

The Effect of the Extracts of Endophytic Fungi on Pancreatic α -Amylase Activity

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Abstract: Inhibitors of pancreatic α -amylase offer an effective strategy to lower the levels of postprandial hyperglycemia by control of starch breakdown. Among 86 fungal endophytes isolated from 15 medicinal plants *Aspergillus terreus*-AF104S, *Aspergillus egypticus*-HT166S and *Penicillium sp.*-CC200 exhibited strong pancreatic amylase inhibitory potential were selected. Endophytes were subjected to ethyl acetate extraction and tested for α -amylase inhibition, in order to assess and evaluate their inhibitory potential on pancreatic α -amylase. Analysis showed concentration dependent enzyme inhibition up to 83% with half inhibition (IC₅₀) values for less 25 mg·mL⁻¹, which is lower than acarbose as control. It was observed 3-fold increasing of V_{max} and maintenance K_m at control level in the presence of extracts *A. terreus*-AF104S and *Penicillium sp.*-CC200, while in presence of extract *A. egypticus*-HT166S K_m was doubled, and V_{max} was maintained at the control level. Kinetic studies allow proposing the competitive mode of α -amylase inhibition by extracts *A. egypticus*-HT166S and uncompetitive inhibition by extracts *A. terreus*-AF104S and *Penicillium sp.*-CC200.

Key words: Endophytic fungi, medicinal plants, α -amylase, bio-active natural products, inhibitory activity.

1. Introduction

Diabetes mellitus, one of the common metabolic disorders that characterized by elevated blood glucose level [1]. Postprandial hyperglycemia caused by consumption of high-carbohydrate diets can progress to full symptomatic type 2 diabetes (DM2) [2]. The control of hyperglycemia is critical in the management of diabetes mellitus since in long term, acute and chronic complications can occur with significant morbidity and mortality [1, 3]. International Diabetes Federation (IDF) has reported that 8.3% (366 million) of the adult diabetic patients worldwide is 366 million in 2011, and it is predicted to increase to 552 million people by 2030 that can be managed by diet control and consumption of various synthetic antidiabetic

drugs [4, 5].

Current therapeutic approach for the control of postprandial hyperglycemia, as the earliest metabolic response in DM2, is to retard and reduce the digestion and absorption of ingested carbohydrates by the inhibition of carbohydrate-hydrolyzing enzymes, such as α -amylase and/or α -glucosidase [6]. Commercial hypoglycemic agents as acarbose, miglitol, voglibose, metformin effectively control blood glucose by competitive and reversible inhibition of α -amylase and α -glucosidase from intestine as well as pancreas, and potentially reduce the progression of diabetes [7]. Despite their efficacy, these drugs are often associated with some undesirable adverse gastrointestinal side effects [6, 7]. Therefore, the management of diabetes without any side effects is still a challenge and plants continue to play an important role in the discovery of new compounds for the treatment of this disease [8, 9].

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At the same time, the endophytic fungi asymptomatic inhabiting various tissues of living plants and producing huge number of unique bioactive substances become an alternative resource of hypoglycemic antidiabetic compounds [10]. It has been reported, the metabolites of endophytic fungi of some medicinal plants have potential as inhibitors of α -glycosidase and α -amylase [11]. Recently, we have isolated a number of endophytic fungi from several local medicinal plants and the strains producing metabolite with high inhibitory activity against pancreatic amylase have been selected [12].

In this context, the aim of this work is to study the effect on the activity of pancreatic α -amylase ethyl acetate extracts of endophytic fungi isolated from anti-diabetic plants *Helianthus tuberosus*, *Celosia cristata* and *Allium filidens*.

2. Materials and Methods

Cultivation of three selected endophytes for obtaining fungal biomass was carried out by submerge fermentation in 500 mL flasks with 100 mL Chapek-Dox medium for 5 days at 26 °C.

For extraction of inhibitory metabolites 5 g of homogenized biomass of each isolate was transferred to a conical flask containing 50 mL of ethyl acetate and left for one day on shaker at room temperature. The mixture was filtered through a paper filter (Whatman No. 1), Na_2SO_4 was added at concentration $40 \mu\text{g}\cdot\text{mL}^{-1}$. Extract was evaporated to dryness on a rotary evaporator, 1 mL of dimethyl sulfoxide (DMSO) was added and stored at -40 °C prior to use.

Determination of α -amylase activity was carried out by the modified method used for measurement in the plant extract [13]. Solution of starch as a substrate was prepared in concentration $1 \text{ g } 10 \text{ mL}^{-1}$ water, boiled for 2 minutes, adjusted to 100 mL with distilled water and used within 2-3 days. For the preparation of iodine reagent 0.5 g of crystalline iodine, 5 g of potassium iodide were dissolved in 250 mL of water; 2 mL of this reagent was adjusted to 100 mL by 0.1 M

HCl and used as working solution. To 2 mL of the prepared starch solution 100 μL pancreatic α -amylase ($13 \text{ u}\cdot\text{mL}^{-1}$ in 0.1 M Na-acetate buffer pH 4.7), 100 μL of the extract endophyte ($20 \text{ mg}\cdot\text{mL}^{-1}$), 2 mL of acetate buffer were added and incubated for 10 minutes at 30 °C. The sample without extract used as control. After incubation the reaction was terminated by adding 10 mL of iodine reagent and the absorbance was measured at 630 nm using a spectrophotometer.

Inhibitory activity was expressed by the formula: $(A_0 - A_t)/A_0 \times 100\%$, where A_0 —absorption of control sample, A_t —the absorption of test sample, respectively.

The mode of inhibition of endophytic extracts on α -amylase action was determined by increasing substrate (starch) concentration. Kinetic parameters, namely Michaelis-Menten constant affinity (K_m) and maximum velocity (V_{\max}) were derived from appropriate Lineweaver-Burk plots [14].

3. Results and Its Discussion

The study of lipid-lowering and anti-diabetic activity of various fractions of extracts of the mycelium 17 endophytic fungi isolated from *Salvadora oleoides Decne* (Salvadoraceae), showed that the extracts of two endophytic fungi *pp. Aspergillus* and *Phoma* significantly decrease blood glucose level [15]. Positively active as inhibitors of α -amylase and α -glycosidase were extracts of 2 from 9 fungal endophytes isolated from the Indian anti-diabetic plants *Momordica charantia* and *Trigonella foenum-graceum*. In the experimental conditions *in vitro* IC₅₀ of two isolates were lower than the control acarbose [16]. According to study of antidiabetic activity of *Syncephalastrum racemosum* isolated from red algae *Gracilaria corticata*, its acetone and methanol extracts inhibited amylase activity for 19.4 and 23.7%, respectively [17].

From the roots, stems and leaves of 15 plants growing in Uzbekistan we obtained 86 endophytic fungal isolates. It was established that the ethyl acetate

extracts of number of isolates at concentration 100 $\mu\text{g}\cdot\text{mL}^{-1}$ inhibited pancreatic α -amylase activity *in vitro* in the range of 2.5% to 85% [12]. Among studied fungal strains as the most active there were selected *Aspergillus terreus*-AF104S isolated from *Allium filidens*, *Aspergillus egypticus*-HT166S from *Helianthus tuberosus* and *Penicillium sp.*-CC200 from *Celosia cristata*.

Studies of dose-dependent inhibitory effects of extracts revealed the highest inhibitory action caused by 100 mg of extracts as well as acarbose, while values of IC₅₀ of all three strains were lower than acarbose (Table 1).

In the study of inhibitory properties of various compounds according to any enzymes it is important to determine their influence on kinetic characteristics of the enzymatic process. In this regard, we have studied the dependence of the rate of amylolytic

reaction on the concentration of substrate in the presence of extracts from *A. terreus*-AF104S, *A. egypticus*-HT166S and *Penicillium sp.*-CC200.

The rate of hydrolysis of starch in the presence of extracts on Lineweaver-Burk plot is showed in Figs. 1-3. Maximum achievable velocity of reaction (on y-axis) decreases in the presence of the extracts of endophytes *Penicillium sp.*-CC200 and *A. terreus*-AF104S (Figs. 1 and 2).

However, the effective value of the apparent Michaelis constant does not change (on the x-axis). As it can be seen from summary Table 2 below, the value of K_m in the presence and in the absence of inhibitory connections is of $8.33 \pm 0.4 \text{ mg}\cdot\text{mL}^{-1}$ for both extracts (Table 2).

The maximum velocity of amylases is reduced more than 3 times (the interval on the y-axis) up to $0.26 \text{ mg}\cdot\text{min}^{-1}$ and $0.28 \text{ mg}\cdot\text{min}^{-1}$ by extracts *Penicillium*

Table 1 Effect of extracts concentration on α -amylase inhibition.

№	Extracts, mg	α -amylase inhibition, %			
		Acarbose- IC ₅₀ -30	<i>A. terreus</i> - AF104S-IC ₅₀ -25	<i>A. egypticus</i> - HT166S-IC ₅₀ -20	<i>Penicillium sp.</i> - CC200-IC ₅₀ -25
1	20	48.5	42.2	46.6	36.0
2	60	74.2	62.5	70.2	68.5
3	100	82.0	70.5	82.0	83.0
4	120	80.0	60.0	80.0	71.2

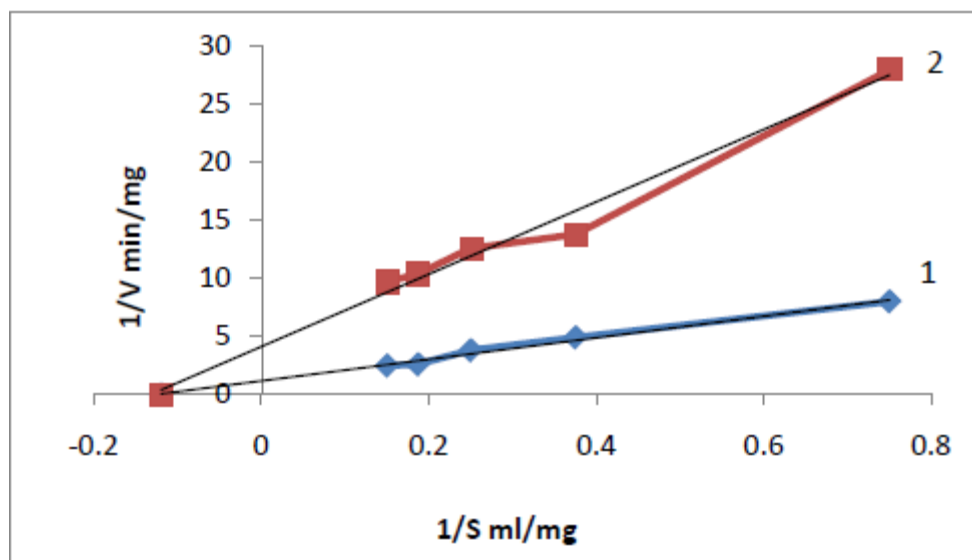


Fig. 1 The influence of *Penicillium sp.*-CC200 extract on activity of α -amylase at different concentration of substrate (1—without extract, 2—with extract).

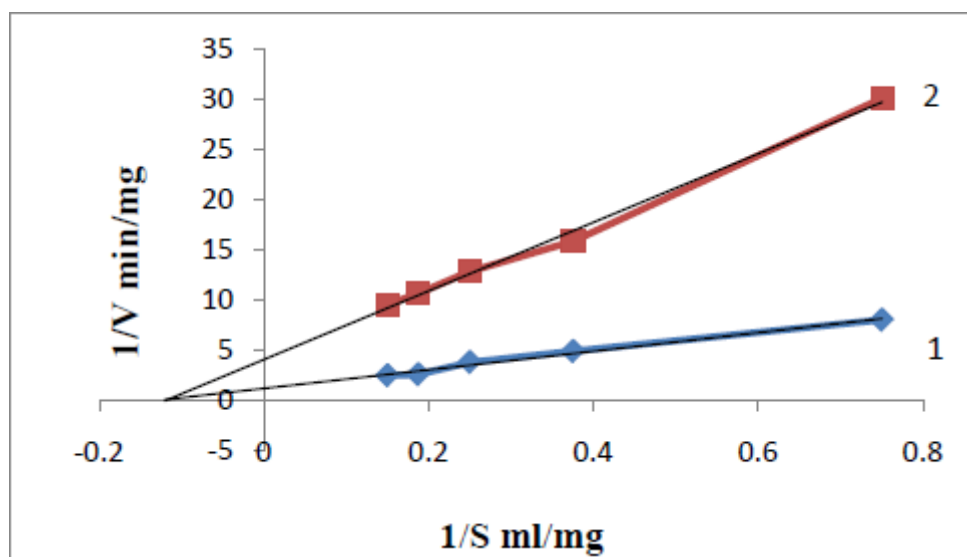


Fig. 2 The influence of *A. terreus*-AF104S extract on activity of α -amylase at different concentration of substrate (1—without extract, 2—with extract).

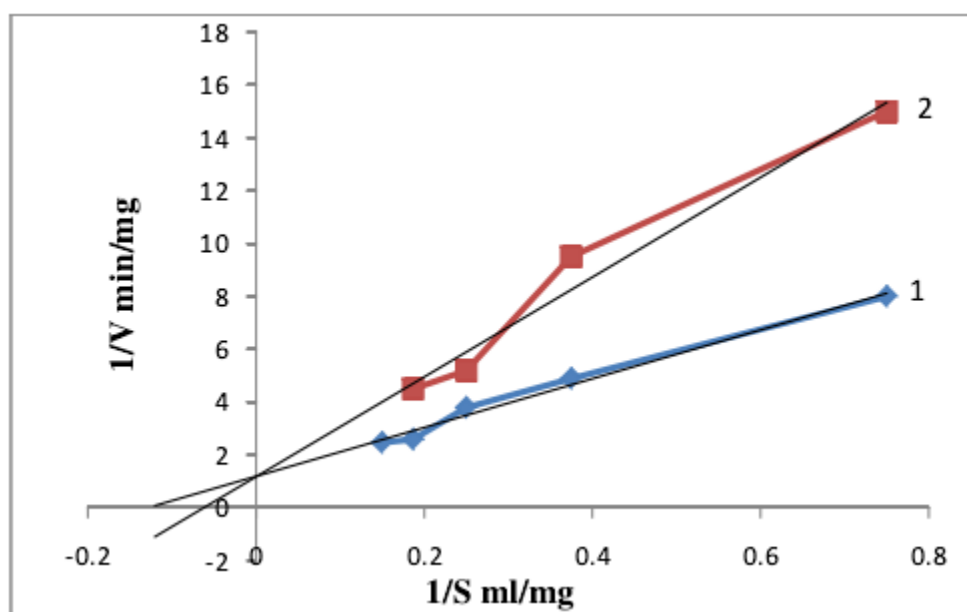


Fig. 3 The influence of *A. egypticus*-HT166S extract on activity of α -amylase at different concentration of substrate (1—without extract, 2—with extract).

Table 2 Effect of inhibitory extracts on catalytic properties of pancreatic α -amylase.

№		Control (without extracts)	Extracts			
			<i>Penicillium</i> <i>sp.-CC200</i>	<i>A. terreus</i> -AF104S	<i>A. egypticus</i> -HT166S	Acarbose
1	K_m , mg·mL ⁻¹	8.33 ± 0.4	8.33 ± 0.4	8.33 ± 0.4	15.8 ± 0.6	55.0 ± 2.0
2	V_{max} , mg min ⁻¹	0.9 ± 0.05	0.26 ± 0.02	0.28 ± 0.02	0.9 ± 0.05	0.9 ± 0.05

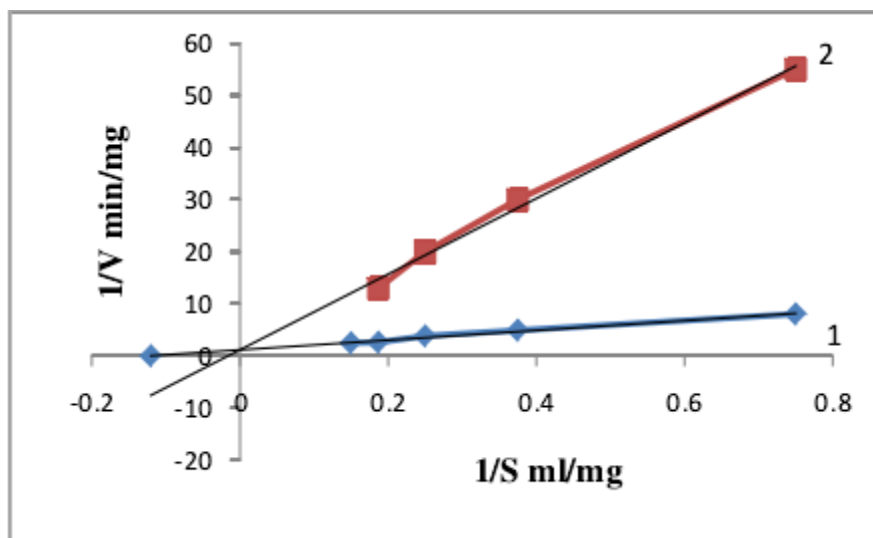


Fig. 4 The influence of acarbose on α -amylase activity at different concentration of substrate (1—without acarbose, 2—with acarbose).

sp.-CC200 and *A. terreus-AF104S*, respectively. In the absence of inhibitory substances maximum velocity of hydrolysis is $0.9 \pm 0.05 \text{ mg} \cdot \text{min}^{-1}$. This tends to suggest an uncompetitive mode of action. Uncompetitive inhibitors bind to enzyme-substrate complex forming an enzyme-substrate-inhibitor complex and delays rate of reaction [18].

As it can be seen from the data presented in Fig. 3, despite a decrease in the activity of amylase, the extract of *A. egypticus-HT166S* had no effect on the value of amylase catalytic constants in the presence of inhibitory compounds, the value of Michaelis constant is increased by 2 times and is $15.8 \pm 0.6 \text{ mg} \cdot \text{mL}^{-1}$ (Table 2). This suggests that the type of inhibition is competitive, i.e. the affinity of the inhibitor to the enzyme is higher than that of starch.

A noticeable change in the kinetic constants for amylase was observed with the commercial inhibitor acarbose (Fig. 4). As it can be seen from the obtained data, despite the decrease of amylase activity, acarbose does not affect the value of the catalytic constants. Michaelis constant is increased almost by 7 times and is $55.0 \pm 2.0 \text{ mg} \cdot \text{mL}^{-1}$ (axis of abscissa). This suggests that the affinity of the inhibitor to the enzyme is higher than that of starch, and the inhibition of the activity occurs at very competitive type.

It should be noted that kinetic characteristics of

inhibitory action by Lineweaver-Burk coordinates for endophytic inhibitors poorly studied, while several scientific reports highlight the inhibitory action on α -amylase of plant phytochemicals [19, 20]. For example, traditionally used antidiabetic medicinal plants of Mauritius were studied for amylases kinetics *in vitro*. In particular, it was found that in the presence of methanol extracts of two plants (*Elaeodendron orientale* and *Antidesma madagascariensis*) decrease in both K_m and the velocity V_{\max} were observed. This tends to suggest an uncompetitive mode of inhibition. In contrast, methanol extracts of *Erythroxylum laurifolium* was found to follow mixed type of inhibition. Mixed inhibitor bind to free and to substrate bound enzyme and interfere with binding and catalysis of substrate, increasing affinity and decreasing reaction rate [18].

There is also report about influence on α -amylases kinetics of *Syncephalastrum* sp. isolated from medicinal plant *Adathoda beddomei*. It was shown that crude extract of mycelium inhibited α -amylase activity for 72.5%. Mode of inhibition of mycelial crude extract on amylase activity was determined by means Lineweaver-Burk plot analysis of data according Michaelis-Menten kinetics and appeared to be competitive (K_m increases whereas V_{\max} remains the same [21]).

4. Conclusion

Thus, the presented data demonstrated that studied extracts of *Penicillium sp.-CC200*, *A. egypticus-HT166S* and *A. terreus-AF104S* isolated from anti-diabetic plants *Helianthus tuberosus*, *Celosia cristata* and *Allium filidens* are active α -amylase inhibitors with uncompetitive and competitive modes of action. Pertaining to the role of α -amylase in the control of postprandial hyperglycemia, selected endophytes could be alternative source for application in development of new antidiabetic strategies.

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