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ISOLATION OF PROTEINACEOUS α-AMYLASE INHIBITOR FROM *BACILLUS PUMILUS* NGP-1

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ABSTRACT

The present study was conducted to screen the proteinaceous α-amylase inhibitory activity of *Bacillus pumilus* NGP-1. The culture free supernatant was precipitated by ammonium sulfate at 10-80% saturation for overnight at 4ºC. The precipitated proteins were desalted by dialysis and the protein content was estimated to be 1.3 mg/ml. The α-amylase inhibitory activity of partially purified proteinaceous α-amylase inhibitor was found to be 56%. The proteinaceous α-amylase inhibitor was characterized by Analytical High Performance Liquid Chromatography. The molecular weight of the α-amylase inhibitor was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis as 29kDa. The results suggested that the proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-1 is first of its kind and may be an important candidate in research of diabetes.

KEYWORDS: ***Post prandial hyperglycemia, α-amylase inhibitor, Diabetes mellitus, Starch blockers,Porcine pancreatic α-amylase.***

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INTRODUCTION

Diabetes is fast gaining the status of a potential epidemic in India.[1,2] Reports reveals that 592 million people are likely to suffer from diabetes by 2025.[3] The majority of diabetes is type II diabetes caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver.[4] Post prandial hyperglycemia is an early defect in type II diabetic patients that leads to severe diabetic complications.[5] The recent approach for controlling postprandial hyperglycemia is to use inhibitors of the enzyme which delays carbohydrate digestion and prolong the overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise. [6,7,8,9] The prevention in the uptake of dietary starch in the body is the prime reason why amylase inhibitors are also called starch blockers. α-amylase inhibitors are classified as proteinaceous and non-proteinaceous amylase inhibitors.[10] Inhibitors like acarbose and miglitol which inhibits glycosidases such as β-glucosidase and α-amylase are currently used in the treatment of diabetes. However, many of these synthetic hypoglycemic agents have their limitations associated with side effects, non-specific interactions and does not reduce complications arising due to diabetes. Conditions such as bloating, abdominal discomfort, diarrhoea and flatulence have been recognized as the main side effects.[11] So the natural products are given due importance in the treatment of diabetes mellitus as they render easy affordability, availability and negligible side effects when compared to their synthetic or chemically synthesized counterparts.[12,13] There are several reports of proteinaceous and non-proteinaceous inhibitors from plants and its effects on blood glucose levels after food uptake [14] but proteinaceous α-amylase inhibitor from microorganism have not studied much. So the study was focused on proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-1.

MATERIALS AND METHODS

Collection of Soil Samples

Soil samples were collected from Valparai, Tamilnadu, India in a sterilized poly bag sealed properly and brought to the laboratory for further analysis. It was stored at 4°C until used.

Isolation of bacteria

One gram of soil was suspended in 100ml sterile distilled water and serially diluted up to10^{-7}. From each dilution 0.1ml of sample was spread plated on nutrient agar plates and incubated at 37°C for 24 hours. Individual colonies were selected based on the colony morphology, appearance and maintained in agar slants.

Screening for α-amylase inhibitory activity

All the isolated bacterial isolates were screened for α-amylase inhibitory activity, according to the method of Suthindhiran *et al*[15] with minor modification. 500µl of inhibitor and 500µl of porcine pancreatic α-amylase solution (0.5 mg/ml) were incubated for 10 minutes at 25ºC. After pre-incubation, 500µl of 1% starch solution was added to each tube. The reaction mixtures were then incubated at 25ºC for 10 minutes. The reaction was stopped with 1ml of dinitrosalicylic acid reagent and incubated in boiling water bath for 5 minutes. The content was cooled to room temperature. The reaction mixture was then diluted after adding 10ml distilled water and absorbance was measured at 540 nm. Control was maintained without the inhibitor. Positive control was done with acarbose. All the reactions were carried out in triplicate value. Percentage of inhibition was calculated using following equation:

$$\% \text{ Inhibition} = \frac{[(A_{540}\text{ Control} - A_{540}\text{ Inhibitor})]}{A_{540}\text{ Control}} \times 100$$

The isolate which showed the highest percentage of inhibition was taken for further study. The protein content of cell free supernatant was estimated by Lowry's *et al*., method.[16]

Production of α-amylase inhibitor

The strain *Bacillus pumilus* NGP-1 was inoculated in 100ml BPM6 production medium (0.5g peptone, 0.15g yeast extract, 0.05g magnesium sulfate, 0.5g sodium chloride, 0.15g beef extract, 0.03g dipotassium hydrogen phosphate, pH 7.4±0.2) and incubated at 37°C for 48 hours. It was then centrifuged at 7,000 rpm for 10 minutes and the cell free supernatant was precipitated by ammonium sulfate at 10-80% saturation and kept for overnight at 4ºC. The precipitated proteins were centrifuged at 12,000 rpm for 30 minutes and the pellet was suspended in 0.02M sodium phosphate buffer (pH 6.9). The protein content was estimated for the ammonium sulfate precipitates before desalting by Lowry's *et al*., method.[16] Desalting of protein was carried out by dialysis (Molecular weight cut-off between 12000 to 14000) against phosphate buffer saline (pH 7.4) with the buffer change at regular interval of an hour. The α-amylase inhibitory activity and protein content of precipitated proteins with 10-80% saturation after desalting were examined and the saturation percentage which exhibited maximum activity with minimum protein content was selected for further study.

Molecular Identification

The bacterial isolate which exhibited the remarkable α-amylase inhibitory activity was identified by 16S rDNA Sequencing.[17] The 16S rRNA gene was amplified using primers 27F (5'AGAGTTTGGATCMTGGCTCAG 3'), 1492R (5' CGGTTACCTTGTTACGACTT 3') and was subjected for sequencing.[18] The obtained nucleotide sequence (393bp) was analyzed for molecular identification (Genbank Accession number: KY101156).

Partial characterization of proteinaceous α-amylase inhibitor

SDS-PAGE analysis

SDS-PAGE of dialyzed protein was carried out with 10% separating gel and 5% stacking gel by the method of Laemmli.[19] The protein sample mixed with loading dye was loaded into each lane along with the protein marker of 14-96 kDa in the adjacent well. The gel was stained

for proteins with Coomassie Brilliant Blue R-250 and the destained gel was visualized for the bands.

Analytical HPLC

The proteinaceous α-amylase inhibitor was subjected to analytical HPLC (Shimadzu C-18) for partial purification of proteins by using UV detector at 220 nm with the flow rate of 1ml/minute. Acetonitrile was used as the solvent.

RESULTS AND DISCUSSION

Totally 37 different bacteria were isolated from soil samples collected from Valparai, Tamilnadu, India. All the bacterial isolates were screened for α-amylase inhibitory activity with a positive control acarbose and the isolate *Bacillus pumilus* NGP-1 showed remarkable inhibitory activity was used for further study. The inhibitory effect of cell free supernatant showed 56.43% of inhibition with protein content (1.6 mg/ml) whereas acarbose showed 41.73% (0.4 mg/ml). The protein content of ammonium sulfate precipitated protein before dialysis showed 1.9 mg/ml. After dialysis 40% ammonium sulfate saturated protein precipitates showed the highest α-amylase inhibitory activity of 56% (1.3 mg/ml) when compared with other percentage of ammonium sulfate saturation. SDS-PAGE of partially purified proteinaceous α-amylase inhibitor showed protein band of 29kDa (Figure 2).The partially purified proteinaceous α-amylase inhibitor was subjected to analytical HPLC at 220nm. The chromatogram reveals the presence of major fraction at the retention time of 2.563, which may be responsible for the observed activity (Figure 3). The result clearly explains that the proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-1 may be a potent inhibitor to control postprandial hyperglycemia. Type 2 diabetes mellitus is one of the most prevalent diseases affecting the population all over the world. Treatment of post prandial hyperglycemia with the use of enzyme inhibitors without side effects is very important in this modern era. Lots of α-amylase inhibitors have been reported from plants [20] but reports from microorganisms are only limited. In the present study *Bacillus pumilis* NGP-1 was isolated from soil sample collected from Valparai, Tamilnadu and screened for proteinaceous α-amylase inhibitory activity. The precipitated protein exhibited a remarkable inhibitory activity with 56% when compared with acarbose which is a commercially available drug. Similar α-amylase inhibitory activity was reported on ethanolic extract of *Pteris vittata* with 70.87%.[21] Acarbose produced from *Actinoplanes* strains is the synthetic drug which is currently used in the treatment of diabetes.[22] Due to adverse effects of these drugs research is focussed on other natural sources.*Streptomyces variabilis* strain PO-178 isolated from Western Ghat possessed similar observations for α-amylase inhibitory activity with 46% of inhibition.[23] Recently other α-amylase inhibitors from *Cladosporium herbarum* F- 828 and *Paenibacillus lentimorbus* have been reported. [24,25] Purified proteinaceous α-amylase inhibitor extracted by ammonium sulphate precipitation from common bean seeds corroborated the present study.[26] α-amylase inhibitor from different seaweeds has been reported.[27] A study done by Murao *et al*.,[28] reported that a large number of actinomycetes and bacteria were successful in producing α-amylase inhibitor. Another study by the same authors examined that subspecies of *Streptomyces diastaticus* subsp. *amylostaticus* No.2476 showed the ability to produce α-amylase inhibitor. This finding supports the earlier report by Zhibin Sun *et al*.,[29] on proteinaceous α-amylase inhibitor AAI-CC5 from *Streptomyces* sp CC5 and also the antidiabetic potential of a peptide from *Aspergillus awamori* by Singh and Kaur.[30] Similarily Prabavathy *et al*., [31] also reported on antidiabetic activity of endophytic fungi isolated from *Adathoda beddomei*. α-amylase inhibitory activity of *Micromonospora* Sp VITSDK3 was also reported by Suthindhiran *et al*.[15] Characterization of the compound with SDS-PAGE revealed the proteinaceous nature of the inhibitor with a molecular weight of 29 kDa. Similarly proteinaceous α-amylase inhibitor Pa1 and Pa2 with molecular weight of 39.6 kDa and 28.1 kDa from *Phaseolus acutifolius* A has been reported.[32] In various studies α-amylase inhibitory peptide of 22 kDa from *Aspergillus awamori*, 18 kDa from *Cladosporium herbarum* were reported.[30,24] Four polypeptide α-amylase inhibitors with molecular masses of about 14 kDa have been reported from wheat.[33] The above studies suggest that a potent α-amylase inhibitor can find a role in drug development. In the present study, a significant α-amylase inhibitory activity from *Bacillus pumilus* NGP-1 has been reported. Further studies are needed to purify and characterize the inhibitor for development into drug for the treatment of type 2 diabetes mellitus.

AACCAAGGCCGCGTGCCTAATAATGCAAGTCGAGCGGACAGAAGGGAGCTTGCTCCCGGATGTTAGCGGC
GGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATAC
CGGATAGTTCCTTGAACCGCATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACGGATGGACCCG
CGGCGCATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCATTG
GACGAAAGTCTGACGGACAAACGCCGCGTGAGTGAAAAGGTTTTGGTAT

Figure 1
16S rDNA Sequence of *Bacillus pumilus* NGP-1

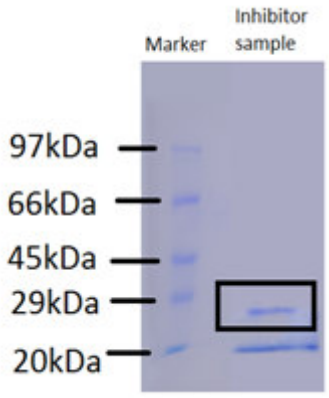

Figure 2
SDS-PAGE analysis of partially purified proteinaceous α-amylase inhibitor

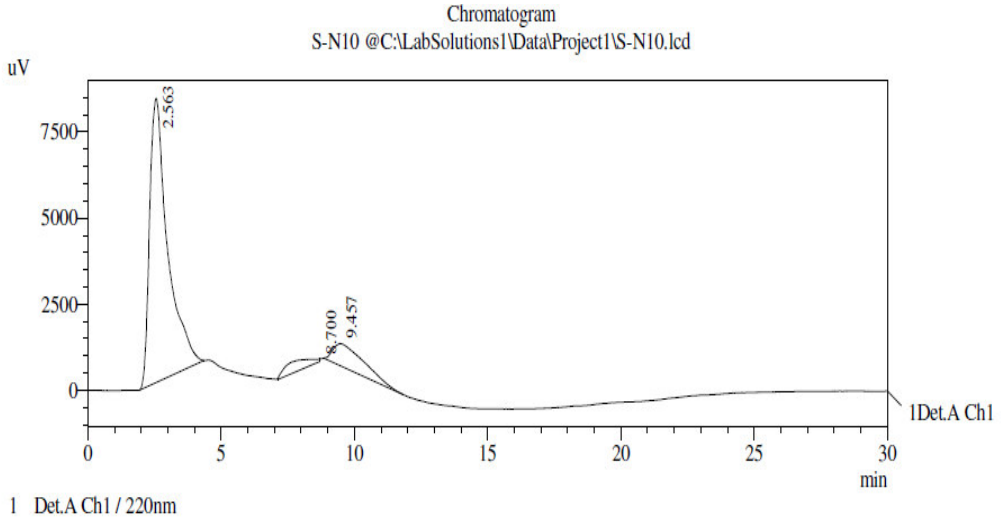

Figure 3
HPLC chromatogram of α-amylase inhibitor

CONCLUSION

The partially purified proteinaceous α-amylase inhibitor from *Bacillus pumilus* NGP-1 showed prominent α-amylase inhibitory activity when compared to acarbose. Proteinaceous α-amylase inhibitor from natural sources is the need of hour due to the side effects caused by the commercially developed drugs. The bioactive compounds from microorganism have not been exploited to that extent. So this present study focused on α-amylase inhibitor from *Bacillus pumilus* NGP-1, which will give an attractive prospect towards the management of diabetes in controlling postprandial hyperglycemia by conducting further research to characterize the biomolecule identified.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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