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Proteinaceous α-amylase inhibitors of *Setaria italica* Linn (Co-6) and its effect on α-amylase from human saliva and *Bacillus* sp

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ABSTRACT

The specific α-amylase inhibitory activity of *Setaria italica* Linn (Co-6) was determined by using heat-treated and ammonium sulphate precipitated fractions of α-amylase isolated from *Bacillus* sp. The α-amylase inhibitor from *S. italica* Linn (Co-6) was precipitated with 1.0 M fraction of ammonium sulphate and used for further purification and characterization. To overcome the endogenous α-amylase activity, the extract was subjected to the heat treatment at 60 ºC. Complete loss of α-amylase inhibitory activity of the millet extract was evidenced above 85 °C. The results of this study suggest that, the use of *S. italica* Linn (Co-6) flour exhibits significant α-amylase inhibitory activity, in a dose dependent manner for Non- Insulin Dependent Diabetes Mellitus (NIDDM).

INTRODUCTION

Alpha amylase is an enzyme that breaks down starch or glycogen. α-amylases are 1-4 glucanohydrolase, which gives a mixture of maltose, maltotriose and dextrin. It is present in plants, animals, bacteria, fungi and human beings (Takagi *et al.*, 1971). In mammals the salivary gland and pancreas are the main sources of the enzyme. It is used for the degradation of dietary starch. Prevention of glucose absorption in the human gastrointestinal tract by inhibiting the activity of carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase, is the recent strategy for controlling postprandial hyperglycemia (Lakshmanasenthil *et al.*, 2014). Cereals, legumes and other plants are found to be a good source of proteinaceous α-amylase inhibitors. Extensive research has been conducted on their properties and biological effects (Garcia-olmeda *et al.*, 1987). α-amylases inhibitory activity has been detected in most cereals, including wheat, ragi, barley, rye, sorghum, maize, oats, pearl millets, triticale and several other plants (Feng *et al*., 1991). Salivary, pancreatic, fungal and bacterial α-amylases exhibit variation in their susceptibility to the inhibitory action of proteinaceous α-amylase inhibitors. High levels of α-amylase inhibitors present in cereals and legumes can impair starch digestion. The action of the inhibitor is restricted only to the salivary amylase, as the proteolytic action of pepsin on the proteinaceous inhibitor, would render them inactive in the intestine. α-amylase inhibitor commonly called as "starch blocker", extracted from a protein in the white kidney bean (*Phaseolus vulgaris*) is supposedly preventing the breakdown of starch molecules. The undigested starch is passed out in the feces. α-amylase inhibitors find therapeutic application in NIDDM, which allows the diabetic patient to consume moderate amount of starch by maintaining the postprandial blood glucose level at 200 mg/100 ml. It also finds positive application in obesity and diabetic retinopathy (Agarwal *et al*., 2012). Foxtail millet (*S. italica*) is a common food in parts of India. *S. italica* not only causes a significant decrease (70%) in blood glucose level of diabetic rats, but also significantly reduced triglycerides, and total/LDL/VLDL cholesterol levels, while exhibiting an increase in HDL cholesterol (Sireesha *et al.*, 2011). With reference to the above study, the minor millet *S. italica* Linn (Co-6) was studied for its α-amylase inhibitory activity.

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MATERIALS AND METHODS

Samples and chemicals

S. italica Linn (Co-6) seeds for the study were collected from the seed collection center (freshly harvested), Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu, India. Human salivary α-amylase was purchased from Sigma-Aldrich Co., USA. The diluted enzyme solution for assay was prepared by dissolving 100 mg of the enzyme (2.5 Units/mg) in 100 ml of 0.2 M phosphate buffer solution of pH 6.9 to achieve one unit of enzyme activity in 0.4 ml of the diluted enzyme solution. The diluted buffered solution of the enzyme was stored at 4 ^{o}C until use.

Isolation of *Bacillus* sp.

Bacillus sp was isolated from decaying rice meal. About 25 g of the meal was homogenized using sterilized buffered peptone water and serially diluted. The diluted sample was then streaked on nutrient agar to isolate *Bacillus* sp. The isolate was identified by Gram staining and MALDI-TOF analysis. Amylase production by the isolate was screened on starch agar plate by iodine test.

Production of α-amylase enzyme from *Bacillus* sp

α-amylase enzyme from *Bacillus* sp was produced by using submerged fermentation method (in BBraun® Fermentor) using amylase production medium (0.4% starch, 0.5% defatted groundnut meal, 0.2% beef extract, 0.5% peptone, 0.2% $(NH_4)_2HPO_4$, 0.05% Na_2CO_3, 0.05% $MgSO_4$, 0.005% $MnCl_2$ and 0.02% K_2SO_4) for 24 hrs at 37 °C (300 rpm, pH 6.8). The enzyme assay was carried out for the determination of enzyme activity by the modified method described by Kokiladevi *et al*. (2005).

Partial purification of α-amylase enzyme

After 6 hrs of fermentation, the culture filtrate was subjected to centrifugation at 10,000 rpm for 10 min. The precipitate or the sediment was filtered through a membrane filter (2µm). The filtrate was subjected to partial purification with ammonium sulphate (0.5, 1.0, 1.5 and 2.5M) and the ammonium sulphate precipitates were designated as AA AS 0.5, AA AS 1.0, AA AS 1.5 and AA AS 2.5 respectively. The precipitates were then reconstituted in ice cold 0.2 M Phosphate buffer (pH 6.8) and subjected to dialysis using the same buffer overnight at 4°C. The dialysate was kept at -20°C and then lyophilized. Buffer reconstituted lyophilized powder (10mg/100 ml) was used as a source of α-amylase.

Extraction of α-amylase inhibitors

About 100 g of *S. italica* Linn (Co-6) flour was dissolved in 300 ml of 0.15M NaCl and kept for 1 hrs at room temperature. After centrifugation at 10,000 rpm for 30 min, the supernatant was subjected to heat treatment at 60 °C for 10 min and again centrifuged. The soluble components obtained by heat treatment are the heat soluble proteins. This fraction was subjected to ammonium sulphate precipitation at 0.5M, 1.0M, 1.5M and 2.5M and were designated as AAI AS 0.5, AAI AS 1.0, AAI AS 1.5 and AAI AS 2.5 respectively. The precipitates were then suspended in 0.2 M Phosphate buffer (pH 6.8) and dialyzed thoroughly against the same buffer for 24 hrs at 4 ^{o}C. The dialysate was kept at -20 °C in a deep freezer and then lyophilized. The lyophilized powder was reconstituted in cold phosphate buffer of pH 6.8 (10 mg/100 ml) and used as a source of α-amylase inhibitor for further studies.

Enzyme inhibitor assay

A total of 500µl of inhibitor solution and 500µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006M sodium chloride) and 25 µL α-amylase (0.4 IU) was incubated at 25°C for 10 min. After pre-incubation, 500µl of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5s intervals. The reaction mixtures were then incubated at 25 °C for 10 min. The reaction was stopped with 1.0ml of dinitrosalicylic acid reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was diluted by adding 10 ml of distilled water and then the absorbance was measured at 540nm using a UV- Vis spectrophotometer (UV-1100, Shanghai MAPADA Instruments Co., Ltd., China). One unit of enzyme activity (I.U) is defined as the amount of enzyme causing the release of 1µ mole of maltose in 1 min under the assay condition. The reducing sugar released from starch was estimated as maltose (1 mg/ml) equivalent from the standard graph. The α-amylase inhibitory activity was calculated by following formula (Lakshmanasenthil *et al*., 2014):

$$\text{Inhibitory activity (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

Effect of temperature on the activity of enzyme inhibitor

One ml of α-amylase inhibitor was incubated at 60, 70, 80, 90 and 100 ^{o}C for one hrs in a digitally controlled heating block. The heat-treated samples were subjected to enzyme inhibitor assay.

RESULTS

Production of α-amylase by submerged fermentation process

The isolate was found to be Gram positive rods. By MALDI-TOF analysis, the isolate was identified to be *Bacillus* sp. The screened *Bacillus* sp. was used for the production of α-amylase by submerged batch fermentation. The enzyme production was determined (in I.U) and the maximum enzyme activity was observed at 6th hr of fermentation (Table 1).

Partial purification of α-amylase

The culture filtrate was subjected to ammonium sulphate precipitation and dialysis. Maximum enzyme activity was found in AA AS 1.5M dialyzed fraction (Table 2). Dialyzed fraction was lyophilized and then stored at -20°C in a deep freezer for further use (as a source of enzyme).

Table 1: Production of α-amylase by submerged fermentation process.

Hours of submerged fermentation	α-amylase activity in I.U/ml of culture filtrate
3	1.2 ± 0.08
4	2.1 ± 0.06
5	3.0 ± 0.08
6	4.2 ± 0.03
7	3.8 ± 0.03
8	2.8 ± 0.06
9	1.9 ± 0.08
10	1.3 ± 0.04

Table 2: Partial Purification of α-amylase from *Bacillus* sp.

Type of fraction	α-amylase activity in I.U/ml
Culture filtrate	4.2 ± 0.05
AA AS 0.5M	10.2 ± 0.03
AA AS 1.0 M	16.8 ± 0.06
AA AS 1.5M	29.2 ± 0.05
AA AS 2.5M	5.3 ± 0.03

α- amylase inhibitory activity

Protein concentrations and inhibitory activity of the inhibitor in the crude extract, heat soluble protein fraction and the dialysates were determined. The dialysates obtained after precipitation with four different concentrations of ammonium sulphate showed significant difference in inhibitory activity against α-amylase. The crude extract and AAI AS 0.5 (0.5M) fraction exhibited minimum inhibitory activity. After heat treatment, a threefold increase in the specific inhibitory activity was evidenced. This might be due to the denaturation of endogenous α-amylase in the millet. The AAI AS 1.0 fraction exhibited the highest inhibitory activity against α-amylase (Table 3).

Table 3: Inhibition of Human α-amylase and *Bacillus* sp. α-amylase by various inhibitor fractions.

Type of Inhibitor fraction	% Inhibition on Human salivary α-amylase enzyme	% Inhibition on *Bacillus* sp α-amylase enzyme
Crude extract	17.5 ± 0.02	2.5 ± 0.04
Heat treated fraction	52.5 ± 0.03	76.2 ± 0.05
AAI AS 0.5M	18.7 ± 0.04	21.0 ± 0.03
AAI AS 1.0M	91.2 ± 0.06	94.4 ± 0.04
AAI AS 1.5M	58.0 ± 0.05	59.8 ± 0.04
AAI AS 2.5M	22.0 ± 0.05	26.2 ± 0.06

Table 4: Effect of temperature on the activity of enzyme inhibitor.

Temperature in °C	% Inhibition of crude fraction	% Inhibition of AAI AS 1.0 M fraction
Control (28°C)	20.2 ± 0.03	23.0 ± 0.04
37°C	53.1 ± 0.02	57.8 ± 0.04
50°C	59.9 ± 0.04	63.0 ± 0.05
60°C	62.2 ± 0.02	90.7 ± 0.04
70°C	40.0 ± 0.04	51.2 ± 0.04
80°C	33.0 ± 0.03	40.0 ± 0.04
90°C	15.8 ± 0.05	19.8 ± 0.05
100°C	ND	ND

ND – Not detected

Effect of temperature on the activity of enzyme inhibitor

The dialysate of AAI AS 1.0 was incubated at 28°C (Control), 37, 50, 60, 70, 80, 90 and 100°C for 1hr in a digitally controlled heating block. A drastic reduction in inhibitory activity was evidenced upon incubation at 100°C for 1 hr. A significant increase in inhibitory activity was observed in the sample incubated at 60°C (Table 4).

DISCUSSION

The screening of natural resources can lead to the development of potent and specific inhibitors for α-amylase. The enzyme plays a key role in carbohydrate metabolism of microorganisms, plants and animals. Moreover, several insects, especially those similar to the seed weevils that feed on starchy seeds during larval and/or adult stages, depend on their α-amylase for survival.

Traditional medicinal plants are continuously being explored in search of new anti-diabetic drugs which will be safer, specific and effective hypoglycemic agents (Lakshmanasenthil *et al*., 2014). In this study, antidiabetic activity of *S. italica* Linn (Co-6) extensively studied in an attempt to screen for new inhibitors of α-amylase.

Research on starch digestion, as a target for control of starch-dependent insects have been focused in recent years after studying the detrimental effect of α-amylase inhibitors from *Phaseolus vulgaris* seeds to the development of cowpea weevil *Callosobruchus maculatus* and Azuki bean weevil *Callosobruchus chinensis.* The specific proteinaceous inhibitors of α-amylase are wide spread in kidney beans (*Phaseolus vulgaris*). The inhibitor from white bean has been obtained in a high state of purity and named phaseolamin (Marshall and Lauda, 1975).

The absence of mammalian α-amylase inhibition activity in rice extracts has been reported. However, Saunders and Yetter (1977) detected lower levels of inhibitory activity from rice endosperm extracts toward insect α-amylase. Like most other cereals, rice contains other types of hydrolytic enzyme inhibitors, such as cysteine protease inhibitor and a serine protease inhibitor (Tashiro and Maki, 1978**)**.

Similar results were reported by Silano *et al.*, (1987) that the wheat albumin fraction contains a hetergeneous group of protein that can inhibit α-amylase from different sources. A large number of proteins in the wheat albumin fractions display inhibition towards α-amylases from different origin (Deponte *et al*., 1976).

The seeds of the Mexican crop plant *Amaranthus hypocondriacus* are found to contain an α-amylase inhibitor (AAI) which is a 32 residue long polypeptide. AAI strongly inhibits the α-amylase activity of insect larvae but does not exhibit inhibitory activity on proteases and mammalian α-amylases (Chagolla-Lopez *et al.*, 1994).

The above studies suggest that a potent α-amylase inhibitor can find a role in drug development and pest management in agriculture. In the present study, a significant α-amylase inhibitory activity has been reported. Further studies are needed to purify the inhibitor to homogeneity, to characterize the purified inhibitor and to study the kinetics of the inhibitor for possible development in to a drug for NIDDM.

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