

## PCR Based Detection of Insulin Like Protein from *Dolichos lablab* L.

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In the current research work, the genetic region corresponding to both alpha and beta chains of insulin like protein from *Dolichos lablab* L was identified using PCR. Earlier study by Venâncio *et al.*, (2003) has revealed that the amino acid sequence of alpha and beta chains of bovine insulin has significant similarity to amino acid sequence of insulin like protein from legume *Vigna unguiculata* (cowpea). Although the amino acid sequence was available for *V. unguiculata*, the corresponding DNA sequence was not available in the database. Considering the resemblance in protein sequence, primers were designed using nucleotide sequences of alpha and beta chains of bovine insulin and were used for amplification of these chains from *V. unguiculata* and *D. lablab*. Genomic DNA extraction was carried out by the conventional ethanol precipitation method followed by purification using preparative AGE. Standardization on PCR amplification of alpha and Beta chain of Insulin from *D. lablab* L. and *V. unguiculata* was carried out. Results obtained for the PCR amplification of alpha and beta chains were validated by the re-amplification of gene, where the previously obtained PCR product was used as template DNA. All the above experiments suggest that the DNA sequence of both chains of insulin gene is probably highly conserved amongst different organisms as the primer designed using bovine insulin DNA sequence could amplify the same gene in plants like *V. unguiculata* and *D. lablab* L.

*Key words: Insulin like protein, Bean, PCR based detection, Dolichos lablab*

Leguminous plants are outstanding for their high levels of bioactive compounds, which can impact glucose metabolism by various mechanisms. Antidiabetic activity of leguminous plants is due to bioactive compounds such as genistein and daidzein, alpha amylase inhibitors, alpha-glucosidase inhibitors (Getek *et al.*, 2014).

Multiple reports have shown presence of Insulin like proteins in various plants including bitter gourd, spinach and important food legumes legume such as soybean and Cowpea. (Khanna *et al.*, 1981, Collier *et al.*, 1986, Hirano H. (1996) Oliveira *et al.* (1999).

Protein involved in carbohydrate transport to fruits was detected in empty pods and seed coats of cowpea (*Vigna unguiculata*) by Western blotting (Venâncio *et al.*, 2003). Insulin-like proteins are synthesized during the embryo development stage in certain edible leguminous plants such as *Glycine max*, *Vigna radiata* and *Vigna unguiculata*. The insulin is expressed only when embryo development occurs in legume seeds. The expression of insulin during germination is in a demarcated pattern. Insulin-like proteins are expressed only during seed germination and found to be absent during the dormant phase (Pathak *et al.*, 2011).

Orally active fraction of Insulin like protein was purified from *Costus igneus* which was found to have similar therapeutic effectiveness like animal insulin. The characterization of Insulin like protein showed that it is structurally different from insulin but functionally similar (Joshi *et al.*, 2013).

In the developing countries, the number of people suffering from diverse types of DM is incessantly aggregating. Therefore, it is inevitable to have alternative sources of insulin.

With enormous increase in patients, developing economical sources of insulin is a foremost challenge for countries like India. Being a pivot of diverse metabolites, plants can unquestionably serve as an alternative source of insulin like polypeptides. Previously reported research on hypoglycaemic activity of plants definitely ascertains that plants can also be used for synthesizing orally active, functionally similar insulin like proteins. (Joshi *et al.*, 2013).

Insulin like proteins were extracted using a modified, less laborious protocol from germinating seeds of *Dolichos lablab* L. Presence of insulin like protein was confirmed by SDS PAGE, HPLC. The efficacy of the purified insulin was assessed using a yeast glucose uptake assay, where the effectiveness was comparable with the standard metronidazole. (Palekar *et al.*, 2019)

During present work, genomic DNA extracted from 48 hours germinated seeds of *Dolichos lablab* L was used as a template DNA for the detection of genetic sequence of alpha and beta chains of insulin, as the maximum amount of insulin like protein was reported in germinating seeds of *Dolichos lablab* L. Primers were designed on the basis of DNA sequence of alpha and beta chains of bovine insulin as Vennancio *et al.*, (2003) reported that at amino acid sequence level bovine insulin has significant similarity to insulin like protein of *Vigna unguiculata*. Although nucleotide sequence of insulin like protein of *V. unguiculata* was not available, nucleotide sequence of bovine insulin reported by Venâncio *et al.*, (2003) was used as reference for primer synthesis. PCR reaction was standardized using *Dolichos lablab* L. and *Vigna unguiculata* genomic DNA to amplify the nucleotide sequence of insulin like protein.

## MATERIALS AND METHODS

Authentic dry seeds of *Dolichos lablab* L. were procured from Agricultural Research Station, Navsari. The authentic, healthy, dry Seeds for *Vigna unguiculata* were procured from the local market. The seeds were germinated for 48 hours as highest insulin like protein was obtained in this phase as per our previous study. (Palekar *et al.*, 2019)

PCR Reagents such as Taq Polymerase and Taq Buffer were procured from Sigma Aldrich Pvt. Ltd. Primers were designed and obtained from Sigma Aldrich Pvt. Ltd. 10mM dNTP mix was purchased from Thermo scientific Pvt. Ltd. 100bp Ladder was used for current study which was acquired from Invitrogen Pvt. Ltd.

The DNA sequence for alpha and beta chains of Bovine insulin (GI: 18776263) which was available in the NCBI nucleotide database, was used for the design of the primers to amplify the genes for these chains from *V. unguiculata* and *D. lablab* L.

### Genomic DNA extraction:

Genomic DNA extraction was carried out by the conventional in house protocol using ethanol precipitation. Germinating seeds were macerated in 10 folds of cold Tris acetate buffer containing 5 % Sodium Lauryl Sulphate, 10 mM EDTA and 1% sodium chloride. The extract was filtered using four layers of cheese cloth and filtrate was subjected to precipitation using ice cold Ethanol. Genomic DNA pellet was collected by centrifugation.

The genomic DNA was solubilized in Tris EDTA buffer and purified by Agarose gel electrophoresis to remove traces of RNA.

### Optimization of PCR for amplification of Beta chain of Insulin from *D. lablab* L. & *V. unguiculata*:

The calculated T<sub>m</sub> for forward and reverse primers for Beta chain gene based on primer length and GC content was 47 °C and 48 °C respectively. Based on the T<sub>m</sub>, a gradient PCR reaction was set up with annealing temperature varying from 43-45 °C to finalize optimal temperature. A typical PCR reaction was set up with final concentration of components as 1x PCR buffer, Forward Primer (5' TTC GTC AAC CAG CAT CTG 3') and Reverse Primer (5' GGC CTT GGG CGT GTA 3') for Beta chain (0.25uM) along with dNTP mix (50uM), Taq polymerase (1U) and gDNA (10ng) of *D. lablab* L. and *V. unguiculata* as templates. A non-

template reaction was also set as negative control. PCR cycle program was set as 94°C for 5 min for initial denaturation followed by 35 cycles of 94°C for 30 sec, 43-45°C for 30 sec, 72°C for 30 sec and final extension of 72°C for 7 min. Expected amplicon size was 87 bp.

### Optimization of PCR for amplification of Alpha chain of Insulin from *D. lablab* L. and *V. unguiculata*

As discussed earlier, the forward (5' GGC ATC GTG GAG CAG TGC 3') and reverse (5' GTT ACA GTA GTT CTC CAG CTG 3') primers for the amplification of alpha chain of insulin from *D. lablab* L. and *V. unguiculata* were designed using bovine insulin alpha chain DNA sequence. The calculated T<sub>m</sub> for these primers were 54°C (Forward) and 52°C (Reverse). To determine the optimal annealing temperature for the amplification of this chain a gradient PCR reaction was set up where the annealing temperature was varied from 45-47°C in 3 independent reactions. A typical PCR reaction was set up with final concentration of components as mentioned earlier for Beta chain. A non-template reaction was also set as negative control. PCR cycle program was set as 94°C for 5 min for initial denaturation followed by 35 cycles of 94°C for 30 sec, 45-47°C for 30 sec, 72°C for 30 sec and final extension of 72°C for 7 min. Expected amplicon size was 63 bp. Post amplification the amplicon was loaded on 3.5% agarose gel.

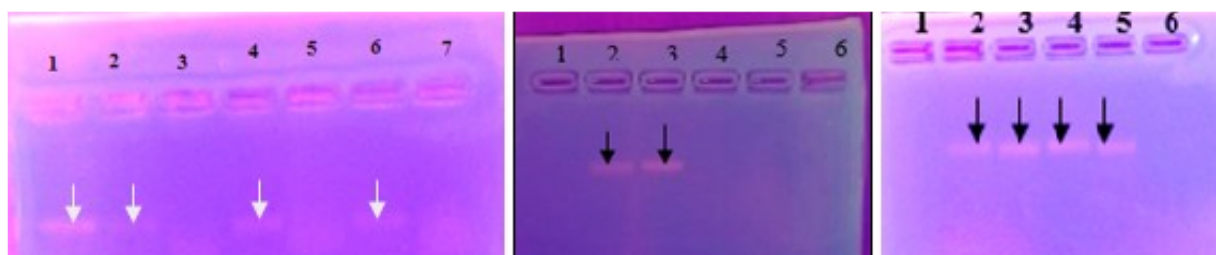


Fig 1-A

Fig 1-B

Fig 1-C

**Figure 1-A: Insulin  $\beta$  chain amplicon on 4% agarose gel;** (Lane 1 – *V. unguiculata* 43 °C , Lane 2 – *D. lablab* L 43 °C , Lane 3 – *V. unguiculata* 44 °C , Lane 4 – *D. lablab* L 44 °C, Lane 5 – *V. unguiculata* 45 °C, Lane 6 – *D. lablab* L 45 °C , Lane 7 –negative control)

**Figure 1-B: Amplification of Insulin  $\beta$  chain gene from *V. unguiculata* and *D. lablab* L. post 40 PCR cycles at 44 °C annealing temperature, loaded on 3.5% agarose gel;** (Lane 1 – negative, Lane 2 – Insulin Gene amplified from *V. unguiculata* ,Lane 3 -Insulin Gene Amplified from *D. lablab* L, Lane 4 – 6 blank)

**Figure 1-C: Re-amplification of insulin  $\beta$  chain amplicon loaded on 3.5% agarose gel** (Lane 1 negative Control, Lane 2 – 5  $\mu$ l Re-amplified Product of *V. unguiculata*, Lane 3 – 25  $\mu$ l Re-amplified Product of *V. unguiculata*, Lane 4 – 5  $\mu$ l Re-amplified Product of *D. lablab* L., Lane 5 – 25  $\mu$ l Re-amplified Product of *D. lablab* L., Lane 6 – blank)

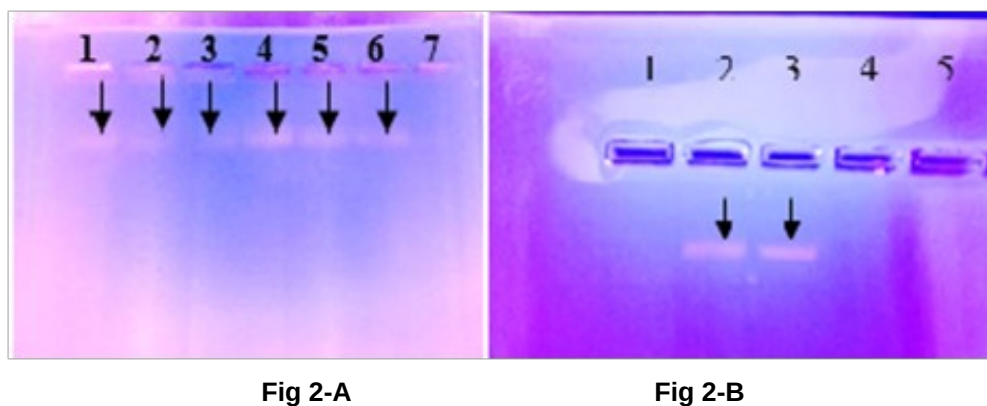


Fig 2-A

Fig 2-B

**Figure 2-A: Insulin  $\alpha$  chain gene amplification from *V. unguiculata* and *D. lablab* L., Amplicon loaded on 3.5% agarose gel; Lane 1 – *V. unguiculata* amplified at 45°C, Lane 2 – *D. lablab* L. amplified at 45°C, Lane 3 – *V. unguiculata* amplified at 46°C, Lane 4 – *D. lablab* L. amplified at 46°C, Lane 5 – *V. unguiculata* amplified at 47°C, Lane 6 – *D. lablab* L. amplified at 47°C, Lane 7 – negative.**

**Figure 2-B: Insulin  $\alpha$  chain amplicon from PCR with (45°C) primer annealing temperature, loaded on 3.5% agarose gel; Lane 1 – negative Control, Lane 2 – Amplicon -*V. unguiculata*, Lane 3 – Amplicon -*D. lablab* L., Lane 4 & 5- blank**

## RESULTS AND DISCUSSION

PCR amplification of gene for insulin like protein from plants *V. unguiculata* and *Dolichos lablab* L. was successfully carried out using the primers of bovine insulin gene as reported by Venancio *et al.* (2003). Results obtained for the PCR amplification of alpha and beta chain (Fig.1-A, B, C and Fig.2-A, B) were validated by the re-amplification of the gene, where the previously obtained PCR product was used as template DNA which confirmed that there was no non specific binding of primers. After complete standardization and optimization of the annealing temperature, PCR amplification of insulin gene was obtained.

All the above experiments suggest that the DNA sequence of both chains of insulin gene is probably highly conserved amongst different organisms as the primer designed using bovine insulin DNA sequence as reference could amplify the same gene in plants like *V. unguiculata* and *D. lablab* L.

## CONCLUSION

The above mentioned PCR protocol can be used as tool for amplification of insulin alpha and beta chain from other legumes. This can be used for assessment of other legumes for envisaging the antidiabetic potential. Further validation of the PCR studies have to be done

by sequencing the amplicon of insulin like protein gene in plants.

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