Induction, immobilization, modification and natural inhibitors of \( \alpha \)-glucosidase from *Penicillium chrysogenum*

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Abstract
\( \alpha \)-glucosidase (EC: 3.2.1.20) was isolated from *Penicillium chrysogenum*. The enzyme was enhanced by plant growth regulators such as gibberellic acid (GA\(_3\)), benzylaminopurine (BAP) and kinetin. Dansyl chloride inhibited the enzyme at 1, 2, 3, 4 and 5 mM with \( T_{0.5} \) 67, 52.2, 34.4 and 23.3 min, respectively. The substrate offered partial protection for the enzyme against dansyl chloride inhibition. The enzyme was activated by Ca\(^{2+}\) and Mg\(^{2+}\). However, Pb\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\) and Hg\(^{2+}\) inhibited \( \alpha \)-glucosidase activity. The enzyme was immobilized on Ca alginate and the optimal concentration for 3% w/v. The optimal concentration of CaCl\(_2\) was recorded at 3 mM. The optimal CaCl\(_2\) concentration and the optimum time for immobilization was 3mM and 4hr. The enzyme was inhibited by aqueous extracts of *Datura stramonium*, *Trigonella foenum-graecum*, *Hyoscymus muticus* and *Cynodon dactylon*. The IC\(_{50}\) values for the four extracts were 59.1, 73.6, 68.5 and 77.1 \( \mu \)g ml\(^{-1}\), respectively.

Keywords: P chrysogenum; glucosidase; Induction; Modification; Immobilization; Inhibition

Introduction
It is difficult to recover the soluble enzyme from the reaction mixture. Thus, it cannot be applied for catalyzing more reactions, however, the procedure of immobilization can make it feasible. The loss of some activity for the immobilized biological catalyst in repeated use is a common phenomenon [1].

The aim of immobilization of any enzyme is to increase its stability against different factors of incubation. This due to the fact that the mobility of immobilized enzyme is restricted. Thus, the immobilized enzyme could act under various environmental conditions with little loss of activity compared to the free enzyme [2].

Immobilized \( \alpha \)-glucosidase may be applied repeatedly since it retains its activity. Thus, there is intensive work to use the immobilized enzymes to be retained in a biochemical reactor and allow operation in continuous way and consequently lower cost of its use [3].

Material and methods

**Materials**
Glycerol and other chemicals mentioned in the investigation were purchased from Sigma Chemicals (Sigma Aldrich, Steinheim, Germany). All media cultures including Plate Count Agar (PCA) and others were obtained from Merck Company (Merck, Darmstadt, Germany).

**Experimental**
*Penicillium chrysogenum* Thom ATCC 10106 was provided by Salwa A. Khalaf, Prof. of Microbiology, Botany Department, Faculty of Science, Zagazig University.
Growth medium

Modified Czapak’s dox agar (CDA)

This medium used for growth of *P. chrysogenum* at a final pH 7.3 was described by Eaton et al. [4] includes the following in g/L: 2g sodium nitrate, 30g glucose, 1g potassium dihydrogen phosphate, 0.5g potassium, 0.5g chloride, 0.5g magnesium sulphate, 20g agar, 0.01g ferrous sulfate and 1L distilled water. Boil to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure (121ºC) for a period of 15 min. Mix well before dispensing. The volume of required acid for 100 ml of sterilized medium was approximately 1ml. The medium was mixed well and poured into sterile petri plates. Cultural characteristics were observed after an incubation at 25ºC - 30ºC for 48-72 h and were kept in the refrigerator at 4ºC for storage.

Potato-dextrose agar medium (PDA)

This medium used for the inoculum preparation at final pH 5.6 was that Vanderzant and Splittstoesser [5]. It included of the following in g/L: 20g dextrose, 4g potato extract, 15g agar and 1L distilled water. Boil to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121ºC) for 15 min. Mix well before dispensing. The volume of required acid for 100 ml of sterilized medium was approximately 1ml. The medium should not be heated after the addition of acid. Cultural characteristics recorded after incubation at 25°C to 30°C days. Rate of recovery is considered as 100% for the growth of *P. chrysogenum* on Sabouraud Dextrose Agar.

The effect of phytohormones on α-glucosidase activity

Three phytohormones including kinetin, gibberellic acid (GA$_3$) and benzyl amino purine were tested at different concentrations (20, 40, 60, 80 and 100 µmole) followed by measuring α-glucosidase activity spectrophotometrically.

Modification of α-glucosidase.

Stock solution of DnsCl in acetonitrile were freshly prepared 0.5 ml of the enzyme was incubated for 1h at 4 0C with different concentrations of DnsCl (1, 2, 3, 4 and 5 mM) in 100 mM Tris (pH 7). The enzyme was incubated in 100 mM Tris (pH 7) containing various concentrations of DnsCl for 15 min. Terminate the reaction by adding 20 mM β-mercaptoethanol and 30 mM lysine.

Effect of various cations on α-glucosidase activity

Different metal cations were tested regarding their effects on α-glucosidase activity. These cations were Zn$^{2+}$, Cd$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Na$^{2+}$ and Ca$^{2+}$ and tested at 5 mM in the reaction medium followed by measuring enzyme activity.

Immobilization of α-glucosidase

α-glucosidase was immobilized by alginate) Sodium alginate (5 % w/v) in 150 mM sodium phosphate buffer (pH 8.0) was prepared by heating at 50 ºC then cooled down to room temperature. The solution containing enzyme was mixed with sodium alginate solution in total volume 10 ml. The mixture was put into a syringe, and beads were formed by dropping the solution into 100 mM CaCl$_2$ solution after gentle stirring for 2 h at 4ºC. The beads were then filtered and washed thoroughly with distilled water, dried using filter paper (whatman no.1). The beads were exposure to the open air for a period of 1 h before being used. The filtered solution of CaCl$_2$ was used for determination of α-glucosidase activity.

Determination of α-glucosidase immobilization yield.

The yield of immobilization was identified as the yield for enzyme which was immobilized by entrapment and expressed by the following equation:

\[
\text{Immobilization yield} = \frac{\text{Activity added}}{\text{Activity of immobilized enzyme}} \times 100.
\]

Reusability of immobilized α-glucosidase.

Reusability was done by measuring the enzyme activity throughout 10 cycles.

Effect of extracts from various plants on α-glucosidase

Experimental

*Daturna stramonium* L. is belonging to family: Solonaceae and was collected from Mansoura University area. *Trigoella foenum-graecum* L. belongs to family: Fabaceae and was brought from the local market of Mansoura. *Hyoscyamus muticus* L. belongs to family : Solanaceae and was collected from Wadi Hagul region of the northern part of the Eastern Desert of Egypt, (The Isthmic Desert). *Cynodon dactylon* L. belongs to family: Poaceae and was collected from cultivated area of Mansoura Governorate.

Preparation of plant extracts

Plant leaves were air-dried and extracted aqueously by distilled water then various concentrations (20, 40, 60, 80, 100 µg ml$^{-1}$) were prepared and tested for their effects on α-glucosidase activity.

Results

Effect of plant growth regulators on α-glucosidase activity from *P. chrysogenum*

The results in Fig. 1 reveal that the three tested phytohormones GA$_3$, BAP and kinetin induced α-glucosidase activity and GA$_3$
was the best inducer followed by BAP and kinetin. Also, it was remarkable that there was continuous increment in α-glucosidase activity with increasing the time of incubation. GA₃ as growth regulator induced the activity of α-glucosidase. GA₃ induced the activities of other enzymes such as phosphoenol pyruvate carboxylase [6], myrosinase [7], NADH-glutamate synthetase [8], sucrose synthase [9] and acid phosphatase [10]. The enhancement of α-glucosidase by GA₃ might be attributed to enhancing of α-glucosidase synthesis through controlling the transcription and translation.

Benzylaminopurine (BAP) induced α-glucosidase of *P. chrysogenum*. BAP enhanced the activities of other enzymes including glutamate oxaloacetic acid transaminase [11], and RNA polymerase [12]. α-glucosidase activity was induced in presence of zeatin in the growth medium. Also, L-asparaginase activity of *Aspergillus niger* was found to be induced by zeatin in growth medium [13]. The induction of α-glucosidase by zeatin could be explained on the bases that zeatin may act at the transcriptional or posttranscriptional level including posttranslational regulation, however, this require more investigation for clarification of the actual mechanism of induction.

**Effect of dansyl chloride on thermostability of α-glucosidase from *P. chrysogenum***

Dansyl chloride is a reagent used for the detection of lysyl residue in the protein [14]. The obtained results in Fig. 2 show that dansyl chloride inhibited α-glucosidase activity at all tested concentrations. The inhibition was dependent on both the concentration and the time of incubation. The T₀.₅ values were 67, 52.2, 34.4 and 23.3 min. Plotting log conc. against log t₀.₅ (Fig. 3) resulted in straight line with slope 0.7. The effect of substrate of α-glucosidase on the enzyme protein against dansyl chloride was carried out using 5 and 10 mM substrate in presence of dansyl chloride (Fig. 4). The resulting reveal that the substrate of α-glucosidase offered appreciable protection against dansyl chloride and the protection was dependent on the substrate concentration. Dansyl chloride is known as a reagent for lysyl group in proteins [15]. In this investigation dansyl chloride inhibited α-glucosidase activity revealing that lysyl residue is present in the active site and taking essential part in the enzyme catalysis.

Generally, modification of enzyme chemically plays a role in probing the mechanism of enzyme activity. This technique is applied for identification of the amino acid in the enzyme molecules which are responsible for the catalytic properties of the entire protein. During the chemical modification, the enzyme activity may be altered [16]. Generally, it was observed that the chemical modification of the enzyme could selectively target the residues specifically at the active site, as demonstrated by substrate protection. The obtained results in this paper are valuable for determination of the active groups which are taking part for enzyme catalysis for possible regulation of the enzyme activity.
Protection of $\alpha$-glucosidase by its substrate against inactivation by dansyl chloride.

Effect of different cations on $\alpha$-glucosidase activity from P. chrysogenum

The effect of different metal cations on $\alpha$-glucosidase activity was studied. The cations tested were Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Pd$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Hg$^{2+}$. These cations were examined at 5 mM in the reaction mixture. The enzyme activity was expressed as units mg$^{-1}$ protein (Fig. 5). The relative activity was also calculated. The results revealed that Ca$^{2+}$ was the best activator for $\alpha$-glucosidase whereas Hg$^{2+}$ was the most suppressive cations reduced the activity to 1.2 units mg$^{-1}$ protein. The other remaining cations expressed variable inhibitory effect on the enzyme activity. $\alpha$-glucosidase was activated by Ca$^{2+}$ and Mg$^{2+}$, however Pb$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Ni$^{2+}$ and Hg$^{2+}$ were inhibitors. The activation of $\alpha$-glucosidase by Ca$^{2+}$ could be due to the in forcing of the interactions inside $\alpha$-glucosidase molecules and by the binding of Ca$^{2+}$ to the autolysis site. Ca$^{2+}$ activated other enzymes such as phytase [17] and glucose isomerase [18].

The inhibition of $\alpha$-glucosidase by Cd$^{2+}$ and Hg$^{2+}$ might be due to oxidation of sulfhydryl groups of $\alpha$-glucosidase. Heavy metals could inhibit $\alpha$-glucosidase activity by binding to sulfhydryl groups in proteins or might disrupt enzyme protein structure or disable of an essential element [19].

Immobilization $\alpha$-glucosidase activity from P. chrysogenum on calcium alginate

The pure $\alpha$-glucosidase was immobilized on calcium alginate and the immobilized yield was calculated. The results in Fig. 6 indicate that immobilization yield increased up to 3 % w/v of calcium alginate then declined at 4 and 5 % w/v where the immobilization yield were 31 % w/v and 28 % w/v, respectively. This critical point $\alpha$-glucosidase activity is at its maximum and this temperature is called optimal temperature. $\alpha$-Glucosidase was immobilized using Ca alginate and the optimal concentration was 3% w/v. After which the immobilization decreased at the higher concentrations. The best time for immobilization of $\alpha$-glucosidase was 4h. The present results revealed that $\alpha$-glucosidase immobilized on Ca alginate was reusable for 10 cycle where the enzyme retained 50% of its activity. The immobilized $\alpha$-glucosidase in the present investigation could be preferred to avoid costly purification processes and to increase catalytic stability as mentioned by [3].

Effect of CaCl$_2$ immobilization of $\alpha$-glucosidase activity from P. chrysogenum

The recorded results in Fig. 7 show continuous increase in the immobilization yield with the increase in CaCl$_2$ concentration.
up to 3 mM followed by reduction at 4 and 5 mM where the immobilization yield values were 62 and 54 %, respectively.

**Figure 7** Effect of CaCl$_2$ on immobilization yield of α-glucosidase activity from P. chrysogenum

**Effect of immobilized time of immobilization yield on α-glucosidase activity from P. chrysogenum.**

The results in Fig. 8 indicate continuous increase in the immobilization yield up to 4 h after which the immobilization yield decreased at 6 and 5 h where the immobilization yield was 52.8% and 40.4%, respectively.

**Figure 8** Effect of immobilized time on immobilization yield of α-glucosidase activity from P. chrysogenum.

**Reusability of immobilized α-glucosidase enzyme from P. chrysogenum.**

This experiment was done to study the reusability of alginate-immobilized α-glucosidase throughout 10 cycles (Fig. 9). The results of the reusability experiment reveal that α-glucosidase activity decreased gradually throughout the 10 cycles, however 50% of its initial activity after the 10th cycle.

**Figure 9** Reusability of immobilized α-glucosidase enzyme.

**Effect of plant extracts on α-glucosidase activity from P. chrysogenum.**

In trial to find a natural inhibitors for α-glucosidase activity it was decided to study the influence of extracts from various plant species on α-glucosidase activity. The tested plants Datura stramonium L., Trigonella foenum-graecum L., Hyoscyamus muticus L. and Cynodon dactylon L. Each plant extract was examined at different concentrations 20, 40, 60, 80 and 100 µg ml$^{-1}$.

The illustrated results in Fig. 10 showed the influence of these plant extracts extract on α-glucosidase activity. These results showed continuous inhibition of the enzyme activity by the plant 1 extract in a concentration dependent manner. The activity was expressed as units mg$^{-1}$ protein and the relative activity was calculated. The IC$_{50}$ of three plant extracts were 59.1, 73.6, 68.5 and 77.1 µg ml$^{-1}$, respectively. The aqueous extracts from tested plants ( Datura stramonium, Trigonella foenum-graecum, Hyoscyamus muticus and Cynodon dactylon ) inhibited α-glucosidase activity. The inhibition of α-glucosidase activity by the four tested plants might be due to the presence of several phytochemicals, saponin and tannin in these extracts. Previous studies on inhibitors of α-glucosidase inhibitors isolated from various medicinal plants suggested that flavonoids and potential inhibitors for α-glucosidase [20].

**Conclusion**

The present results revealed successful method for induction of α-glucosidase and its immobilization on alginate. In addition, the results introduce four plant extracts as a natural candidates for inhibiting of α-glucosidase. However, it needs to know the
main compounds of which exhibit the bioactivity of these extracts.

**Author’s contributions**

Hamed M. El-S hora, Mohsen E. Ibrahim and Mohammad W. Alfakhary performed the experiments together and participated in manuscript for publications.

**Conlicts of interest**

None.

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**References**

[1] Brena BM, Batista-Viera F. Immobilization of enzymes and cells. Humana Press; 2006. Immobilization of enzymes.
[2] Bai YX. Covalent immobilization of triacylglycerol lipase onto functionalized novel mesoporous silica supports. Journal of Biotechnology. 2006;125:574–582.
[3] Tanino KK, Kalcsits L, Silim S, Kendall E, Gray GR. Temperature-driven plasticity in growth cessation and dormancy development in deciduous woody plants: a working hypothesis suggesting how molecular and cellular function is affected by temperature during dormancy induction. Plant Molecular Biology. 2010;p. 1–2.
[4] Eaton AD, Clesceri LS, Greenberg AE. Standard Methods for the Examination of Water and Waste water. Washington, USA: American Public Health. Association; 1998. 20th Ed.
[5] Vanderzant C, Splittstoesser DF. Compendium of methods for the microbiological examination of food. Washington, D.C.; 1992. 3rd ed. A. Pub Hea.
[6] Bihzad MA, El-Shora HM. Phosphoenolpyruvate carboxylase from Rumex dentatus, a C3-plant. Journal of Plant Physiology. 1996;149:669–676.
[7] El-Shora HM, Ahmed M, El-Shobaky MM, El-Atrozy. Activity of purified bacterial myrosinase and its essential residues. International Journal of Current Microbiology Applied Science. 2016;5:567–578.
[8] El-Shora H. Effect of growth regulators and group modifiers on NADH-glutamate synthase of narrow cotyledons. Journal of Biological Science. 2001;1:597–602.
[9] Kaur S, Gupta AK, Kaur N. Effect of GA3, kinetin and indole acetic acid on carbohydrate metabolism in chickpea seedlings germinating under water stress; Plant Growth Regulators; 2000.
[10] El-Shora HM, Metwally M. Effect of phytohormones and group selective reagents on acid phosphatase from Cladosporium cladosporioides. Asian Journal of Biotechnology. 2009;1:1–11.
[11] Abdel-Ghaffar F, Marzouk M, Ashour MB, Mosaad MN. Effects of Eimeria labbeana and E. stiedai infection on the activity of some enzymes in the serum and liver of their hosts. Parasitology Research. 1990;5:440–443.
[12] Ananiev EV, Barsky VE, Ilyin YV, Churikov NA. Localization of nucleoli in Drosophila melanogaster polytene chromosomes. Chromosoma. 2003;81:619–628.
[13] El-Shora HM, Youssef MM, Salwa AK. Induction and immobilization of asparaginase from Aspergillus niger. Biochemical Letter. 2005;1:35–46.
[14] El-Shora HM, Khalaf SA, Zaki MO. Active residues and immobilization of cyanide hydratase from Cladosporium oxysporum. International Journal of Science Research. 2015;7:455–465.

[15] El-Shora HM, Metwally MA. Production, purification and characterization of proteases from whey by some fungi. Annals of Microbiology. 2008;58:495–502.

[16] Kaiser ET, Lawrence DS, Rokita SE. The chemical modification of enzymatic specificity. Annals Review Biochemistry. 1985;54:565–595.

[17] El-Shora HM, Abo-Kassem EM. Regulation and characterization of phytase activity in Latuca sativa cotyledons; 2000.

[18] El-Shora HM, El-Shobaky AM, Ghoneim JE. Activity of glucose isomerase from Bacillus thuringiensis under Different Treatments. Inter of Journal of Current Microbiology Applied Science. 2016;2:579–589.

[19] Srivastava PK, Kayastha AM. Significance of sulfhydryl groups in the activity of urease from pigeonpea (Cajanus cajan L.) seeds. Plant Science. 2000;1:149–158.

[20] Kwon YI, Apostolidis E, Shetty K. Evaluation of paper Capsicum annuum, for management of natural product, Mini-Reviews in Medical Chemistry; 2007.