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Orally active hypoglycemic protein from *Costus igneus* N. E. Br.: An *in vitro* and *in vivo* study

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ABSTRACT

Plants have been used for the treatment of diabetes since time immemorial. In the present study, insulin-like protein (ILP) is purified from *Costus igneus* belonging to family *Costaceae* from Western ghats of India. The ILP showed cross reactivity with murine anti-insulin antibodies hence was purified by affinity chromatography using anti-insulin antibodies. The characterization of ILP showed that it is structurally different from insulin but functionally similar. The ILP showed a hypoglycemic activity in an *in vitro* assay with insulin responsive cell line RIN 5f. Interestingly ILP showed significant decrease in blood glucose level when administered orally in oral glucose tolerance test. This was compared to insulin a positive control given intraperitoneally in streptozotocine induced diabetic mice. There was no toxic effect seen on animals after administrating the ILP. Therefore we conclude that the ILP purified in the present study from *C. igneus* is a novel protein having hypoglycemic activity.

1. Introduction

Diabetes mellitus is the most common metabolic disorder affecting more than 200 million people worldwide. It is a syndrome characterized by hyperglycemia, altered lipid metabolism [1]. There is an absolute or relative deficiency of circulating levels of the hormone 'insulin'. The peptide hormone, insulin first discovered in 1921 [2] is still the ultimate treatment of diabetes mellitus.

Plants have been widely used for the treatment of Diabetes. Many secondary metabolites have been implicated in the anti-diabetic properties of plants [3]. Although the presence of insulin-like substances/proteins was reported in plant materials like onions, lettuce, green bean leaves etc., way back in 1923, not much attention was paid to these results [4]. However, there was a renewed interest in plant derived insulin when presence of insulin was reported from the fruits of *Momordica charantia* (bitter gourd) [5,6]. Three orally active hypoglycemic peptides: MC-1, MC-2 and MC-3 sharing a common heptapeptide sequence (KTNMKHM) found to be important for the hypoglycemic property, were isolated from this plant [7].

Peptides isolated from a number of higher plants, Fungi and a Cyanobacterium, *Spirulina maxima* were shown to be cross reactive with human anti insulin antibodies by ELISA and western blot [8–10]. An insulin like peptide showing sequence homology to bovine insulin has been found in *Vigna unguiculata* and it was shown to be involved in carbohydrate transport to fruits [11]. A similar peptide has been isolated from the leaves of *Bauhinia variegata*, a popular antidiabetic plant in Brazil. The peptide showed hypoglycemic property in alloxan-induced diabetic Swiss mice [12]. Recently a protein showing distinct functional and structural homology to insulin was characterised from embryo axes of maize. This 5.7 kDa protein, with a well-defined α-helix structure, induced selective synthesis of DNA as well as ribosomal proteins, just like mammalian insulin [13].

Family Zingiberaceae comprises of several genera that show anti-diabetic activity like turmeric [14], ginger [15] and *Costus speciosus* [16]. The plant *Costus igneus* N. E. Br. (formerly known as *Costus pictus* D. Don) belongs to the family *Costaceae*, recently separated from family *Zingiberaceae*. It grows in tropical climate. People traditionally consume 2–3 leaves of this plant twice a day for management of diabetes, which has led to it being named as "insulin plant" [17].

The ethanolic extract of *C. igneus* has shown a potent antidiabetic effect in alloxan induced diabetic rats [18]. Recently Shetty and coworkers [19] reported that regular consumption of its leaves in conjunction with other modalities of treatment effectively provided glycemic control in diabetic patients. The risk of diabetic complications was avoided and no adverse effects due to the

Abbreviations: *C. igneus*, *Costus igneus*; CD, Circular dichroism.

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consumption of insulin plant leaves were reported. Aqueous extracts *C. pictus* have also been shown to stimulate insulin secretion from Islets of Langerhans of mouse and human *in vitro* without decreasing cell viability or compromising membrane integrity [20]. However the effective principle(s) contributing to anti-diabetic properties have not been identified.

In this study, an insulin-like peptide was purified from the leaf extracts of the *C. igneus* plants and its hypoglycemic property was investigated *in vitro* in a glucose responsive cell line. Studies were also carried out using this protein *in vivo* in streptozotocin induced diabetic mice. Oral hypoglycemic property of an insulin like peptide from *C. igneus* in murine model of diabetes has been demonstrated.

2. Materials and methods

2.1. Isolation of insulin-like protein (ILP) from plant extract

Stock plants of *C. igneus* R. E. Br. were collected from FRLHT nursery, Bangalore. They were cultivated in the Mulshi Taluka of Maharashtra state. The leaves of these plants were washed, dried and homogenized at 4 °C in a cooled mortar using 0.05 M homogenizing buffer containing sulphuric acid and 60% ethanol (1: 4 dilution; w/v). The pH of the homogenate was adjusted to 3.0 using liquid ammonia [6]. The proteins were precipitated using four volumes of chilled acetone for 24 h. The precipitate was then dialysed and concentrated through Amicon (Milipore, USA) concentrator. Protein was quantified with Bradford's assay [21] and was stored at −20 °C till further use.

2.2. Purification and characterization of ILP from plant extract

Immunoaffinity column was prepared using murine anti insulin antibodies (1:500) immobilized on the sodium alginate beads. The crude protein sample (8.25 mg/500 μl) was loaded on the column and kept for 5–10 min. The column was washed with a 30× bead volume of PBS (pH 7.2). The protein was eluted using elution buffer (0.2 M Glycine–HCl, pH 2.5) [22–24]. These fractions (1 ml) were immediately neutralized with 1 M Tris buffer (pH 9.0) and absorbance was measured at 280 nm. The Bradford reagent was used for estimating the protein concentration [21].

2.3. Cross reactivity testing

The dot blot was performed to test cross reactivity with murine anti-insulin antibodies. 20 μg of sample were loaded on the membrane directly and air dried. Human recombinant insulin (Biocon, India) was used as positive control. The membrane was probed with murine anti-insulin antibody raised in guinea pig (Sigma, USA) and reaction was visualized with 3, 3′-Diaminobenzidene tetrahydrochloride (DAB) (Sigma, USA) as substrate.

2.4. SDS–PAGE

The purity of protein was checked on 12% SDS–PAGE by the method of Laemmli [25]. The crude extract, purified sample, and human insulin were resolved along with molecular weight marker. The proteins were stained with 0.25% Coomassie brilliant blue – R250.

2.5. Western Blotting

The proteins separated on SDS–PAGE were transferred to nitrocellulose membrane using western blot unit (MerkMillipore, India). The membrane was incubated with anti-insulin antibody (1:500) overnight at 4 °C. The membrane was further probed with HRP labeled secondary antibody and developed using DAB.

2.6. ELISA assay

To quantitate the ILP, a modified ELISA assay was performed [10]. Briefly, a 96-well microtiter plate (Tarsons, India) was coated with 10–40 μg of insulin solution as standard (Biocon, India), The protein samples from each step of purification were incubated at 4 °C overnight in 100 μl 0.05 M carbonate/bicarbonate buffer, pH 9.6. After washing with TPBS (0.05% Tween 20 in 0.1 M phosphate, 0.5 M NaCl, pH 7.6) and blocking with1% gelatin in TPBS the samples were incubated with 50 μl of anti-insulin antibody (1:500) for 2 h at room temperature. The wells were washed with TPBS and incubated with 50 μl of a biotinylated anti-IgG antibody (1:500) for 2 h at room temperature. The plates were washed again with TPBS and developed using substrate TMB/H_2O_2. The reaction was stopped by adding 50 μl 3 N H_2SO_4 and the absorbance was measured at 405 nm [26]. The amount of ILP present was determined from standard curve.

2.7. MALDI-TOFF

The accurate molecular mass of the ILP was determined by MALDI-TOFF (Waters Micromass Q-TOF Ultima Global) using 1 μg/μl protein concentration, where the pure protein sample was dissolved in PBS.

2.8. Circular dichroism (CD)

The secondary structure of the purified protein was analyzed by CD spectroscopy on a Jasco J-720 spectropolarimeter. The analysis was done by Yang's reference and limit of wavelength was set at 190–300 nm in the far UV region.

2.9. Biological activity of ILP

2.9.1. In vitro glucose tolerance test

The hypoglycemic property of the purified protein was tested on Insulin responsive cell line RIN5f (National Center for Cell Science, Pune).10^4 cells/well were seeded in a 24-well plate with DMEM (Dulbecco's Minimal Essential Medium, Hi Media, India), containing 10% FBS (Fetal Bovine Serum, Sigma, USA) and 0.1% antibiotic solution (Sigma, USA). The cells were allowed to adhere overnight at 37 °C and 5% CO_2. The DMEM was discarded and wells were washed with Hank's balanced salt solution (HBSS). 500 mM glucose and ILP (25, 50 and 100 μg) were added and the cells were incubated for 180 min. Aliquots of media (200 μl) were collected at 0, 30, 60, 90, 120, 150 and 180 min. The amount of glucose not utilized by cells was estimated using DNSA method. The amount of glucose in the wells was expressed in milimoles of glucose per 100 μl of aliquots collected [27].

2.9.2. In vivo study

2.9.2.1. Induction of diabetes mellitus. The male Swiss mice (6–8 weeks old), weighing 25 ± 2 g, maintained at an ambient temperature of 25 ± 2 °C with standard food and water *ad libitum* were made diabetic with a single intra-peritonial injection of streptozotocin (STZ) (3 mg/25 g of body weight) in citrate buffer, pH 4.5 [28]. The institutional animal ethical guidelines were followed throughout the study.

2.9.2.2. Oral glucose tolerance test (OGTT). The oral glucose tolerance test was performed on overnight fasted diabetic and normoglycemic mice. The animals were divided into four groups such as control (receiving vehicle), insulin (positive control), animals