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Studies on the Amylase Inhibitors from the Seeds of Adenanthera Pavonina

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An α-Amylase inhibitor was isolated and purified employing ammonium sulphate fractionation, molecular sieve chromatography on sephadex G-10 and G-50 and HPLC from the seeds of *Adenanthera pavonina*. The molecular weight was found to be 10 - 12 kDa as determined by gel-permeation chromatography on Sephadex G-100. The specific inhibitor activity, fold purity and the yield obtained for *Adenanthera pavonina* amylase inhibitor was 32.12, 51 and 13.07, respectively. The purified inhibitor was heat stable and retained more than 52% activity at 65 °C. The optimum pH obtained for purified inhibitor was 6.3 and 100% Zone of inhibition was observed when it was added on the plated organisms. The *Adenanthera pavonina* amylase inhibitor inhibited the activity of human salivary α-amylase and inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes and related nutritional problems results in obesity.

Keywords: Amylase Inhibitors, Isolation, Characterization, *Adenanthera pavonina* seeds**.**

Plants contain proteins with inhibitor activity against animals α-amyalses (Shainkin and Birk, 1970; Silano *et al.,* 1973) occur widely in plants. These amylase inhibitors make plants less palatable by inhibiting α-amyalses of animals thereby preventing the digestion and absorption of dietary starch. This is useful for treating metabolic disorders such as obesity and diabetes mellitus (Ali *et al.,* 2006; Asma Ali *et al.,* 2012). In plants, these enzyme inhibitors are storage or reserve proteins functions as regulators of endogenous enzyme or defensive agents against the attacks of animal predators and insect or microbial pests (Octavio and Rigden, 2002; Octivio and Rigden, 2000; Richardson, 1991). α -amylase inhibitors are attractive candidates in people with diabetes reduce the glucose peaks that can occur after a meal, slowing the speed with which alpha amylase can convert starch to simple sugars until the body can deal with it as well as in plants as a defence mechanism against insect pests highly dependent on starch as energy source (Franco *et al.,* 2000; Nagy-Gasztonyi *et al.,* 2010).

Adenanthera pavonina is a perennial and non-climbing species of leguminous tree. Its uses include food and drink, traditional medicine and timber. It is commonly called Red Lucky Seed and it is cultivated for forage. It is also grown as medicinal plant, an ornamental garden plant or urban tree. The young leaves and seeds can be cooked and eaten. This tree is used for making soap and a red dye can be obtained from the wood. Since the seeds are used as medicine and work on amylase inhibitor is scanty, the present work is undertaken. In the present study, partial purification and characterization of amylase inhibitor is described.

MATERIALS AND METHODS

Seeds of *Adenanthera pavonina* were procured from in and around of Puthige Panchayath of Kasaragod District of Kerala, India.

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Chemicals

Bovine Serum Albumin, α-amylase, Starch, Sephedex G-10 and G-50 were obtained from Sigma chemical company, USA. All other chemicals used were of technical grade.

METHODS

Preparation of acetone powder

The *Adenanthera pavonina* seeds were soaked, dehulled and acetone powder (10 %) was prepared according to the method of Wetter (1957). Soaked and dehulled seeds were blended in a homogenizer for 5 mins with chilled acetone and filtered using suction pump. The cake obtained was dried at 37° C, powdered and stored at 4°C until further use.

Preparation of crude extract

A 10 % extracts of acetone powder of *Adenanthera pavonina* was prepared by stirring on a magnetic stirrer using sodium phosphate buffer pH 7.0 for 1.5 hr followed by centrifugation at 10,000 rpm for 15 mins at 4° C. The supernatant was collected and used for qualitative and quantitative analysis of proteins/peptides for amylase inhibitory activity and antioxidant activity.

Ammonium sulphate fractionation

The crude extract was subjected to 0 - 90% ammonium sulphate precipitation. Solid powdered ammonium sulphate was added slowly with constant stirring over magnetic stirrer for 30 min at 4°C. The stirring was continued after the addition of ammonium sulphate salt for one hour. The solution was allowed to stand for 1hr. The precipitated protein was recovered by centrifugation at 10,000 rpm for 30min. The protein pellet was dissolved in small amount of 0.025 M sodium phosphate buffer, pH. 7.0.

Sephadex G -10 and G-50 gel-filtration chromatography

Sephadex G-10 and G-50 were allowed to swell in excess of distilled water on a boiling water bath for 5 hrs, separately. The gels were decanted to remove fines and then equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, separately. The gels were packed into columns of size 1.0 cm X 110.0 cm under gravity. The columns were equilibrated with two bed volumes of 0.05M sodium phosphate buffer, pH 7.0 and fractions were collected at a flow rate of 10 ml/hr. The concentrated Ammonium sulphate fractionation was loaded on to the Sephadex G-10 gel and the proteins were eluted with same buffer with a fraction volume of 2.0 ml. Quantitative analysis of proteins, amylase and protease inhibitor activity was done. The fractions containing amylase inhibitor activity were pooled and subjected to sephadex G-50 chromatography. The elution buffer and fraction volume were similar to sephadex G-10 chromatography. Quantitative analysis of proteins and amylase inhibitor activity was done and the peak fractions containing amylase inhibitor activity were subjected to further purification by RP-HPLC.

RP-HPLC

RP-HPLC is carried out on Reversed-phase octadecylsilica (C18) column using binary solvent system with binary gradient capability and a UV detector. Buffer A is 0.1% (v/v) TFA in water and Buffer B is 100% acetinitrile containing 0.1% (v/v) TFA. Column Equilibration and Blank Run was carried out using Buffer A with a flow rate of 1 mL/min at 220 and 280 nm respectively. Once the stable line is obtained, the sephadex G-50 precipitated sample was injected and eluted the sample with a linear gradient from 0 to 100% buffer B for 30 min.

Amylase activity

Quantitatively Amylase activity was determined by measuring liberated maltose using method of Bernfeld (1955). A typical Amylase assay mixture consists of 0.1 ml (100µg) of amylase enzyme with 0.9 ml of sodium phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 ml of 1% soluble starch at 37°C and stopped after 10 minutes by the addition of (DNS) dinitrosalisylic acid reagent (1.0 ml). After incubation in boiling water bath for 15 minutes, the contents of the test tubes were cooled and the volume in each case was made to 6.0 ml by the addition of distilled water. Absorbance of the mixture was measured at 540 nm using UV-Visible spectrophotometer. The amylase activity was defined as liberation of 1mg of maltose from starch at pH 7.0 at 37°C in 10 minutes. The amylase activity was defined as liberation of 1 ¼mole of maltose formed per min at pH 7.0 at 37°C.

Amylase inhibitor activity

Amylase inhibitor activity was determined by measuring reduction in maltose liberated by salivary amylase using (DNS) dinitrosalisylic

acid (Fossum *et al.,* 1974; Bernfeld, 1955). A typical Amylase inhibitory assay mixture consists of 0.1 M (100µg) of amylase enzyme with 0.9 ml of sodium phosphate buffer (pH 7.0) with *Adenanthera pavonina* amylase inhibitor incubated for 10 minutes at room temperature. The reaction was initiated by the addition of 1.0 ml of 1% soluble starch at 37°C. The reaction was stopped after 10 minutes by the addition of DNS (1.0 ml). Absorbance of the mixture was measured at 540 nm as described above. The amylase inhibitory unit was defined as the number of amylase units inhibited under the assay conditions.

Molecular weight determination

The apparent molecular mass of the purified amylase inhibitor was determined according to the method of Andrews (1970) using Sephadex G-100 (1.13 x 100 cm) pre-equilibrated with 0.025 M sodium phosphate buffer pH 7.0, at a flow rate of 10 ml/h. The column was calibrated with proteins of different molecular mass such as cytochrome-c (12.3 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa) and ²-amylase (200 kDa). Blue Dextran (2000 kDa) was used to determine the void volume (Vo). The molecular weight of the purified amylase inhibitor was determined from the plot of log molecular weight versus K_{av}.

Effect of pH and temperature

The effect of pH on the activity of the purified amylase inhibitor was studied using the buffers of pH 3 – 10. The pH stability was also determined by preincubating the purified amylase inhibitor with different buffers for 30 min. Amylase inhibitor assay was performed as described earlier.The effect of temperature on the activity purified amylase inhibitor was studied at different temperatures ranging between 0 - 90 ºC. The temperature stability of purified amylase inhibitor was studied by pre-incubating, purified amylase inhibitor at different temperatures (0 - 90 ºC) for 30 min. The incubated samples were rapidly cooled and assayed at room temperature. Amylase inhibitor assay was performed as described earlier.

Antimicrobial activity

Antibacterial activity

Preparation of inoculums

Stock cultures of three bacterial strains; *E.coli*, *Klebisella sps* and *Staphylococcus aureus* were maintained at 4°C on slopes of nutrient agar. Active cultures for experiment were prepared by transferring a loop full of cells from the stock cultures to the test tubes of sterile water.

Antibacterial activity by well diffusion method

Antibacterial properties of purified peptides were determined using agar well diffusion method. Approximately 20ml of molten and cooled media (nutrient agar) was poured in sterilized petri dishes. The bacterial test organisms (*E.coli*, *Klebsiella* and *Staphylococcus aureus*) were swabbed uniformly on respected petridishes containing nutrient broth using sterile cotton swabs. Wells were prepared in the agar plates; the wells were labelled and were loaded with 50µl of diluted purified peptides and standard streptomycin. The plates were examined for evidence of zones of inhibition.

Polyacrylamide gel electrophoresis

An anionic disc gel electrophoresis was carried out essentially according to the method of Davis and Ornstein (1964). A discontinuous gel system consisting of 12% separating gel and 4% spacer gel was used. The electrophoresis was carried out in cold applying a current of 50 – 10 mA for 4 hours using tris–glycine (pH 8.3) as electrode buffer and bromophenol blue as marker dye.

RESULTS AND DISCUSSION

Purification

All the purification procedures were performed at 4^0C unless otherwise stated. Crude extract of the soaked seeds of *Adenanthera pavonina* was subjected to ammonium sulphate fractionation (0 – 90%). The precipitated protein obtained was recovered by centrifugation at 10000 rpm at 4^0c. The precipitated protein was dissolved in small volume of 0.025M sodium phosphate buffer; pH 7.0 applied on to a Sephadex G-10 chromatography and eluted using same buffer. The fractions were analyzed both for proteins and amylase inhibitor activity. Three fractions were obtained and these fractions were labeled as Fraction –I, II and III. (Fig.1). The fraction - I containing amylase inhibitor activity was pooled and concentrated using ammonium sulphate. The concentrated amylase inhibitor fraction was applied on to a Sephadex G-50 chromatography (Fig.2). Four fractions were obtained and were

labeled as Fraction –I, II, III and IV. The fraction - II containing amylase inhibitor activity was further subjected to purification by RP-HPLC (Fig.3). A α-amylase inhibitor was purified employing ammonium sulphate precipitation, ethanol fractionation, chromatographic separation on Sephadex and reversed phase-high profile liquid chromatography (Ho and Whitaker, 1993; Kokiladevi *et al.,* 2005; Hivrale *et al.,* 2011; Mridu Gupta *et al.,* 2014).

The molecular weight of amylase inhibitor from the seeds of *Adenanthera pavonina* as determined by gel-permeation chromatography on Sephadex G-100 was found to be 13.6 kDa. Alpha amylase inhibitor with a different molecular weight has been reported from bean cultivars, white kidney beans and *Phaseolus vulgaris* (Mridu Gupta *et al.,* 2014; Yamaguchi, 1993; Wato *et al.,* 2000). In the present studies the maximum activity for the HPLC purified α-amylase inhibitor was observed

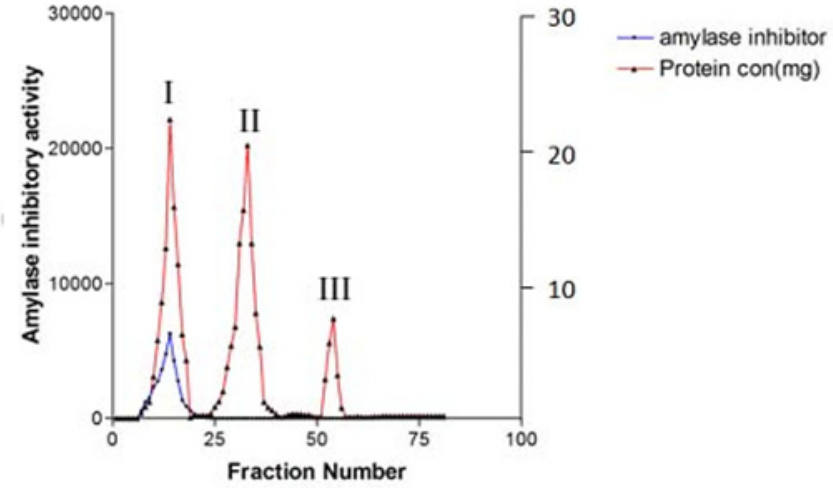


Fig. 1. Sephadex G-10 Chromatography of amylase inhibitor activity from the seeds of *Adenanthera pavonina*

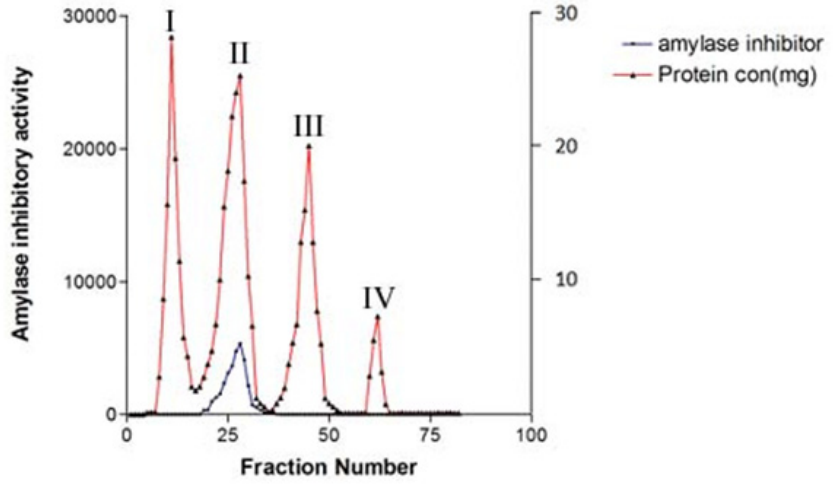


Fig. 2. Sephadex G-50 Chromatography of Sephadex G-10 amylase inhibitor fraction from the seeds of *Adenanthera pavonina*

at 25 °C– 45 °C. Similar results were observed for bean seed and rye α-amylase inhibitor (Frels and Rupnow, 1985; Power and Whitaker, 1977; Granum, 1978). The purified amylase inhibitor from *Adenanthera pavonina* was inhibited the human salivary amylase. The α-amylase inhibitor purified from various plant sources was found to be effective on human salivary amylase (Yoshikawa *et al.,* 1999; Iulek *et al.,* 2000; Heidari *et al.,* 2005; Hivrale *et al.,* 2011).Natural antioxidants present in plants are responsible for inhibition or prevention of the injurious effects of oxidative stress caused by free radicals in the body. The HPLC purified fraction also showed the anti-oxidant activity

Anti-bacterial activity of purified inhibitor was tested on three bacterial strains, *E.coli, Klebsiella* and *Staphylococcus aureus* using well diffusion method as described earlier. An inhibition zone was observed only for E,coli. The normal growth and development was suppressed by *Adenanthera pavonina* amylase inhibitor.

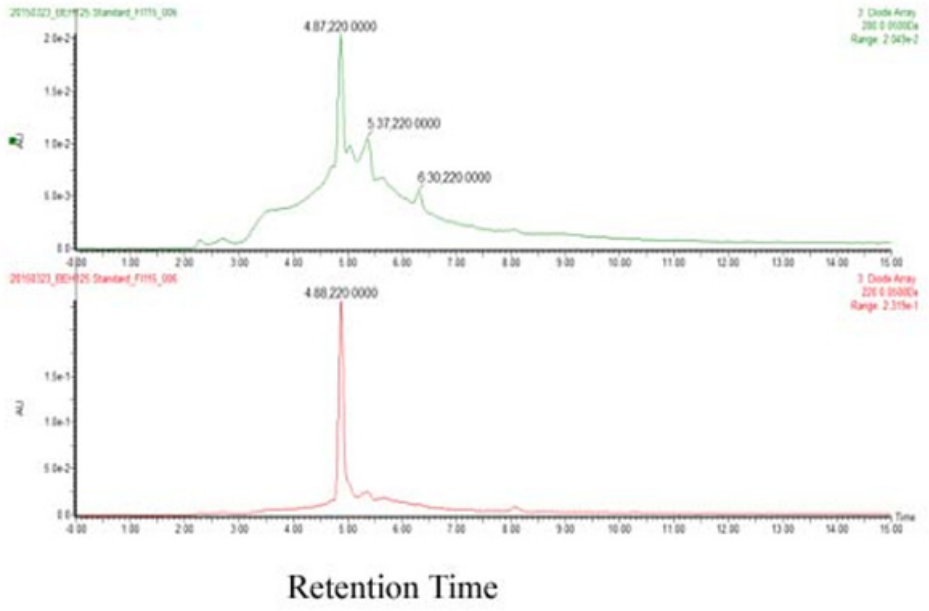
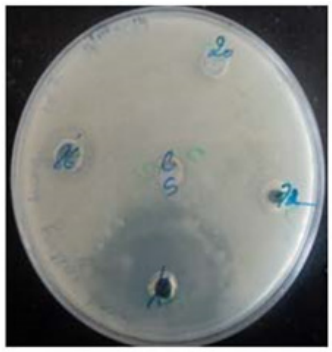


Fig. 3. Chromatogram of Elution profile of Sephadex G-50 Amylase inhibitor fraction on RP-HPLC



Amylase Inhibitory studies on Streptococcus

Fig. 4. Antibacterial activity of Amylase inhibitor

Effect of temperature and pH on Mucuna amylase inhibitor

The velocity of the inhibition reaction for amylase inhibitor of *Adenanthera pavonina* was determined at different temperature ranging between 4^0C to 100^0C. The optimum temperature obtained from the graph was 45^0C. *Adenanthera pavonina* amylase inhibitor was stable between temperatures 4^0C to 65^0C.The inhibitor activities of the amylase inhibitor in the different buffers were determined with amylase as enzyme. The optimum pH obtained from the graph was 6.2. The *Adenanthera pavonina* amylase inhibitor was stable between pH 2.0 to 10.

Inhibition Studies

Adenanthera pavonina amylase inhibitor inhibited both salivary amylase and the growth of streptococcus (Fig.4). The results indicate that amylase inhibitor from the seeds of *Adenanthera pavonina* is potent antimicrobial agent and inhibition of mammalian amylases suggests its potential in treatment of diabetes.

CONCLUSION

The α-amylase inhibitor was partially purified from the seeds of *Adenanthera pavonina*. The partially purified inhibitor showed the molecular weight of 13.6 kDa. Purified amylase inhibitor was found to inhibit the activity of human salivary α-amylase. Inhibitory activity of α-amylase inhibitor against mammalian amylases could suggest its potential in treatment of diabetes.

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