Screening of Actinomycetes for dipeptidyl peptidase-4 inhibitors production

Chandrasen Chandwad, Sudhakar Gutte

Department of Microbiology, Research Center, Mrs. Kesharabai Sonajirao Kshirsagar Alias Kaku Art, Science and Commerce College, Beed, Pin 431122, Maharashtra, India

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**ABSTRACT**

Hyperglycemia or high blood sugar is the most common cause of diabetes. Diabetes mellitus is the most common and fastest growing disease in the world. One of the therapies to treat diabetes is inhibition of dipeptidyl peptidase-4 inhibition by inhibitors. Dipeptidyl-peptidase 4 is a membrane glycoprotein having serine exopeptidase activity, which is cleaves X-proline or X-Alanine residue at N-terminus of peptides. Dipeptidyl peptidase-4 degrade glucagon-like peptide-1, which is the main cause for high blood glucose level, hence inhibitors of Dipeptidyl peptidase-4 have emerged as oral anti-diabetic agents. Actinomycetes are potential sources of enzyme inhibitors, drugs, amino acids, vitamins, pharmaceutical important chemicals etc. Present work mainly highlights the isolation of actinomycetes from soil samples and screening of extracts for dipeptidyl peptidase-4 inhibition activity. Isolation of actinomycetes was carried out by different methods and pretreatments. Isolated actinomycetes grown in fermentation condition and broth extracted with Isopropanol alcohol and ethyl acetate and obtain solid material after evaporation of solvents. The metabolites of each isolate were tested for inhibition of dipeptidyl peptidase-4 inhibition using spectroscopic method. Dipeptidyl peptidase-4 activity is measured by fluorescence of cleaved substrate, which is proportional to the enzymatic activity present. Total 130 actinomycetes strains were isolated. Among 130 extracts of actinomycetes, four extracts have shown positive results for dipeptidyl peptidase-4 inhibition. The actinomycetes strains that produce inhibitory compounds are A-9, A-12, C-4 and D-6. These results show that actinomycetes are a potential source for dipeptidyl peptidase-4 inhibitors, which may lead to valuable novel drugs for diabetic treatment.

**INTRODUCTION**

Diabetes is a growing global health problem. Diabetes mellitus leads to cardiovascular disorders, muscular disorders and obesity etc. High blood sugar, or hyperglycemia, is a major concern, and can affect people with both type 1 and type 2 diabetes (Drucker, 2003). One of the approaches for treatment and control of diabetes is involved inhibition of dipeptidyl peptidase-4 (DPP4) by inhibitors (Lin et al., 2019). DPP-4 is a membrane glycoprotein having serine exopeptidase activity (Holst, 2002). After ingestion of food,
gut releases incretins that gives signal for secretion insulin (Kieffer and Habener, 1999). Role of incretins, Glucagon Like Peptide-1 (GLP-1) and Gastrointestinal inhibitory peptide (GIP) in glucose homeostasis is depicted in Figure 1.

GLP-1 is inactivated by the DPP-4 enzyme and that affect on the insulation secretion and glucose metabolism (Zeng et al., 2016). DPP-4 effect on blood glucose level has shown in Figure 2.

GLP-1 is inactive by the DPP-4 enzyme and hence it is no longer available for signaling to secretion of insulin (Drucker DJ et al.). Active GLP-1 and inactive GLP-1 and DDP action are shown in Figure 3.

New drug discovery approach is based on the design on the GLP-1 action and DPP 4 action (Wang et al., 2017). There are two approaches for the drug discovery as 1. GLP-1 stabilization 2. DPP-4 inhibition (Ahrén, 2003). Approach for drug discovery and current marketed drugs are depicted in Figure 4 (Li et al., 2018).

There are two sources of DPP-4 inhibitors; one is chemically synthesized and other natural source (Chandwad and Gutte, 2019). Natural source includes the microorganisms and plant (Singh et al., 2017). Actinomycetes produces important bioactive compounds like enzymes, enzyme inhibitors, antibiotics, amino acid, anti-cancerous agents, anti-diabetic drugs, anti-obesity drugs, and all of them have great economic importance (Imada, 2005). In literature several enzyme inhibitors producing actinomycetes are found from the different habitats including soil and marine environment (Raja et al., 2010). Searches for drugs and other important chemicals have been remarkably successful and approximately two thirds of naturally occurring antibiotics have been isolated from actinomycetes.

There is a need of new and novel DDP-4 inhibitors, so it can lead to the development of novel drug for diabetes treatment. Hence present work mainly focused on the isolation of actinomycetes and screening for DPP-4 inhibitors, which may lead to development of new drug for obesity treatment.

MATERIALS AND METHODS

Soil sampling and processing

Five black soil samples were collected from different agricultural land. Soil samples were collected in a sterile plastic bag after removing 2-3 inch surface layer of soil by using sterile spatula (Mathew et al., 2017).

Soil samples were air dried in laminar airflow unit for a day at room temperature and stored at 4°C until processed (Kumar and Jadeja, 2016).

Isolation of actinomycetes

Collected soil samples were pretreated at different conditions and isolation carried out on the selective medium with and without antibiotics. Combinations of physical and chemical methods were used for selective isolation of actinomycetes species.

Medium for actinomycetes isolation

Yeast Extracts Malt Extract Dextrose Agar (YMA) used and the same media with antibiotic Cycloheximide (50 μg/mL) and Nystatin (50 μg/mL) (Chandwad and Gutte, 2019).

Physical and chemical treatment combination (dry heat and calcium carbonate) and preservation of actinomycetes

One gram each of soil samples weighed in a flask and heat dried in oven at 35 to 45°C for 20 minutes. 1 gram dried sample was diluted to 9 ml by sterile normal saline water with 1 % calcium carbonate and incubated at 30°C for 72 hrs in incubator. Treated soil sample were centrifuged at room temperature at 4000 rpm for 4 minutes. The supernatant is serially diluted and each dilution plated on with and without antibiotics YMA medium using 100μl suspension. Plates were incubated at 28°C for 10 days. Dry and muddy characters of colony considered as actinomycetes which is cross checked by hyphal growth by using microscopy. Actinomycetes were subcultured by picking pure isolated colony on the YMA slants; slants incubated 28°C for 10 days, after full growth slants were stored at 4°C.

Shake flasks fermentation process

The grown culture on YMA slants scraped with 5 ml normal saline solution and 100 μl suspension inoculated in seed medium, 25 ml medium in 250 ml flask consists of soybean meal 1.5% (w/v), 2 % Dextrose, 1 % Glycerol, and flasks incubated at 30°C for 48 hrs at 200 rpm on rotary shaker. Grown culture 10 % used for 25 ml production medium inoculation. 25 ml production medium in 250 ml flasks consists of soybean meal 2.5%(w/v),0.5 % Yeast Extract,2 % Dextrose and 1 % Glycerol. Production flasks incubated for 8 days at 28°C on a rotary shaker at 220 rpm (Imada, 2005).

Metabolites extraction with solvents

After completion of 8 days fermentation cycle, broth was harvested and stirred for 30 minutes to homogenize, and equal volume of Isopropyl alcohol (IPA) was added and stirred for 2 hrs. The solution was filtered through cloth and celite filter aid, and crude material obtained by evaporation of IPA under vacuum in Rota evaporator. Crude materials were
Figure 1: Role of Incretins in Glucose homeostasis

Figure 2: DPP-4 effect on blood glucose level

Figure 3: Active GLP-1 and inactive GLP-1 and DDP action
Preliminary screening of extracts for DPP-4 inhibition

DPP-4 inhibition assay was carried out by monitoring cleavage of H-Gly-Pro-AMC by measuring color at 460 nm using spectrophotometer. The principle of the assay is that the substrate, H-Gly-Pro-AMC is cleaved by DPP-4 to give fluorescent product which is measured by the spectrophotometer. Generation of the fluorescence of test sample is based on the action of compound present in the test solution; the presence of the inhibitor is indicated by the reduced action of the enzyme on the substrate and thereby difference in the absorbance at 460 nm (Sigma kit MAK203).

Preparation of metabolites solution

Dissolved 2 mg crude extract in 0.1 ml DMSO used for the assay inhibition assay.

Preparation of DPP-4 Substrate

2 μl substrate added in 23 μl Assay buffer.

Preparation of DPP-4 enzyme

1 μl DPP-4 added in 24 μl Assay buffer.

Preparation of inhibitor Control (Sitagliptin)

2.5 μl DPP-4 inhibitor dissolved in 97.5 μl of Assay buffer.

Assay Protocol

Assay protocol and details are shown in Table 1. Each blank, test and positive control reaction incubated for 10 minutes at 37 °C. Plates were protected from the light during the incubation. The relative activity was measured at the end of 10 minutes incubation time and is expressed as percentage ratio of enzyme activity in the presence of inhibitors and absence of enzyme inhibitors (Raja et al., 2010; Mathew et al., 2017; Chandwad and Gutte, 2019). The % inhibition was calculated according to the formula:

\[ \text{Inhibition} = \frac{\Delta A_{460 \text{nm}} (\text{Inhibited test}) - \Delta A_{460 \text{nm}} (\text{Inhibited test})}{\Delta A_{460 \text{nm}} (\text{Uninhibited test})} \times 100 \]

Determination of IC\text{50}

To determine the 50 % inhibitory activity, applied extracts concentration in the range of 0.1 to 2 mg in the inhibitory assay. IC\text{50} can easily determine on the graph plotting enzyme activity Vs extract concentration.

RESULTS AND DISCUSSION

Actinomycetes isolation results

A total 130 actinomycetes strains were isolated from soil sample by using the pretreatment and using selective nutrient medium with and without antibiotics.

DPP-4 inhibitory activity of extracts of actinomycetes

The inhibition assay was performed by monitoring and measuring the appearance of fluorescence in the assay test tube at 460 nm using spectrophotometer after incubation for 10 minutes at 37°C. The principle of the assay is that the substrate is cleavedged
Table 1: Assay protocol

| Description and components | Blank | Positive Inhibitory control | Test sample |
|----------------------------|-------|------------------------------|-------------|
| Enzyme solution            | 10 µl | 10 µl                        | 10 µl       |
| DMSO                       | 10 µl | 10 µl                        | -           |
| Assay Buffer               | 970 µl| 960 µl                       | 970 µl      |
| Substrate solution         | 10 µl | 10 µl                        | 10 µl       |
| Metabolite                 | -     | -                            | 10 µl       |
| DPP-4 inhibitor (control)  | -     | 10 µl                        | -           |

by DPP-4 enzymes to give a cleaved fluorescence cleaved product. Generation of the fluorescence of test sample is based on the action of DPP-4 and inhibitors present in the test solution; the presence of the enzyme inhibitor is indicated by the reduced action of the enzyme on the substrate and thereby difference in the fluorescence of solution and intensity was measured at 460 nm in spectroscopy. The extract of isolate A9, A12, C4, C6, shown inhibitory activity as 20, 30, 60 and 40 % respectively. The extracts shown 20 to 60 % inhibition of DPP-4 are shown in the Graph 1.

Graph 1: Representation of % inhibition of DPP-4

DPP-4 inhibitory activity of inhibitors and IC_{50} determination

50 % inhibitory activity of A12, C4 and C6 of extracts determined using various concentrations of extracts in the assay. Extract of isolate A12, C4 and C6 having 50 % inhibitory activity at 2.5mg,1.4 mg and 1.8 mg respectively, whereas the Sitagliptin (standard) has shown 50 % inhibitory activity at below 0.1 mg concentration. Further investigation research is under progress on for fermentation process optimization and purification of compounds.

CONCLUSION

From the present study, it is noticed that actinomycetes found in soil are rich of bioactive compounds including enzyme inhibitors and such bioactive compounds and actinomycetes can be isolate from soil of different habitat. Present research confirmed that actinomycetes produce potential DPP-4 inhibitors that can be explored as drug for diabetes treatment.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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