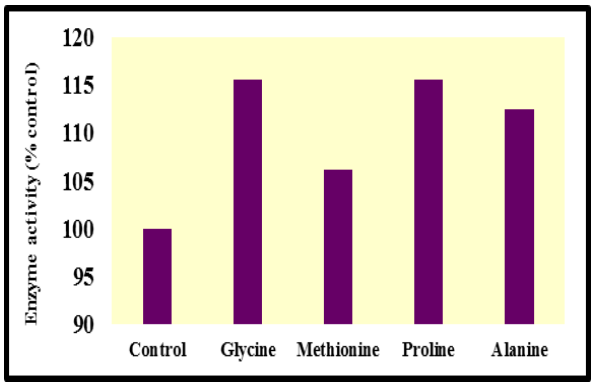


**Figure 6:** Effect of some amino acids on maltase activity at 60 ºC for 1 hr.

*Immobilization of maltase from marrow cotyledons on chitin*

The enzyme expressed appreciable activities throughout 6 cycles; each cycle was for one hour **(Fig. 7)**. It is noticed that the immobilized maltase was active for all five cycles and retained 162.4 U after cycle number 6.

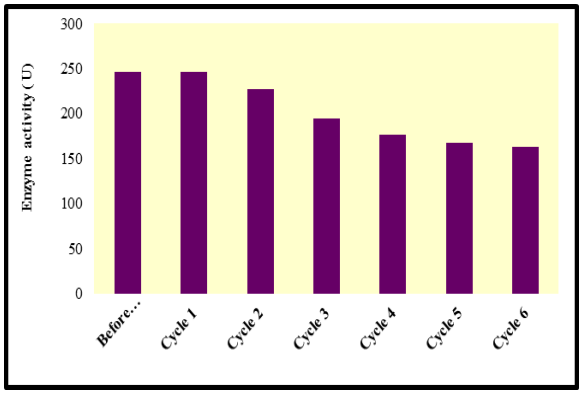


**Figure 7:** Reusability of immobilized maltase on chitin

*Effect of immobilization on pH of optimum maltase activity*

The results in **Fig. 8** indicated that the pH was shifted after immobilization from 6.0 in case of the free maltase to 9.0 in immobilized maltase.

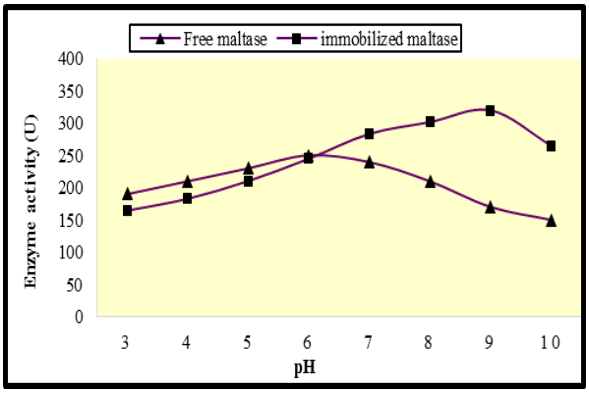


**Figure 8:** Optimum pH of free and immobilized maltase.

*Effect of immobilization on the temperature of optimum maltase activity*

The results in **Fig. 9** showed that the optimal temperature of maltase was shifted from 50 ℃ in free maltase to 60 ℃ in immobilized maltase.

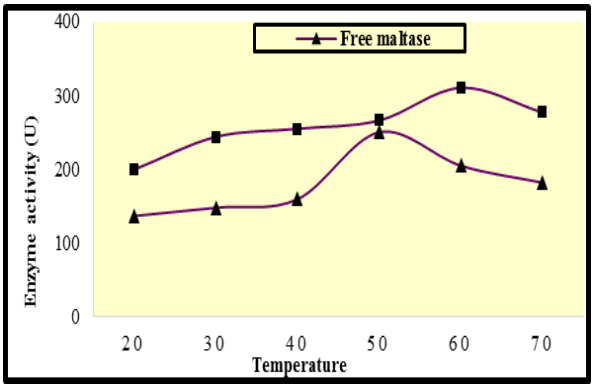


**Figure 9:** Optimum temperature of free and immobilized maltase.

*Effect of metal ions on maltase activity in free and immobilized maltase*

The results in **Fig. 10** revealed that the $K^{2+}$, $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$, and $Zn^{2+}$ activated immobilized maltase with a higher rate compared with the free maltase. On the other hand, $Hg^{2+}$ and $Cu^{2+}$ inhibited the immobilized maltase activity with a lower rate compared to that observed for the free maltase.

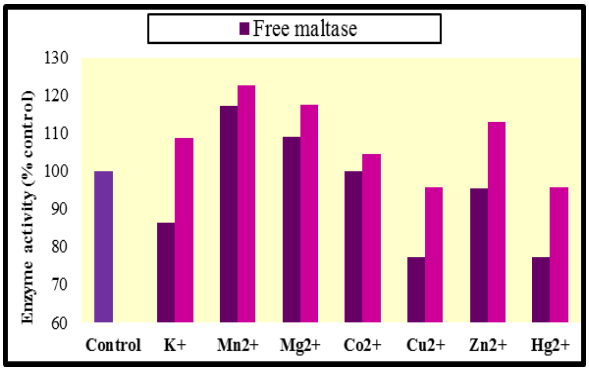


**Figure 10:** Effect of metal ion on free and immobilized enzyme activity.

*Effect of sorbitol on free and immobilized maltase activity*

The activity of free maltase increased from 105% up to 117% of control at 40 mM up to 70 mM sorbitol (**Fig. 11)** and the activity of immobilized maltase increased from 110% up to 125% of control at 10 mM up to 50 mM sorbitol (**Fig. 12)**

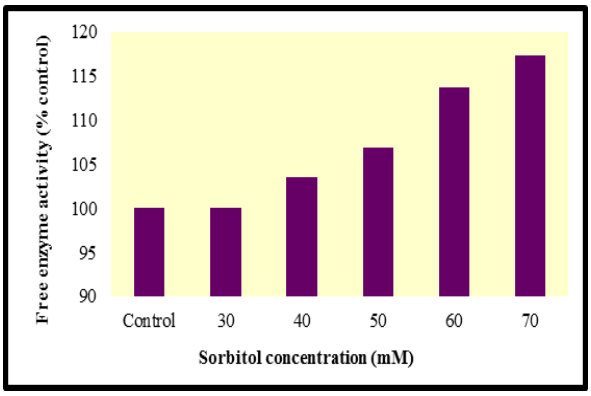


**Figure 11:** Effect of different concentrations of sorbitol on free maltase.

*Effect of mannitol on free and immobilized maltase activity*

The activity of free maltase increased from 110% up to 125% of control at 60 mM up to 90 mM mannitol (**Fig. 13)** and the activity of immobilized maltase increased from 110% up to 140% of control at 10 mM up to 50 mM mannitol (**Fig. 14)**

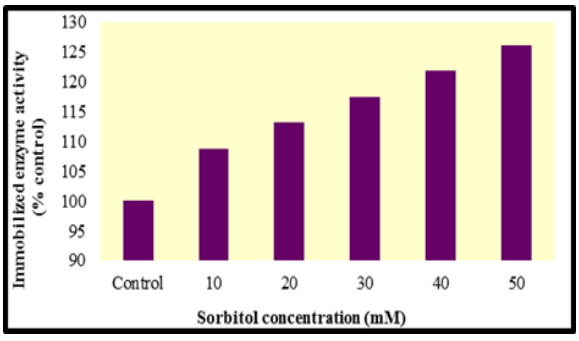


**Figure 12:** Effect of different concentrations of sorbitol on immobilized maltase.

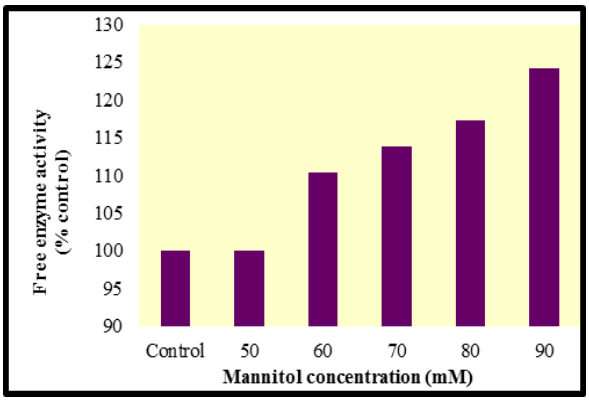


**Figure 13:** Effect of different concentrations of mannitol on free maltase.

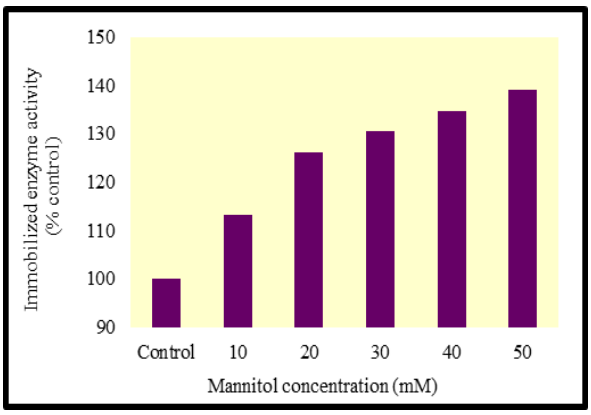


**Figure 14:** Effect of different concentrations of mannitol on immobilized maltase activity.

## Discussion

The goal of the present investigation was to assess some kinetic features of maltase under immobilization with chitin. The specific activity of the final preparation of maltase from cotyledons of 5-day old seedlings of *C. pepo* was 385 U $mg^{-1}$ proteins. The specific activity of α-glucosidase from germinating millet seeds was 76 U $mg^{-1}$ proteins (Yamasaki *et al.*, 2005), and from *Schizosaccharomyces pombe* was 22.5 U $mg^{-1}$ proteins (Okuyama *et al.*, 2005).

EDTA as chelating agent activated maltase whereas o-phenanthroline had no influence. It was found that EDTA slightly increased the thermal stability of polyphenol oxidase (Weemaes *et al.*, 1997). The enhancement in the enzyme activity by EDTA could be attributed to the fact that the maltase enzyme is partially purified. Therefore, it is possible that EDTA can chelate some inhibiting cations in the enzyme preparation. o-Phenanthroline had no effect on maltase in the present investigation. This result reveals that maltase is strongly resistant to chelating agents. However, this compound inhibited other enzymes such as protease (El-Shora and Metwally, 2008b).

Studying the effect of some surfactants on enzyme activity showed that Triton X-100 and Tween-80 inhibited the enzyme activity. This could be due to the denaturation of the enzyme protein by these compounds. Mandviwala and Khire (2000) reported that Triton X-100 and Tween-80 inhibited the phytase enzyme by inducing conformational changes in the enzyme molecules which render the enzyme not active. Also, El-Shora (2001) reported that Triton X-100 and Tween-80 inhibited urease activity isolated from *Chenopodium album*. However, Triton X-100 and Tween-80 activated other plant enzymes such as protease (El-Shora and Taha, 2010; Ibraheem and Malomo, 2017). The addition of BSA, nicotinic acid to the reaction mixture of maltase led to the activation of the enzyme. The BSA may result in the activation of maltase by keeping the tertiary structure of the enzyme protein and consequently improving the stability of the enzyme configuration. Wehtje *et al.* (1993) stated that the impact of albumin might be attributed to its protein nature that results in the stabilization and prevention of the enzyme deactivation.

Polyethylene glycol (PEG-6000) activated maltase at the concentrations between 20-30 mM. These findings are in harmony with those reported for the tyrosinase enzyme (El-Shora and Metwally, 2008a). It was suggested that PEG binds to the free amino groups situated in the amino acid side chains of the proteins particularly lysine (Santos *et al.*, 2018).

The reinforcement in the hydrophobic interactions among nonpolar amino acids inside the enzyme molecules improved the resistance of the enzyme to the inactivation and therefore increased its activity (Imai *et al.*, 2018). Sodium fluoride, succinic anhydride, phthalic anhydride, 2-mercaptoethanol, and diphenylamine inhibited maltase activity. The inhibition of maltase by the two examined anhydrides succinic and phthalic could be due to changes in the enzyme configuration by these compounds (Yahong *et al.*, 2011). Regarding the storage stability of maltase at 25 °C, it was found that the enzyme kept 43.5 % of its initial activity after the 5th day of incubation.

It was found that all the tested amino acids (glycine, methionine, proline, and alanine) activated the enzyme. Furthermore, glycine and proline were the best activators and methionine was the worst activator. The activation by these amino acids could be attributed to the fact that these amino acids could act as osmolytes. The amino acids were found as osmolytes for tyrosinase from mushroom (Zolghadri *et al.*, 2019). It was found that proline stimulates the renaturation of an unfolded protein of citrate synthase (Choudhary *et al.*, 2016). Also, osmolytes, which are molecules that protect organisms against stress, have found to stabilize proteins (Lin and Timasheff, 1994).

In general, the precise nature of the response of the enzymes to amino acids is unknown, although it may be related to the maintenance of conformational characteristics and integrity of the proteins by the solutes (Paleg *et al.*, 1981). The nature of the enzyme, hydrophobic character and the degree of interaction with the additives determine the stabilizing effect of the additives (Lozano *et al.*, 1993).

The expected improvement in the enzyme stability towards different deactivating force is one of the central reasons for

enzyme immobilization, where, the immobilization of the enzyme reduces the conformational mobility of the molecules. Accordingly, the immobilized enzyme could act in harsh environmental conditions where the loss in its activity is less than the loss in the activity of free counterpart (Bai *et al.*, 2006).

Maltase enzyme from *C. pepo* was successfully immobilized in a system of chitin. The immobilized enzyme expressed good stability during repeated use in 6 cycles. However, the slight decline of the enzyme activity during successive 6 cycles could be the result of enzyme inhibition due to the cross-linking of the immobilized enzyme with the product of the enzyme reaction. It should be stated that good stability could significantly decrease the cost of practical utilization.

The optimum pH values were 6.0 and 9.0 for the free and immobilized maltase, respectively. However, the pH profile of the immobilized enzyme revealed slightly enhanced stability, in comparison to the free enzyme, which means that the immobilization method preserved the enzyme activity. The extensive distortion of the native conformation of the enzyme protein by extreme pH was thought to be prevented by immobilization of the enzyme on the surface of the carrier (El-Shora and Youssef, 2008).

The immobilization procedure could protect the enzyme active conformation from distortion or damage by heat exchange and so, the optimum temperature for maltase activity upon immobilization was shifted from 50 °C to 60 °C (Bai *et al.*, 2006). It has been revealed that the stability of the enzyme can be improved by treatment with additives and immobilization (Zucca *et al.*, 2016). The improvement in the stability of maltase by immobilization makes it proper for application in industrial processes performed at elevated temperature as the immobilized enzyme could work in harsh environmental conditions with less activity loss compared to the free counterpart. The immobilized maltase was more resistant to inhibition by some cations than the free enzyme. It could be noted that immobilization gives the enzyme a kind of protection against the inhibitory action of these cations, but the mechanism of this protection requires more investigation.

Both mannitol and sorbitol were good stabilizers for free and immobilized maltase at 60 $^{O}$C. The immobilized enzyme was more stable than the free one in the presence of any of the two compounds (Zdarta *et al.*, 2018). Mannitol was reported to protect enzymes from thermal denaturation such as phytase (El-Shora *et al.*, 2004), and lipase (El-Shora and Metwally, 2006). In addition, mannitol affects osmoregulation and storage. It regulates the reducing power and services as a compatible solute in both plants (Loescher *et al.*, 1992) and algae (Davison and Reed, 1985).

## Conclusion

It can be concluded that the immobilization of maltase on chitin improves its properties and its reusability.

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