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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

treated with ILP intraperitoneally and orally respectively. Glucose (2 g/kg body weight) was given orally and immediately the ILP was administered orally (50 μg/200 μl) to the mice. The peripheral blood glucose level by tail prick was measured at 0, 30, 60, 90, 120, 150 and 180 min using Accuchek Active Glucometer (Roche Diagnostics GmbH, Germany). Insulin [70 μg (2 IU)/25 g] body weight was administered subcutaneously as a positive control [29]. The blood glucose level was measured in milligram per deciliter [30]. Similar experiment was performed on another group of normoglycemic and diabetic mice where the ILP (50 μg/200 μl) was injected intraperitonially immediately after glucose.

3. Results

In our attempt to isolate hypoglycemic component from *C. igneus,* we have purified a low molecular weight protein with oral hypoglycemic activity.

3.1. Purification and characterisation of ILP from C. igneus

The ILP was purified from acidified ethanolic extract followed by affinity column chromatography. The purity of the protein was confirmed by SDS–PAGE (Fig. 1a). The approximate molecular weight was found to be 11 kDa. Further, the cross reactivity of ILP with anti-insulin antibodies was confirmed by Western blot analysis (Fig. 1b). The specific activity of the protein was calculated by ELISA using human insulin as standard. Table 1 shows fold purification and specific activity at each purification step. After the final purification step, the protein concentration was 3 mg/ml. Thus 2.37-fold purification was achieved as compared to crude extract.

The exact molecular weight of the protein was found to be 56118 dalton using MALDI-TOFF. The circular dichroism spectra of the protein showed 31.1% alpha helical structure and 68.9% beta sheet structure using analysis by Yang's reference.

3.2. In vitro hypoglycemic activity of the ILP from C. igneus

The purified ILP was first tested for its hypoglycemic property on insulin responsive RIN5f cell lines. The utilization of glucose by cells in presence of various concentrations of ILP (25, 50, 100 μg) was calculated over 3 h. The human insulin was used as a positive control. The cells without treatment served as control. The dose-dependent rapid decrease in the glucose concentration in the supernatant was observed in presence of ILP, indicating an effective glucose uptake by the cells (Fig. 2). The decrease in glucose concentration was more pronounced in the wells treated with 50 and 100 μg of protein compared to insulin (25 μg). This indicated that the protein we have purified has insulin like activity. Furthermore, the decrease in glucose concentration was much better than insulin between 60–120 min after application, proving the rapid hypoglycemic effect of ILP (Fig. 2).

3.3. In vivo hypoglycemic activity of the insulin-like protein from C. igneus

Since good hypoglycemic activity was observed in *in vitro* bioassay, we decided to extend the assay in *in vivo* mice model. The minimum required dose of ILP needed to get effective response was found to be from *in vitro* bioassay (Fig. 2). Therefore first, the normal mice were treated with 50 μg of protein intraperitoneally and orally. 70 μg of human insulin given intraperitoneally was taken as a positive control. The OGTT was preformed for three hours. The ILP given orally was able to lower the blood glucose level within 120 min, making animals hypoglycemic whereas both intraperitoneal insulin and ILP effects were gradual and did not cause acute hypoglycemia as compared to oral ILP (Fig. 3). This result confirms the rapid oral hypoglycemic property of the ILP.

Then we extended our study in diabetic mice. The animals were treated after their blood glucose level reached around 550 mg/dl. The animals were divided into four groups as mentioned earlier. Again we saw a rapid decrease in the blood glucose level in the animals treated with ILP as compared to the insulin positive control. In the first 30 min., insulin brought down glucose level to 490 mg/dl while ILP brought down the glucose level to 450 mg/dl (Fig. 4). Interestingly we found comparable hypoglycemic activity for ILP when supplemented orally as well as intraperitoneally even in the diabetic mice and the lowering of glucose level was even better than insulin (Fig. 4).

The animals were kept under observation to see any side effect of the protein administered for a week. There was no toxic effect seen on animals after administrating the purified protein at the dose mentioned.

4. Discussion

Small peptides are known to play diverse roles in plants; as mediators of cell-to-cell communications, through extracellular ligand-receptor interactions [31,13], in defense response, enhancement of callus growth and meristem organization, self-incompatibility, leaf shape and organ abscission [31,32]. In plants, growth and development processes involve metabolism of stored starch into glucose, like mobilization of liver glycogen into glucose in animals. Animal insulin has improved seed germination [33–35], accelerated synthesis of ribosomal proteins in germinating maize embryos [36] and increased the activity of glyoxysomal enzymes

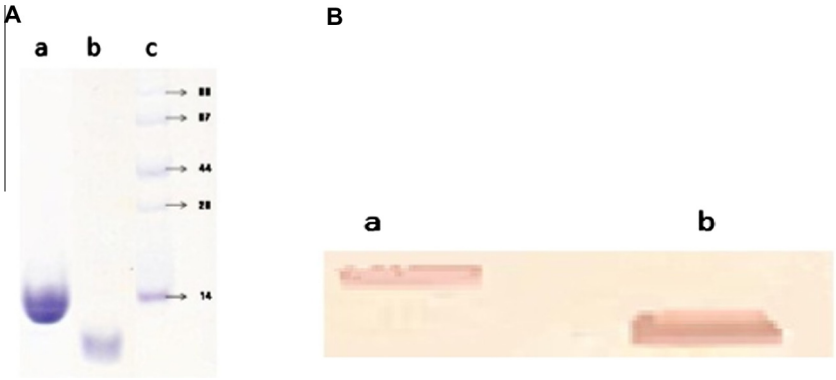

Fig. 1. Purification and identification of ILP from *Costus igneus*. (A) SDS–PAGE of Purified ILP from *Costus igneus*. Lane a ILP; Lane b Insulin (positive control); Lane c Molecular Weight Marker (B) Western Blot of ILP from *Costus igneus* Lane a ILP, Lane b insulin (positive control).

Table 1
Protein purification Steps.

Step	Protein (mg/ml)	Specific Activity	% Yield	Fold Purification
Crude	30.7	0.35	100	1
Concentrated	36	0.63	97.52	1.8
Pure Protein	3	0.83	6.09	2.37

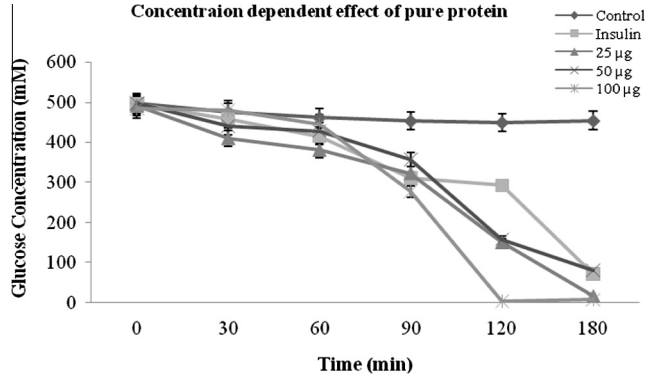

Fig. 2. *In vitro* Glucose Uptake Assay in RIN 5f Cell Line. ♦, control; ■, insulin; ▲, 25 µg ILP; ×, 50 µg ILP; *, 100 µg ILP.

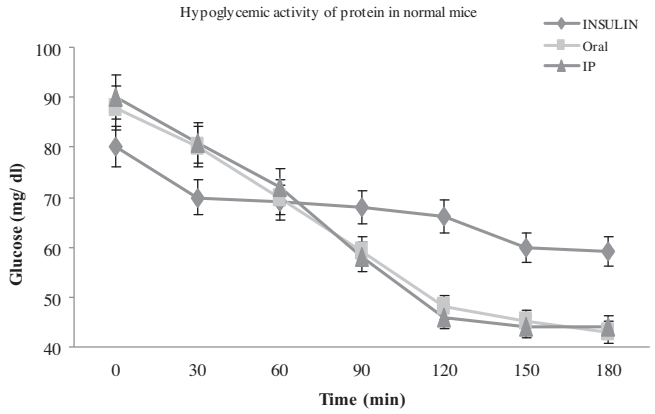

Fig. 3. *In vivo* Hypoglycemic Activity of ILP in Normoglycemic Swiss Mice. ♦, insulin; □, orally given ILP; ▲, ILP given i.p. (The data represents mean ± SD, $n = 6$).

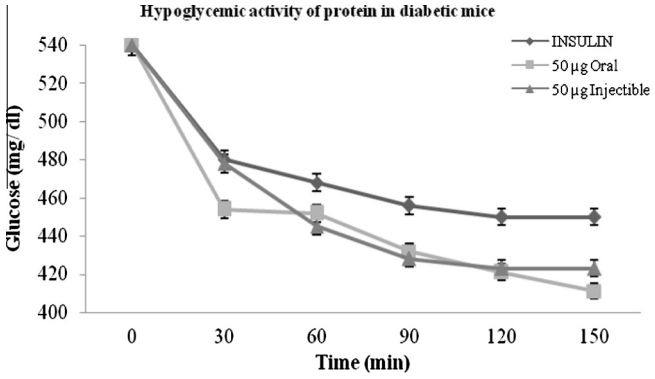

Fig. 4. *In vivo* Hypoglycemic Activity of ILP in STZ-Induced Diabetic Swiss Mice. ♦, insulin; □, orally given ILP; ▲, ILP given i.p. (The data represents mean ± SD, $n = 6$).

[33]. In plant chloroplasts, starch is broken down into glucose, then transported to cytoplasm via a glucose transporter. In *Bauhinia*, insulin-like protein has been localized to chloroplast membrane [12].

Insulin and other Insulin-like Growth Factors (IGFs) evolved very early and are found from the lowest to the highest life forms. The presence of insulin-like peptides having immunoreactivity with anti-insulin antibodies is reported in plants such as Bitter Gourd [5,6], *B. variegata* [12], Maize [36], *Canavalia ensiformis* [37] and *Vigna unguiculata* [11]

In this study, the low molecular weight protein having cross reactivity with anti-insulin antibodies was isolated from the leaf tissues of *C. igneus* in an acidified ethanol solution [5,11,38]. The protein was purified using affinity column prepared with murine anti-insulin antibodies immobilized on sodium alginate beads due to epitope identity to insulin. The immunoreactivity of the ILP with murine anti-insulin antibody was confirmed by Western Blot analysis (Fig. 1b). The molecular mass of the ILP was found to be 5.7 kDa by MALDI-TOFF. This corelates well with other reports such as *L. gibba* (6 kDa) [39], *Vigna unguiculata* (6 kDa) [11], *B. variegata* (5.7 kDa) [12]. This is the first report showing presence of insulin immunoreactive proteins in *Zingiberaceae-Costaceae*. The biophysical characterization of ILP showed that it is structurally different from Insulin but functionally similar to insulin.

In India, *C. speciosus* and *C. igneus* (*C. pictus*) are the two commonly found species of *Costus*. *C. pictus* leaf extracts showed good inhibitory activity against α-glucosidase and α-amylase enzymes [40]. Although the effector molecules are not characterized, the results prove the potential of *Costus* plants in controlling the postprandial hyperglycemia. The methanolic leaf extracts (200 mg/kg) of *C. igneus* lowered blood glucose level in diabetic mice in 30 days, better than glibenclamide (0.5 mg/kg), [41]. Rhizome extracts of *C. igneus* exhibited anti-oxidant activity, stimulated glycolytic enzymes and controlled gluconeogensis in diabetic rats [42]. Bioactive compounds were quercetin and diosgenin.

Recently, the aqueous extract of rhizome of *C. speciosus* was shown to be effective in decreasing plasma glucose level and improving pancreatic protein and DNA content in diabetic rats. It also increased release of plasma insulin and C-peptide [43].

The methanolic extract of *C. pictus* leaves has been shown to increase the insulin secretion in *in vitro* cultures of MIN 6 cells at basal level but the extract did not release insulin after glucose stimulation of MIN 6 cells [20]. However in our studies the ILP isolated from *C. igneus* was able to reduce glucose level *in vitro* in a concentration dependent manner in RIN 5f cell line. Since the ILP had a long-lasting hypoglycemic effect, beyond 1 h post-application, it has good therapeutic potential.

Encouraged with *in vitro* results, the hypoglycemic property was checked in normal and STZ-induced diabetic mice. The ILP was administered along with glucose load and blood glucose clearance was recorded for three hours. Interestingly both intraperitoneal and oral route showed significant decrease in blood glucose level compared to insulin as a positive control. We observed time dependent decrease in glucose level in acutely diabetic mice upon glucose stimulation, indicating that the ILP isolated in this study acts like insulin. There are comparable reports in Alloxan diabetic rats where methanolic leaf extract of *C. igneus* (200 mg/kg) showed hypoglycemic effect after intra-peritoneal administration for 30 days [41]. In yet another study, Eliza et al. [43] reported that hexane extract of *C. speciosus* rhizome was hypoglycemic, improved protein and DNA content of pancreas and increased release of plasma insulin and C-peptide contents in streptozotocin-induced diabetic rats [43].

In fact, in our experiments, oral administration of ILP was able to reduce blood glucose level better than the intraperitoneal insulin administration. Therefore we conclude that the ILP purified in

the present study from *C. igneus* is a novel protein having oral hypoglycemic activity. We hypothesize that the protein purified is functionally similar to animal insulin, although the physiological role of this protein in plant is still unknown.

Proteins and polypeptides would not add a great advantage to the current anti-diabetic therapy in spite of their ability to act like insulin. The reason is that they have to be administered by injection. Thus, no novel advantage can be seen in exchanging insulin with an "insulin-like" agent if both have to be given parenterally. A true advantage of an "insulin-like" agent is that it would be effective when orally administered. Our insulin-like protein does exactly that. It gave an excellent response when the protein was fed orally to the diabetic as well as normal mice as well as when *in vitro* assay was performed.

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