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Kinetics of Hypoglycemic α-Glucosidase Inhibitory Protein

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The activity of α-glucosidase enzyme (EC 3.2.1.20) was inhibited by 40.3% by an extracellular inhibitory protein which isolated from the culture filtrate of *Aspergillus brasiliensis* ATCC-16404. The maximum activity of this protein and fungal mycelial dry weight were obtained at 28°C, 7 days, 6.5, 400 µl/ml, 140 rpm, and rice straw as the optimum incubation temperature, incubation period, pH value, inoculum size, agitation speed, and raw material respectively. The α-glucosidase inhibitory protein (AGIP) was purified and separated electrically as a single band at 40 KDa. There 17 amino acids of the purified protein were determined at different concentrations, where threonine has a highest percentage (90%) and aspartic acid has a lower percentage (24%). Kinetics of AGIP were determined, where V_{max}, K_m, k_{cat}, and catalytic activity values were exactly calculated using Lineweaver-Burk plot compared with those of acarbose as an inhibitor standard. The maximum catalytic activity (7.808 $M^{-1}S^{-1}$) at 0.1 mg/mol was higher than that of acarbose (7.783 $M^{-1}S^{-1}$) at the same concentration.

Keywords: Antidiabetics, diabetes mellitus, enzyme kinetics, protein purification.

Diabetes mellitus (DM) is a chronic disease that arises due to several metabolic disorders of vital processes in human body. The main character of this disease is blood hyperglycemia due to either completely impaired/ insufficient insulin secretion or insufficient insulin absorption due to some disorders in the receptors-insulin attachment[1]. Although DM can be controlled by specific antidiabetic agents, it has serious complications with uncontrolled post-prandial blood glucose level, such as diabetic foot due to serious neuropathy, renal failure, blindness due to serious retinopathy, and cardiovascular diseases[2, 3]. Therefore, DM control is an urgent medical approach to maintain a body health and reduce the developed mortality. There are different antidiabetic mechanisms according to the type of DM. Among these mechanisms is an inhibition of starch-hydrolyzing enzymes that uses with type 2 of DM to reduce glucose liberation in the blood, such as α-amylase (3.2.1.1.), α-glucosidase (EC 3.2.1.20), and β-glucosidase (3.2.1.21)[4, 5]. There is specific function for each starch-hydrolyzing enzyme; for instance, α-amylase converts the starch polysaccharide to oligo and disaccharides, which in turn convert to glucose molecules by α-glucosidase enzyme[6]. The medication market has different pharmaceutical products of α-glucosidase inhibitors, such as acarbose that inhibits α-amylase and α-glucosidase enzymes[7], miglitol and voglibose that inhibit α-glucosidase only[8-10]. All of them are powerful hypoglycemic agents because they can reduce an intestinal starch absorption[11]. Although these products have the same antidiabetic mechanism, but they have different pharmacological features; acarbose and voglibose no have absorbed through the intestine and are excreted through the fecal route; however, miglitol is easily absorbed from the upper part of the intestine and excreted through the urinogenital tract. Nevertheless, all of them have low tissue affinity and variable protein binding[10]. Although

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α-glucosidase inhibitors are useful products as antidiabetics, they have some adverse events such as flatulence, diarrhea, and abdominal pain due to accumulation of undigested disaccharides in the intestine[12]. Interestingly, the liver functions should be checked along the treatment course of α-glucosidase inhibitors because they may be affected. Although the antidiabetic efficiency of α-glucosidase inhibitors, they can't be used as monotherapy due to an insufficient effect especially with uncontrolled type 2 of DM; hence, insulin or sulfonylureas may be combined to full control hyperglycemia[13]. Uncontrolled DM has common symptoms such as increased thirst, polyuria, blurring of vision, recurrent infections, and weight loss. However, in the severe cases other serious symptoms are developed especially with the children and adolescents, such as glycosuria, ketonuria, drowsiness, coma, and death[14]. This study aims to produce and characterize a hypoglycemic AGIP that produced as a secondary metabolite by *Aspergillus brasiliensis* ATCC-16404.

MATERIALS AND METHODS

Subculturing and maintenance of test microorganism

The test microorganism that used in this work is well identified filamentous fungal strain called *A. brasiliensis*, which brought from American Type Culture Collection Laboratory (ATCC) with a code number (16404) to be *A. brasiliensis* ATCC-16404. The subculturing of this strain was carried out by using potato dextrose agar (PDA-Difco) as a nutritive medium, and then incubated at 28°C for 5-7 days. The strain was maintained in the refrigerator at 4°C, and the subculturing had been repeated every one month.

Preparation of fungal cell free extract

The conical flask (250 ml) contained 100 ml sabouraud broth medium (Oxoid) (pH 6.5) was prepared and autoclaved, and then inoculated with *A. brasiliensis* ATCC-16404 culture aged at 7 days. The inoculation had been carried out with shaking at 28°C and 140 rpm for 7 days. The fungal mycelia were separated and discarded by filtration a fungal suspension through a filter paper (Whatman No. 41). The free-mycelial filtrate was centrifuged at 15 000 xg for 10 min at 4°C to discard the pellet and collect the supernatant contained an extracellular α-glucosidase inhibitor (cell free extract).

Assay of α-glucosidase inhibitory protein activity

The activity of AGIP was colorimetrically measured at 405 nm by using UV-Spectrophotometer[15]. The α-glucosidase enzyme and its substrate viz, *p*-nitrophenyl-α-d-glucopyranoside (SIGMA) were used. The substrate solution was prepared by dissolving 0.5 mg substrate in 0.1 M phosphate buffer (pH 6.8). On the other hand, the enzyme solution was prepared by dissolving 0.2 u/ml α-glucosidase in 0.1 M phosphate buffer (pH 6.8). The mixture of an enzymatic reaction was prepared by addition 50 µl enzyme solution and 20 µl cell free extract contained an enzyme inhibitor. The mixture was incubated at 37°C for 5 min to allow an enzyme inhibition. The mixture was supplemented with 30 µl substrate and then incubated at 37°C for 20 min to start the enzymatic reaction, which then stopped by addition 100 µl of 0.1 M sodium carbonate solution (pH 9.8). The control mixture was prepared by addition 50 µl enzyme solution and 30 µl substrate, and then incubated at 37°C for 20 min. The absorbance of *p*-nitrophenol as a product of an enzymatic reaction was measured. The blank solution was a phosphate buffer (pH 6.8). The inhibition ratio was calculated by using the following formula:

$$\text{Inhibition ratio} = \frac{OD\ (sample) - OD\ (blank)}{OD\ (control) - OD\ (blank)} \times 100$$

Optimization of AGIP activity

Effect of incubation period

Ten conical flasks (250 ml) contained 100 ml sabouraud broth media (Oxoid) at pH 6.5 were inoculated with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 28°C and 140 rpm for different incubation periods (3, 5, 7, 9, 11, 13, 15, 17, 19, and 21 days). The AGIP activity and fungal dry weight were determined at each incubation period.

Effect of incubation temperature

Ten conical flasks (250 ml) contained 100 ml sabouraud broth media (Oxoid) at pH 6.5 were inoculated with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 140 rpm for different incubation temperature (20, 22, 24, 26, 28, 30, 32, 34, 36, and 38°C) for 7 days.

The AGIP activity and fungal dry weight were determined at each incubation temperature.

Effect of pH

Ten conical flasks (250 ml) contained 100 ml sabouraud broth media (Oxoid). Each flask has been adjusted at a definite pH (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, and 8.5). All flasks were inoculated with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 140 rpm and 28°C for 7 days. The AGIP activity and fungal dry weight were determined at each pH value.

Effect of inoculum size

Ten conical flasks (250 ml) contained 100 ml sabouraud broth media (Oxoid) at pH 6.5 were inoculated by different inocula (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 µl) with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 28°C and 140 rpm for 7 days. The AGIP activity and fungal dry weight were determined at each inoculum size.

Effect of agitation speed

Ten conical flasks (250 ml) contained 100 ml sabouraud broth media (Oxoid) at pH 6.5 were inoculated with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 28°C and at different agitation speeds (60, 80, 100, 120, 140, 160, 180, 200, 220, and 240 rpm) for 7 days. The AGIP activity and fungal dry weight were determined at each agitation speed.

Effect of raw material

Five conical flasks (250 ml) were supplemented with 5 g of five different raw materials (rice straw, molasses, wheat husk powder, potato peel powder, and milk straw) dissolved in 100 ml distilled water. The mixture pH was adjusted at 6.5. All flasks were inoculated with *A. brasiliensis* ATCC-16404 aged at 7 days, and then incubated with shaking at 28°C and at 140 rpm for 7 days. The AGIP activity and fungal dry weight were determined at each raw material.

Purification of AGIP

There 100 g rice straw were added to 2000 ml distilled water to make a fermentation batch adjusted at pH 6.5. The batch was autoclaved, cooled, and then inoculated with 400 µl/ml *A. brasiliensis* ATCC-16404, and then incubated with shaking at 28°C and 140 rpm for 7 days. The fungal cell free extract was obtained as described above. The proteins of fungal cell free extract included AGIP were precipitated by using saturated ammonium sulfate, which supplemented gently and sequentially at concentrations range (10 to 90%) at 4°C. The mixture was centrifuged at 15 000 xg for 10 min, where the supernatant was discarded and the precipitated proteins were dissolved in 5 ml phosphate buffer (pH 7.5). The total protein content of the cell free extract (control) and of each fraction was estimated according to Lowery *et al*.[16]. The AGIP activity was assayed for each fraction. The active fractions were pooled and passed through di-ethyl-amino-ethyl cellulose and sephadex G 200 to obtain a purified α-glucosidase inhibitory protein[17, 18].

Determination the molecular weight and amino acids sequence of AGIP

The molecular weight of AGIP was determined by using sodium-dodecyl-sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) according to Blackshear[19]. The amino acids sequence of α-glucosidase inhibitory protein was determined by using HPLC according to Golden *et al*.[20].

Determination the kinetic parameters of AGIP

The kinetic parameters (V_{max}, K_m, k_{cat}, and catalytic activity) of AGIP were determined according to Kim *et al*.[21]. Where, constant concentration of α-glucosidase enzyme was incubated with different concentrations of *p*-nitrophenyl-α-d-glucopyranoside at 37°C for 15 min, in the absence or presence of AGIP. The kinetic parameters of α-glucosidase inhibitory protein were determined by using Lineweaver-Burk plots[22] and Michaelis-Menten equation and its reciprocal. All the determinations were performed in triplicate.

Michaelis-Menten equation

$$v_o = \frac{Vmax\,[S]}{Km + [S]}$$

Reciprocal of Michaelis-Menten equation

$$\frac{1}{v_o} = \frac{Km}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax}$$

RESULTS AND DISCUSSION

A. brasiliensis ATCC-16404 was screened to inhibit the activity of α-glucosidase enzyme, where the inhibition ratio was 40.3%. The α-glucosidase inhibitors can be produced by

fungi, such as *Colletotrichum* sp.[23], *Aspergillus aculeatus*[24], and *Aspergillus oryzae*[25]. Singh and Kaur[26] stated that, both α-amylase and α-glucosidase inhibitors are produced by an endophytic *Aspergillus awamori* that isolated from *Acacia nilotica*. Although AGIP may be produced as extracellular or intracellular secondary metabolite, no an extracellular activity for AGIP that produced by *A. oryzae* N159-1 was observed. However, only an intracellular activity for AGIP was observed at 48.3%[27]. The recent approach of DM type 2 control has been interested on α-glucosidase inhibition by using either natural or synthetic inhibitors[28-30]. Inhibition of starch hydrolyzing enzymes is one of the antidiabetic mechanisms that reduce the glucose absorption

Table 1. Determination of optimum environmental and nutritional factors of AGIP

Parameters	Effect of incubation period (day)									
	3	5	7	9	11	13	15	17	19	21
Inhibition %	2.4	3.2	3.8	3.4	3.1	2.6	2.1	1.6	1.1	0.7
Dry weight (mg/ml)	1.2	2.9	3.4	3.2	2.8	2.5	2.1	1.8	1.5	1.2
Parameters	Effect of incubation temperature (°C)									
	20	22	24	26	28	30	32	34	36	38
Inhibition %	1.9	2.4	3.0	3.6	4.0	3.7	3.4	2.8	2.3	1.7
Dry weight (mg/ml)	1.5	1.9	2.4	2.9	3.6	3.1	2.6	2.2	1.7	1.3
Parameters	Effect of pH values									
	4	4.5	5	5.5	6	6.5	7	7.5	8	8.5
Inhibition %	2.2	2.5	2.9	3.3	3.5	3.9	3.7	3.2	2.6	2.1
Dry weight (mg/ml)	1.6	2.2	2.6	2.9	3.2	3.5	3.1	2.7	2.1	1.8
Parameters	Effect of inoculum size (µl/ml)									
	50	100	150	200	250	300	350	400	450	500
Inhibition %	2.0	2.4	2.7	3.0	3.4	3.6	3.9	4.1	3.7	3.3
Dry weight (mg/ml)	1.2	1.6	1.9	2.4	2.8	3.0	3.3	3.7	3.2	2.7
Parameters	Effect of agitation speed (rpm)									
	60	80	100	120	140	160	180	200	220	240
Inhibition %	2.2	2.7	3.2	3.7	4.1	3.6	3.1	2.7	2.4	2.1
Dry weight (mg/ml)	1.4	1.8	2.7	3.4	3.7	3.1	2.7	2.4	2.1	1.6
Parameters	Effect of raw materials									
	Rice straw		Molasses husk		Wheat peel		Potato straw		Milk	
Inhibition %	4.3		4.1		3.8		3.2		2.7	
Dry weight (mg/ml)	3.4		3.0		2.5		2.1		1.7	

Table 2. Purification table of AGIP

$(NH_4)_2SO_4$ (%)	Activity (u)	Total protein (mg/ml)	Specific activity (u/mg)	Purification fold	Yield (%)
Control	220	170	1.3	1.0	100
10	0.0	4.7	0.0	0.0	0.0
20	0.0	5.2	0.0	0.0	0.0
30	0.0	5.8	0.0	0.0	0.0
40	180	6.2	29	22.3	81.8
50	150	6.8	22	16.9	68.1
60	110	7.1	15.5	11.9	50
70	0.0	7.4	0.0	0.0	0.0
80	0.0	7.6	0.0	0.0	0.0
90	0.0	7.9	0.0	0.0	0.0

and then decrease the blood hyperglycemia. We had to produce a sufficient amount of an extracellular inhibitory protein so we could study the kinetic parameters. The highest activity and productivity were obtained at 28°C, 7 days, 6.5, 400 µl/ml, 140 rpm, and rice straw as optimum incubation temperature, incubation period, pH, inoculum size, agitation speed, and raw material respectively (Table 1). Min-Gu *et al*.[27] reported that, the maximum yield and activity of AGIP that produced by *A. oryzae* N159-1 was obtained at 27°C and 5 days as the beat incubation temperature and incubation period respectively. However, the maximum mycelium dry weight was obtained at 7 days of incubation, Furthermore, tryptic soy broth was recorded as the best growth medium, at which AGIP activity was 65.9%, followed by yeast extract-malt extract, yeast-peptone-dextrose, and potato-dextrose media at 52.1%, 49.1%, and 48.1% respectively.

The fermentation batch was prepared according to the determined optimum conditions, and then the cell free extract contained an extracellular AGIP was obtained. The purification process was carried out by using both ion-exchange and gel filtration column chromatographies. All proteins included active ones were precipitated by using saturated ammonium sulfate at different concentrations (10–90%). The total activity of AGIP (u), total protein content (mg/ml), specific activity (u/mg), purification fold, and yield percentage were calculated at each fraction of ammonium sulfate concentration and fungal cell free extract (control). The results proved that, the AGIP was precipitated at three ammonium sulfate concentrations (40–60%); however, the highest activity (180 u/ml) and specific activity (29 u/mg) was observed at 40% (Table 2). The active fractions were pooled and passed through ion-exchange and gel filtration column chromatographies to obtain a purified protein. Min-Gu *et al*.[27] stated that, there different AGIPs that produced by *A. oryzae* N159-1 were purified by sephadex G 25, and seprated electrically at different molecular weights (3–50 KDa). The activities of these proteins were measured, where the highest one (69.9%) was observed with a lower molecular weighted protein at 3 KDa. The peptide nature of this protein was detected by using the pepsin enzyme under controlled conditions, where the activity of AGIP was increased.

The purified AGIP was separated as a single band at 40 KDa by using SDS-PAGE (Figure 1). Min-Gu *et al*.[27] stated that, the purified AGIP that produced by *A. oryzae* N159-1 was separated as a single band at 36 KDa. The amino acids sequuence and their percentages of the purified AGIP were determined by using HPLC (Figure 2), where 17 amino acids were present; Asp acid (24%), Glu acid (47%), Ser (35%), Gly (60%), His (28%), Arg (68%), Thr (90%), Ala (72%), Pro (49%), Tyr (29%), Val (33%), Mth (30%), Cys (25%), Ile (43%), Leu (41%), Phe (34%), and Lys (70%).

The values of V_{max} and K_m, of AGIP were exactly determined from Lineweaver-Burk plots by using Michaelis-Menten equation and its reciprocal, and then the values of k_{cat}, and catalytic activity were calculated. These kinetic parameters had been compared with those of acarbose as an inhibitor standard (Table 3). There narrow range of an inhibitor concentration (0.05–0.15 mg/mol) was used. The results proved that, the values of V_{max} and K_m of acarbose and AGIP increased gradually with the increasing of an inhibitor concentrations until 0.1 mg/mol, and then decreased at 0.15 mg/mol. However, the V_{max} and K_m values of acarbose were slightly higher than those of AGIP. Interestingly, the catalytic activity values of AGIP and acarbose at 0.1 mg/mol were 7.808 $M^{-1}S^{-1}$ and

Table 3. Determination of the kinetic parameters of AGIP and acarbose

Kinetic parameters	Acarbose (mg/mol)				AGIP (mg/mol)			
	0.00	0.05	0.1	0.15	0.00	0.05	0.1	0.15
V_{max} (mM/min^{-1})	0.82	1.34	1.92	0.78	0.78	1.17	1.68	0.74
K_m (mM)	1.24	1.72	2.47	0.89	1.05	1.54	2.15	0.82
k_{cat} (Sec^{-1})	0.00	26.8	19.2	5.22	0.00	23.5	16.8	4.94
Catalytic activity ($M^{-1}S^{-1}$)	0.00	15.5	7.78	5.86	0.00	15.2	7.80	6.02

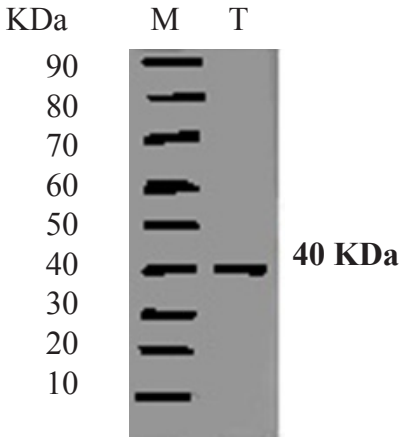

Fig. 1. Agar gel electrophoresis of AGIP

7.783 $M^{-1}S^{-1}$ respectively, where the former was slightly higher than the latter. Sahere *et al*.[31] stated that, the Lineweaver-Burk analysis revealed that inhibition patterns of AGIPs are confused, where $K_m^{app} > K_m$; i.e. K_m is increased with binding of an inhibitor with the free enzyme. On the other hand, $K_m^{app} < K_m$; i.e. K_m is decreased with binding of an inhibitor with enzyme–substrate complex. The binding of an inhibitor with a region on an enzyme rather than an active site, enables that inhibitor to be confused to increase the inhibition specificity of an inhibitor compared with acarbose as a competitive inhibitor[32]. Ghadyale *et al*.[33] and Zhang *et al*.[32] reported that, increasing of carbohydrate concentrations requires increasing of acarbose concentrations as a competitive inhibitor; however, with the confused inhibitors the inhibition activity still lower.

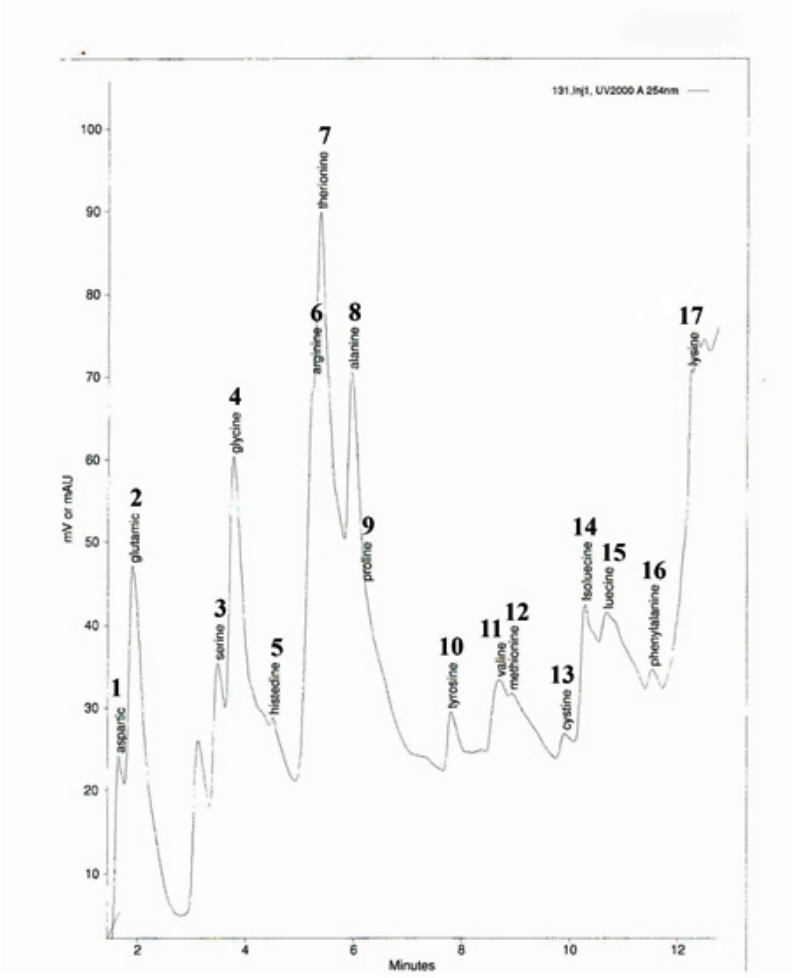

Fig. 2. Amino acids sequencing of AGIP

CONCLUSION

This study proved that α-glucosidase as one of starch hydrolyzing enzymes has been inhibited at 40.3% by AGIP that produced by *A. brasiliensis* ATCC-16404. The maximum activity and productivity of AGIP as well as fungal mycelial dry weight were obtained at optimum environmental conditions. The AGIP was purified and separated electrically as a single band at 40 KDa. The amino acid sequence of the purified AGIP was determined, where therionine and aspartic acid were recorded as higher and lower amino acids concentrations respectively. Both of V_{max} and K_m values of AGIP were determined by linear Lineweaver-Burk plot, and then k_{cat}, and catalytic activity values can be calculated. These kinetic parameters were compared with those of acarbose as a competitive or standard inhibitor.

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