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Purification and Characterization of Amylase Inhibitors from the Seeds of *Senna Alata*

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ABSTRACT: An α-Amylase inhibitor was isolated and purified employing ammonium sulphate fractionation, Sephadex G-10, sephadex G-50 chromatography and HPLC from the seeds of *Senna alata*. The molecular weight determined by SDS – PAGE and Sephadex G-100 chromatography was found to be 10 – 11 kDa. The inhibitor was retained 40% and 65% at pH 2.0 and 10.0 respectively, indicated its stability in a broad pH range. The purified inhibitor was stable in a temperature range from 4˚C to 65˚C. Purified inhibitor also inhibited the human salivary amylase which suggests that the amylase inhibitor of *Senna alata* can be used to treat nutrition related problems.

Key words: Amylase inhibitors, Isolation, Characterization, *Senna alata* seeds

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I. INTRODUCTION

Plants have developed defense mechanisms against insect or insect pests during evolution by producing secondary chemical compounds toxic to or antimetabolic to them. Enzyme inhibitors are one such plants defense compounds preventing pathogens by inhibiting the gut enzymes of insects. α-Amylase inhibitors (α-AI) protect crop plants against insect pests and mammals by inhibiting the activity of their gut α- amylases.[1,2,3] α-amylase inhibitors have been studied from various plants.[4 -9] Insecticidal activity of α-amylase inhibitors are focused on insect weevils since they depend highly on carbohydrate for their energy source required for their growth and development.

Senna alata belongs family Fabaceae (Leguminosae), is generally known as ringworm tree, Christmas candle, candlestick or candle bush. The remarkable biological effectiveness of S. alata was documented for the variety of medical properties from non-infectious relief i.e. constipation, anemia, antidote, dyspepsia, laxative effect, purgative and eventually inhibition of the proliferation of leukemia cells and the treatment of several infections caused by bacteria, protozoa, fungi and virus. The plant is laxative, antibacterial, antitumor, anti-inflammatory, diuretic, analgesic, vulnerary, weakly antifungal, hypoglycemic, and antispasmodic. The leaves, flowers and fruit are mixed in an infusion to treat stomach problems. The plant has insecticidal properties. The seed is laxative and anthelmintic. It is cooked and used as a remedy for intestinal worms.

II. MATERIALS AND METHODS

Materials: *Senna alata* seeds were collected from surroundings of Puthige panchayath of Kasaragod district of Kerala State.

Chemicals: Bovine Serum Albumin, trypsin, BAPNA, α-amylase, Starch, Sephadex G-10 and Sephadex G-50 were purchased from Sigma chemical laboratory, USA. All the other chemicals and reagents used were of technical grade.

Methods:

Preparation of acetone powder and crude extract

The acetone powder (10 %) of soaked seeds of *Senna alata* was prepared according to the method of Wetter.[10] Seeds were blended in a homogenizer with chilled acetone, filtered and the cake obtained was dried at 37^{o} C which was powdered and stored at 4^{o}C until further use. A 10 % extracts of the acetone powder of *Senna alata* was prepared in sodium phosphate buffer pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4^{o} C. The extract was then centrifuged at 10,000 rpm for 15 mins at 4^{o} C. The supernatant obtained was used for qualitative and quantitative analysis of proteins/peptides for protease inhibitor activity, amylase inhibitory activity and antioxidant activity.

Fractionation and purification

Ammonium sulphate precipitation was performed as described by Chandrashekharaiah et al [(10)]. The ammonium sulphate precipitation (0 - 90%) was carried out for the crude extract by the addition of ammonium sulphate. The precipitated protein was recovered by centrifugation at 10,000 rpm for 30min. The protein pellet was dissolved in small amount 0.025 M sodium phosphate buffer, pH. 7.0 and subjected to separation by Sephadex G-10 gel-filtration chromatography.

Gel-filtration chromatography was performed as described by Chandrashekharaiah et al [(10)]. Sephadex G-10 and G-50 were allowed to swell in excess of distilled water, decanted and then equilibrated with 0.05 M sodium phosphate buffer, pH 7.0 separately. The Sephadex G-10 gel was packed into a glass column (1.0 cm X 110.0 cm) under gravity. The packed column was equilibrated with two bed volumes of 0.05M sodium phosphate buffer, pH 7.0 at a flow rate of 10 ml/hr. The 0 – 90% Ammonium sulphate fraction was loaded on to the gel and the proteins were eluted with 50 mM sodium phosphate buffer, pH 7.0 and 2.0 ml fractions were collected. Quantitative analysis of proteins, amylase and protease inhibitor activity was performed. The amylase inhibitor fractions were pooled and subjected to sephadex G-50 chromatography which was performed as described for Sephadex G-10 gel-filtration chromatography. Quantitative analysis of proteins, amylase inhibitor activity was done for all the fractions. The peak fractions containing amylase inhibitor activity were subjected to purification by 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% acetonitrile 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.

Protein estimation: Total protein content of crude extract and all the fraction of sephadex G-10 and G-50 was determined following the method of Lowry et al.[12]

Amylase and Amylase inhibitor assay

Quantitatively Amylase activity was determined by measuring liberated maltose using method of Bernfeld.[13] A typical Amylase assay mixture consists of 0.1 mL (100µg) of amylase enzyme with 0.9 mL of phosphate buffer (pH 7.0). The reaction was initiated by the addition of 1.0 mL of 1% soluble starch at 37°C followed by the addition of DNS after 10 minutes.(1.0 mL). the test tubes were kept in boiling water bath for 15 minutes, cooled and volume was made to 6.0 ml with 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 37^0C in 10 minutes. Amylase inhibitory activity was determined by measuring reduction in maltose liberated by salivary amylase using (DNS) dinitrosalisylic acid.[14, 13] For Amylase inhibitor assay 0.1 ml containing 100µg of amylase enzyme with 0.4 ml of phosphate buffer (pH 7.0) was incubated with 0.5ml of extracts/purified samples of *Senna alata* for 10minutes 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 using UV-Visible spectrophotometer. The amylase inhibitory unit was defined as the number of amylase units inhibited under the assay conditions.

Determination of optimum pH and pH stability

The effect of pH and temperature on the activity of purified inhibitor from the seeds of *Senna alata* performed as described by Chandrashekharaiah[15]. The pH optima and stability of the purified *Senna alata* amylase inhibitor was studied using the buffers of pH 2 – 10. The pH stability was also determined by pre-incubating the purified amylase inhibitor with buffers of different pH for 30 min. Inhibitory activity of the purified Amylase inhibitor was performed as described earlier. The optimum temperature was studied at different temperatures ranging between 0 - 90 ºC. The temperature stability was also studied by pre-incubating purified inhibitor at different temperatures (0 - 90 ºC) for 30 min. The samples were rapidly cooled and assayed at room temperature. Amylase inhibitor activity was determined as described earlier.

III. Results And Discussion

The amylase inhibitor from the seeds of *Senna alata* was purified employing conventional protein purification methods and all the purification methods were performed at 4^0C. The ammonium sulphate fractionation (0 – 90%) for crude protein extract of *Senna alata* seeds was carried out and the protein pellet obtained was loaded onto a Sephadex G-10 column. The proteins were eluted using 0.05 M sodium phosphate

buffer, P^H 7.0 with a fraction volume of 2 ml. Three peak fractions of proteins were obtained, fraction-I, fraction-II and fraction-III. The Sephadex G-10 fraction-I (Fig. 1) containing amylase inhibitor activity was pooled, concentrated and applied on to a Sephadex G-50 column. The proteins were eluted with the buffer and fractions of 2.0 ml were collected at a flow rate of 12 ml/h. The amylase inhibitor activity was eluted in a single peak (Fig.2). The peak containing amylase inhibitor activity was further purified by HPLC (Fig.3.). The *Senna alata* amylase inhibitor was purified to 24.28 fold with a recovery of 61.20% and purified inhibitor showed a specific inhibitor activity of 42.18. An α-amylase inhibitor was purified employing purification techniques such as ammonium sulphate precipitation, ethanol fractionation, chromatographic separation on Sephadex and reversed phase-high profile liquid chromatography.[3,4,16,17]

Molecular Weight

The purified amylase inhibitor from *Senna alata* showed a molecular weight of 10 - 11 kDa (approximately) as estimated using SDS-PAGE and gel filtration chromatography. Feng et al. [18] reported amylase inhibitor with a molecular weight of 14 kDa from Rice. Similarly Alpha amylase inhibitor with a subunit molecular weight of 15,488, 18,620 and 26,302 Daltons from bean cultivars and subunit molecular weights of 7800, 14000 and 22000 kDa white kidney beans have been reported.[3,19]

The inhibitor was retained 40% and 65% at pH 2.0 and 10.0 respectively, indicated its stability in a broad pH range. Nagaraj and Pattabiraman[20] showed that in the alkaline pH range there was a gradual loss of activity. The inhibitor was more stable under acidic condition than in neutral solution. The purified inhibitor was stable in a temperature range from 4˚C to 65˚C. Temperature is one of the most important parameters that affect the rate of enzyme hydrolysis. A heat labile alpha amylase inhibitor was isolated from white kidney beans. [19, 21]

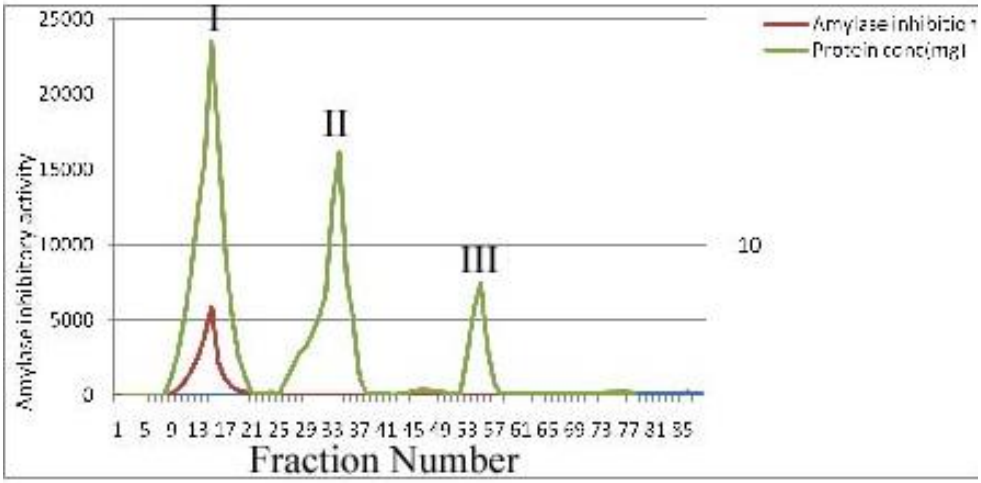

Fig. 1. Purification profile of amylase inhibitor from *Senna alata* on Sephadex G- 10 Chromatography

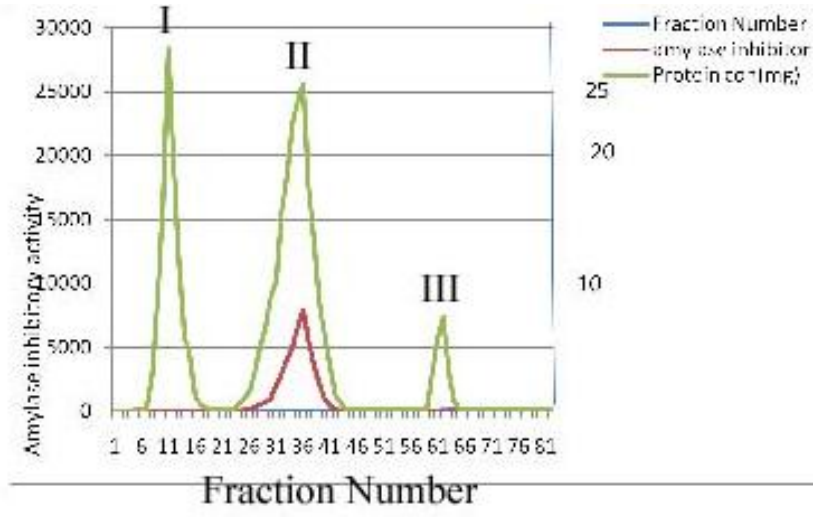

Fig. 2. Purification profile of amylase inhibitor from *Senna alata* on Sephadex G-50 Chromatography

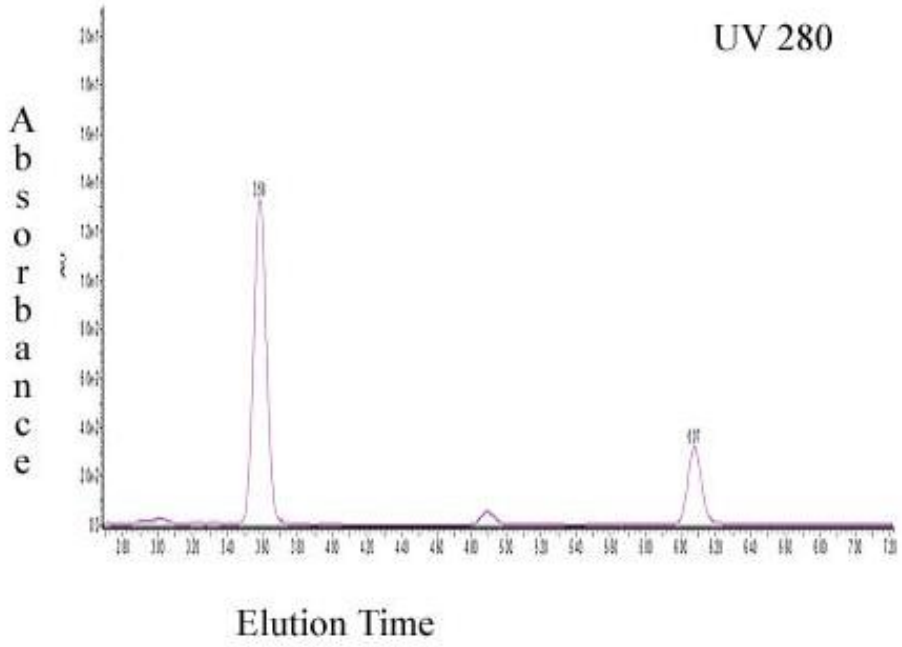

Fig.3. Separation of Sephadex G-50 amylase inhibitor fraction on RP- HPLC

IV. CONCLUSION

The amylase inhibitor with a molecular weight of 10 -11 kDa was purified from the seeds of *Senna alata.* The inhibitory activity on human salivary α-amylase indicated that it can be used to treat diabetes and related nutritional problems.

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Conflict of Interest

I, **Dr K S Chandrashekharaiah**, corresponding author - representative of submitted research article confirm that I **do not have any conflict of interest** in the publication of research article.

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