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Biochemical studies on α-amylase inhibitor extracted from white kidney bean (*Phaseolus vulgaris)*

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ABSTRACT

Crude extract of white kidney beans (Phaseolus vulgaris) showed inhibitory activity against porcine pancreatic α-amylase and human salivary α-amylase. Alpha amylase inhibitors are substances which alter the catalytic action of alpha amylase on starch and consequently slow down or stop the breakdown of starch. The obtained extract was examined qualitatively for phytochemical constituents and total protein content was determined by Lowry method. The crude extract was also studied for α-amylase inhibition activity which showed a linear relationship between the percentage of inhibition and inhibitor concentrations. Optimum temperature was 37ºC, optimum periods for preincubation and incubation were 15 and 60 min. Nearly 90 % of inhibition was occurred after 60 min from the beginning of incubation. Kidney bean crude extract inhibits porcine pancreatic α-amylase and human saliva α-amylase in a non-competitive manner.

Keywords: Inhibitor, Alpha- amylase, kidney beans, non-competitive inhibition.

INTRODUCTION

Phaseolus vulgaris comprises several varieties of beans: white, black, pinto, navy, and northen beans, (Duke 1985).

Amylase inhibitors (also known as starch blockers) have been used to promote weight loss in over weight individuals. The initial amylase inhibitors are not very effective in limiting carbohydrate absorption, (Bo-linn *et al.,* 1982; Carlson *et al.,* 1983; Garrow *et al.,* 1983 and Hollenbeck *et al.,* 1983). However, development of highly concentrated versions of amylase inhibitors, including the extract of the white kidney bean resulted in more potent α-amylase inhibiting activity in vitro (Layer *et al.,* 1986). More effective inhibition of carbohydrate absorption have been showed in humans (Layer *et al.,* 1985; Boivin *et al.,* 1987 and Brugge and Rosenfeld 1987).

Diabetes mellitus is one of the world's major diseases, with an estimation of 347 million adults affected in 2011 (Danaei *et al.,* 2011). Type 2-diabetes mellitus, by far the most common type, is a metabolic disorder of multiple etiology characterized by carbohydrate, lipid and protrein metabolic disorders that includes defect in insulin secretion, almost always with a major contribution of insulin resistance (Alberti and Zimmet, 1998). These abnormalities could lead to lesion such as retinopathy, neuropathy and angiopathy, (Holman *et al.,* 2008). The inhibition of carbohydrate digestive enzymes is considered a therapeutic tool for the treatment of type 2 diabetes (Tundis *et al.,* 2010). The most important digestive enzyme is pancreatic alpha-amylase (EC 3.2.1.1), a calcium metalloenzyme that catalyses the hydrolysis of the alpha-1, 4 glycosidic linkages of starch, amylase,

amylopectin, glycogen and various maltodextrins and is responsible of most of starch digestion in humans.

Common beans have 3-isoforms of alpha amylase inhibitor (Alpha-A1, Alpha-A2, and Alpha-A1L). The alpha-A1 isoform has anti-amylase activity in humans. Alpha-amylase inhibitors with activity against mammalian forms of the enzyme are present in plants and it is suggested that they were developed by plants in order to strengthen their defense against predators.

Plant constituents with enzymatic inhibitory activity include polyphenolic compounds and glycoproteins (Tundis *et al.,* 2010 and Moreno *et al.,* 1990). For example, anthocyanins and ellagitannins present in raspberries and strawberries have been reported to inhibit alpha-glucosidase and alpha-amylase activity, respectively, (McDougal and Stewart, 2005). In addition, flavins and catechins present in green and black teas have been reported to inhibit alpha-amylase and alpha-glucosidase activity as well as retard starch digestion in an in-vitro model, (Koh *et al.,* 2010). Alpha-amylase inhibitors are also present in grains, including wheat and rice (Tundis *et al.,* 2010). However, the greatest body of research has gone into glycoproteins extracted from kidney beans (Phaseolus vulgaris) and more specifically on the proprietary phase 2 product. Weiner *et al.,* (2008) stated that salivary alpha-amylase activity showed a significant inhibition following exposure to cigarette smoke and this may be due to the interaction between aldehydes which present in cigarette smoke and –SH groups of the enzyme. The objective of this study was to evaluate the α-amylase inhibitory potential of P. vulgaris extract.

MATERIALS AND METHODS

Materials:

1. White kidney beans (Phaseolus vulgaris) were purchased from the local market.
2. Porcine pancreatic α-amylase (PPA) was obtained from Sigma Chemical Company, ST. LOUIS, U.S.A.
3. Human saliva α-Amylase.
4. Soluble starch.

Methods:

Preparation of crude α-amylase inhibitor:

α- Amylase inhibitor, crude extract,was extracted from white kidney bean as suggested by Yamaguchi (1991). α- Amylase inhibitor was extracted in a solution of NaCl followed by ammonium sulphate precipitation, resulted supernatant was dialyzed against sodium phosphate (10 mM pH 6.0, containing 15% ammonium sulphate). Obtained extract was kept at 4ºC for further analysis.

Preliminary Phytochemical Screening:

Crude extract of the α-amylase inhibitor was screened for the presence of various phytoconstituents, namely: Tannins, alkaloids, saponins, carbohydrates and /or glucosides and proteins as described in Harbone (1988).

Determination of protein:

Protein concentration of α-amylase inhibitor was determined using the method of Lowry *et al.* (1951).

Alpha-amylase inhibition assay:

The α-amylase inhibitory activity for the crude extract was determined spectrophotometrically assay as reported by Hossain *et al.* (2008). Known volumes of the crude extract (100, 200, 300, 450 and 600 µl) were completed to a total volume of 780 µl by the addition of phosphate buffer 20 mM pH 6.9 containing 6 mM sodium chloride. Porcine pancreatic α-amylase solution (10 µl containing 0.4 units) was added, separately. Samples were preincubated at 37ºC for 15 min, then 200 µl of 1% buffered starch solution were added and the mixture was re-incubated for 1 h. Iodine solution 1% (I_2/KI) was added (100 µl), followed by 5 ml of distilled water. Absorbance of the obtained colour was measured at 565 nm.

Percentage of inhibition was calculated from the equation:

% of inhibition = (A-C)/ (B-C) ×100

Where: A = absorbance of the sample, B = absorbance of blank (containing no enzyme) and C = absorbance of control (containing no starch).

Evaluation the Type of inhibition:

Type of inhibition was evaluated for porcine pancreatic α-amylase and human saliva α-amylase.

1. Crud Extract of α-Amylase Inhibitor:

Previously obtained extract from kidney bean was used during this investigation.

2. Enzyme assay:

Enzyme activity (for both enzymes) was estimated according to **Marshall and Lauda (1975)**, by determination of released reducing sugars from starch as specific substrate. The released reducing sugars were determined by the method of **Somogi (1952)**.

3. Effect of substrate concentration on the velocity of enzymatic activity:

Enzyme activity was assayed using three different concentrations of starch, i.e. 2.5 mg, 6.25 mg and 12.5 mg as specific substrate for porcine pancreatic α-amylase and human saliva α-amylase. Enzyme activity was determined in the absence and in the presence of 100 µl and 150 µl of enzyme inhibitor. The obtained results were used to realize Michaelis and Menten equation (1913). Lineweaver and Burk method (1934) was used to evaluate the type of inhibition.

Stoichiometry of inhibition:

The stoichiometry of inhibitor-amylase interaction was investigated as described by Marshall and Lauda (1975) with a slight modification for the determination of remaining substrate (1% buffered starch) as follows: Porcine pancreatic α-amylase was added to the crude inhibitor extract in digest containing acetate-citrate buffer 6.7 mM pH 5.5, human serum albumin and calcium chloride. Residual of α-amylase activity was determined after durations of preincubation by addition of buffered starch solution; samples were removed for iodine staining measurements.

The formula postulated by Bird and Hopkins (1954) was used to calculate the percentage of inhibition as follows:

% of inhibition = D $[R_0 - R]/R_0 \times 100$

Where: R_0 = Absorbance of substrate-iodine complex in the absence of enzyme (E= 680 nm), R= Absorbance of digest (at the same wave length), and D = Dilution factor of enzyme.

RESULTS AND DISCUSSION

Earlier reports (Hernandez and Jaffe, 1968 and Bowman, 1945), have indicated that specific proteinaceous inhibitors of α-amylase are widespread in kidney beans (Phaseolus vulgaris) and other legumes. Since α-amylase plays an important role in starch breakdown in human beings and animals, the presence of such inhibitors in foodstuffs may be responsible for impaired starch digestion.

α-Amylase inhibitors may be valuable as novel therapeutic and dietetic agents (Puls and Keup, 1973).

The obtained extract from white kidney bean after dialysis is considered as the crude extract of α-amylase inhibitor.

The results of the phytochemical screening conducted on the extract of kidney bean are present in table (1).

Obtained data showed the presence of: tannins, saponins, alkaloids, carbohydrates and / or glycosides and proteins.

Table (1). Phytochemical constituents of α-Amylase Inhibitor crude extract.

Component	Tannins	Saponins	Alkaloids	Glycosides	Proteins
Crude extract	+	+	+	+	+

Several authors attributed the inhibitory effects, of the α-amylase inhibitor, to the presence of some chemical constituents. Kumar *et al.* (2011) showed the presence of carbohydrates, steroids, alkaloids, phenolic compounds, flavonoids, saponins and amino acids in their studies about α-amylase inhibition in Amaranthus spinosus. Kazeem *et al.* (2013) found that flavonoids, reducing sugars, saponins, steroids, tannins and terpenoids are presented in ethanol and water extracts of Picralima nitida leaf.

Boivin *et al.* (1987) and Koh *et al.* (2010) suggested that catechins in green, oolong and black teas may be responsible for its activity against human salivary α-amylase. Phenolic compounds, also, are a large group of structurally diverse naturally occurring compounds that possess at least a phenolic moiety in their structures. Kandra *et al.* (2004) reported that the main inhibitory effects of the tannins are related to its ability to bind strongly with carbohydrates and proteins. They suggested that the interaction between tannins, such galloylated quinic acid, and human α-amylase is correlated with free OH groups in the tannins that are able to participate in hydrogen bonding.

Alpha-amylase inhibition assay:

Percentage of inhibition was measured using the method of Hossain *et al.* (2008).Fixed amount of porcine pancreatic α-amylase was added to different volumes of extract (100, 200, 300, 450, 600 µl) containing different protein concentration (17.42,35,52.3,87.39 and 104.5mgprot.respectively).All samples were pre-incubated for 15 min at 37ºCin order to form the enzyme-Inhibitor complex (EI).

Data are shown in table (2) and fig. (1a and 1b), following points could be deduced:

1-Maximum inhibition (100 %) was occurred when 600 µl (containing 104.5 mg protein) of crude extract was used.

2-Minimum inhibition (19%) was recorded when 100 µl (17.42 mg protein) of crude extract was used.

3-Finally, it seems that IC_{50} occurred when 281.79 µl (49.1 mg protein) from crude inhibitor extract was used.

Table (2). Percentage of inhibition using different concentrations of inhibitor.

Inhibitor concentration		% of inhibition	IC_{50}
volume of inhibitor (µl)	mg protein inhibitor		
100	17.42	19.00	281.79 µl (49.10 mg prot.)
200	35.00	38.90	
300	52.30	56.00	
450	87.39	79.60	
600	104.50	100.00	

IC_{50} = concentration of the extract required to inhibit 50% of α-amylase activity

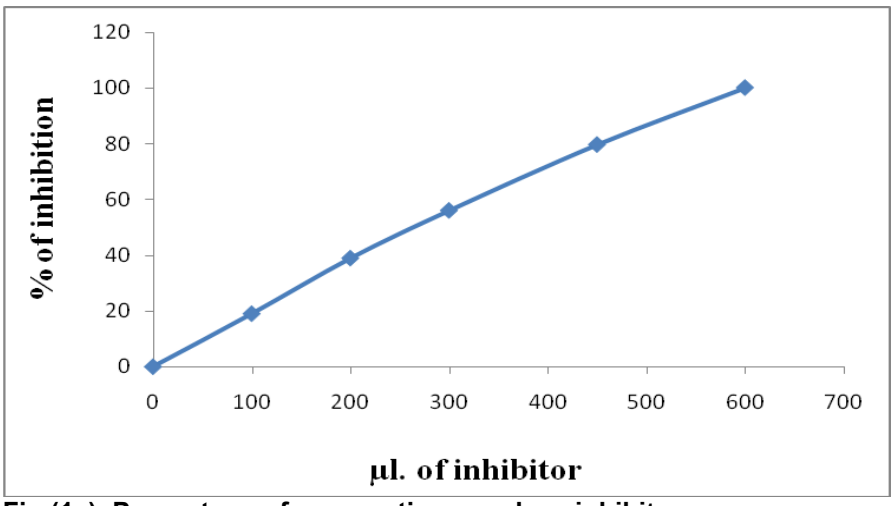

Fig (1a). Percentage of pancreatic α-amylase inhibitor

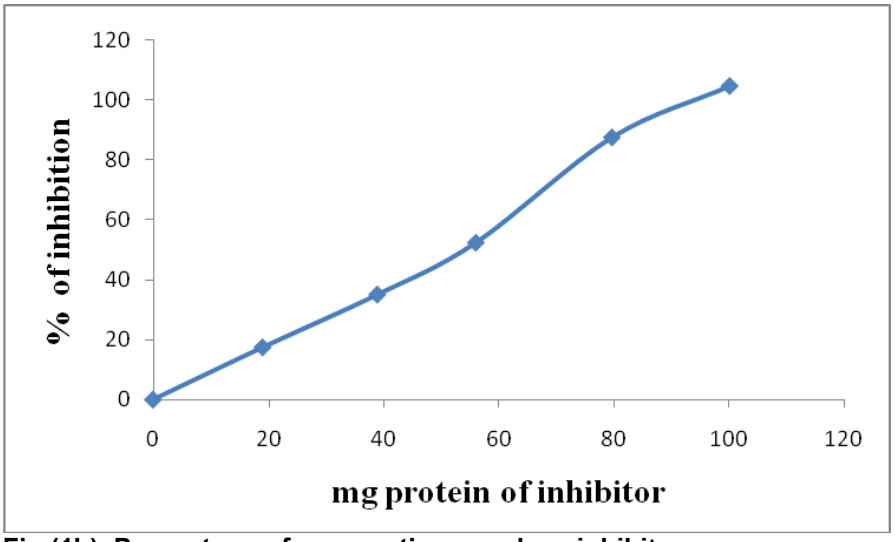

Fig (1b). Percentage of pancreatic α-amylase inhibitor

Type of Inhibition:

α-Amylase enzyme activity was determined for porcine pancreatic α-amylase and human saliva α-amylase by using different concentrations of buffered starch. Enzyme activities were determined in the absence and presence of two levels of α-amylase inhibitor [(100µl – 17.42 mg prot. and 150 µl – 26.13 mg prot.)], then 100 µl of porcine pancreatic α-amylase containing 4 units of enzyme was used in determination of PPA activity, while 125 µl of dilute human saliva was used during the assay of human saliva α-amylase activity.

The obtained data were used to realize the relationship between the concentration of substrate (2.5, 6.25 and 12.5 mg) and enzyme activity to ensure the equation of Michaelis and Menten (1913).

To determine the type of inhibition the method of Lineweaver and Burk (1934) was used, by plotting the reciprocal of different substrate concentrations 1/[S] against the reciprocal of the velocity of enzymatic activity (1/v), in the absence and in the presence of 100 and 150 µl of α-amylase inhibitor crude extract, as shown in figures (2) and (3).

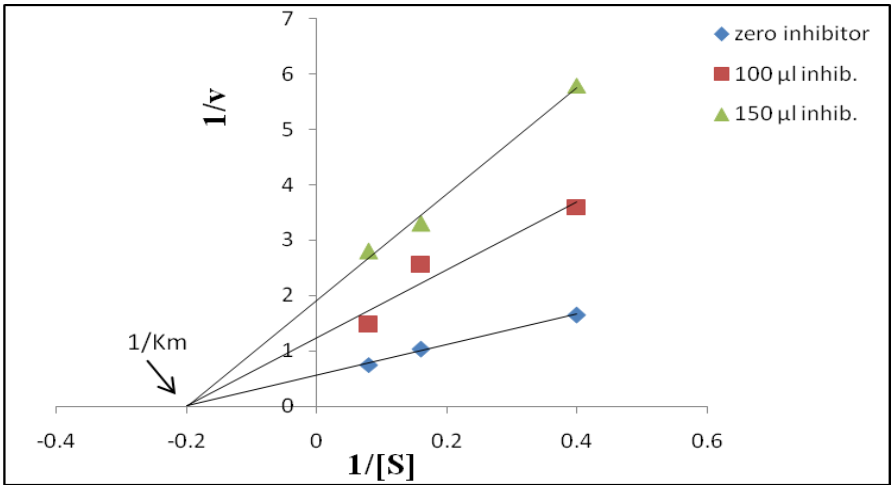

Fig (2). Lineweaver-Burk plot for activity of human saliva α-amylase in the absence and in the presence of inhibitors, 100 µl and 150 µl. The assay digest (5 ml) contained different concentrations of soluble starch (2.5, 6.25 and 12.5 mg), human serum albumin (0.15 mg), Calcium chloride (0.15 mg), sodium phosphate buffer (50 mM, pH 6.9) and enzyme (4 unit), with or without extract. [S] is expressed in mg/ml; V is micromoles of glucose equivalents per ml of enzyme per min.

Our data revealed that the type of inhibition which occurred in used enzymes was non-competitive. Same finding was obtained by Marshall and Lauda (1975), Frels and Rupnow (1985), Subbaramaiah and Sharma (1990), Cengiz *et al.* (2010), Mogale *et al.* (2011) and Kazeem *et al.* (2013).

As reported in Dixon and Webb (1964) inhibitor can influence the enzymatic reaction in such a way that the K_m is apparently increased or V is apparently decreased, or both parameters are apparently changed. The first case is known as competitive inhibition, and the second as non-competitive inhibition. Non competitive inhibition has been interpreted for one-substrate reaction as interference with the conversion of the enzyme-substrate complex into the reaction products, while the formation of the complex is not affected. The quantity of active enzyme present is thus apparently less and this is expressed in a decrease in V and increase in the value of K_m.

K_m values were: 5 mg and 6.6 mg, for human saliva and porcine pancreatic α-amylases, respectively. While V_{max} values were: 2.0, 0.83, and 0.56, for human saliva α-amylase, and were: 0.83, 0.50 and 0.31, for porcine pancreatic α-amylase.

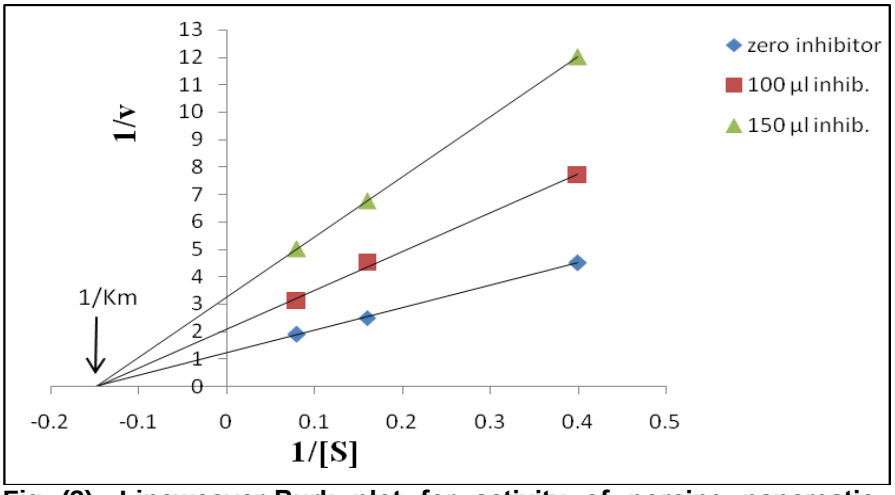

Fig (3). Lineweaver-Burk plot for activity of porcine pancreatic α-amylase in the absence and in the presence of inhibitors, 100 µl and 150 µl. The assay digest (5 ml) contained different concentrations of soluble starch (2.5, 6.25 and 12.5 mg), human serum albumin (0.15 mg), Calcium chloride (0.15 mg), sodium phosphate buffer (50 mM, pH 6.9) and enzyme (4 unit), with or without extract. [S] is expressed in mg/ml; V is micromoles of glucose equivalents per ml of enzyme per min.

The determination of the Stoichiometry of the crude extract:

Crude extract inhibitor, extracted from white Kidney beans (0.6 ml, contg. 107.52 mg. prot.) was pre-incubated with porcine pancreatic alpha-amylase (100 µl contg. 4 unit of enzyme) in digests containing acetate-citrate buffer, pH 5.5, 6.7mM, 0.15mg human serum albumin and 0.15 mg calcium chloride, for 15 min at 37º C. Residual alpha-amylase activity then was assayed using the Iodine staining method, after the addition of soluble starch solution buffered with phosphate buffer (pH6.9, 50 mM). Finally, digests incubated at 37ºC for different periods: 15, 30, 45, 60, 90, 105 and 120 min, (Fig.4).

Data obtained showed a considerable increase in percentage of inhibition. About 20 % of inhibition was obtained after 15 min from the beginning of incubation. Further increase was occurred by increasing period of incubation (nearly 90 %) when the digest was incubated for about 60 min. It could be seen also that, elongation of incubation period resulted in a considerable decrease in inhibition percentage. Fig (4) showed that incubation for 90, 105 and 120min led to an inhibition percentage of about 50, 40 and 35 % respectively. It seems that a reversible reaction was occurred after the formation of the Enzyme-Substrate-Inhibitor complex as illustrated by Bergmeyer (1978). As shown in Fig (4), the rate of reaction began to be constant, after 120 min from the beginning of incubation.

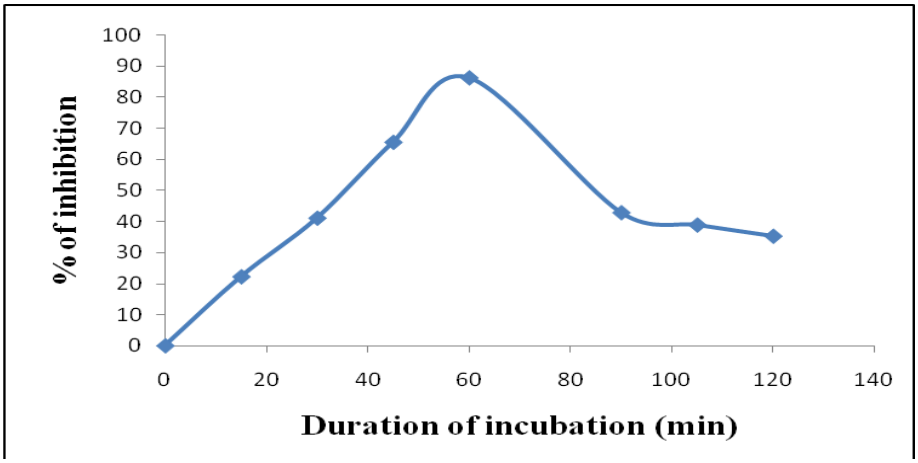

Fig (4). Effect of time of incubation on the activity of α-amylase inhibitor extracted from white kidney bean.

Our results did not agree with those obtained with Marshall and Lauda (1975), where they obtained the maximum of inhibition (45 %) after a period of 60 min from the beginning of incubation. This may be due to the purification of the inhibitor, as they reported, by using 3 different steps of Chromatography: DEAE Cellulose, Sephadex G- 100 and CM- Cellulose, while crude inhibitor was used during this investigation.

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دراسات كيمايئية حيويه على مثبط الالفا-اميليز المستخلص من بذور الفاصوليا.

لـــويس كامـــل تـــادرس، نظمـــى صـــبحى عريـــان، أحمـــد محمـــد يوســـف و نانسى أحمد عبد القادر طه
قسم الكيمياء الزراعية- كلية الزراعة – جامعة المنصورة.

تم استخلاص مثبط الالفا-اميليز من بذور الفاصوليا البيضاء و أظهر مستخلص الفاصوليا قدره تثبيطية تجاه انزيم الالفا-اميليز من مصدرين مختلفين (انزيم الالفا اميليز من بنكرياس الخنزير و انزيم اللعاب) . تقوم مثبطات الالفا-اميليز بتثبيط انزيم الالفا اميليز و بالتالى فانها تأخر عملية تحليل الكربوهيدرات الى سكريات بسيطه (mono and disaccharides). تم تقدير البروتين فى المستخلص و تقدير نشاط المثبط و كانت نسبة التثبيط ١٠٠% عند تركيز ٦٠٠ ميكروليتر(١٠٤.٥ ملجم بروتين)، و كانت IC_{50}عند 281.79 ميكروليتر- ٤٩.١ ملجم بروتين. كما وجد ان نشاط المثبط يقل بعد ٦٠ دقيقه من التحضين على درجة ٣٧ °م، حيث يكون اعلى نشاط عند ٦٠ دقيقه (حوالى ٩٠%) . و عند تقدير نوع التثبيط وجد ان المثبط يثبط انزيمات الالفا-اميليز بطريقه non-competitive .