Expression of canine relaxin precursor protein in *Pichia pastoris* host: A possible tool for early pregnancy diagnosis

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In canines, a physical examination and ultrasound technique remain the most common methods of pregnancy diagnosis. The rapid detection of pregnancy is important in canines. There are several reports of peptide hormone relaxin to be exclusively produced in pregnant bitches and not by non-pregnant or pseudo pregnant bitches. We present here the expression of canine relaxin in *Pichia pastoris* GS115 host. The cDNA prepared using canine placental total RNA was used to amplify 432 bp relaxin gene, which was subsequently cloned in pPICZ-alphaA vector and followed by transformation into *Pichia pastoris* GS115 strain by electroporation. The zeocin resistant recombinant clones were confirmed by colony PCR and sequencing. One of the positive clones was selected for protein expression in culture medium. The supernatant was taken and precipitated using 20% TCA in acetone. The recombinant relaxin precursor protein showed a mol wt of 24 kDa, which is higher than the expected 19 kDa and that could be due to glycosylation in the *Pichia pastoris*. The 24 kDa recombinant protein was detected up to 48 h of post-induction. This is the first report describing the expression of recombinant canine relaxin in *Pichia pastoris*. The polyclonal antiserum against canine relaxin would be useful for detection of pregnancy in bitches and also in assessing the prognosis of canine mammary tumors.

Keywords: Canine, pregnancy diagnosis, *Pichia pastoris*, recombinant protein, relaxin

Introduction

In any animal species, the pregnancy diagnosis is a useful tool for its better management. In bitch, a physical examination and ultrasound technique remain the most common methods of pregnancy diagnosis. Further, the detection of reproductive hormone especially progesterone is of no use as it is present at elevated levels, whether or not the animal is pregnant. There is a need to develop rapid pregnancy detection methodology for the canines. A relevant clinical problem is the lack of an early pregnancy test(s), which would allow monitoring embryonic well-being and diagnosing embryonic death. However, no pregnancy specific proteins are reported in bitches.

There are several reports of peptide hormone called relaxin to be exclusively produced in pregnant bitches and not by non-pregnant or pseudo pregnant bitches1,3. The heterodimeric polypeptide relaxin is a member of the insulin-like superfamily and a hormone of pregnancy in numerous species4. Relaxin was first isolated and purified from corpora lutea of the pig ovary5. It is regarded as a specific marker of pregnancy in dogs1,3. Upon parturition or shortly before spontaneous abortion, relaxin serum concentration decreases to undetectable level, implicating relaxin as a potentially useful indicator for monitoring the onset of parturition in dogs1.

Relaxin is a 6 kDa polypeptide hormone with structural homology to insulin and the insulin-like growth factors. Primates and non-primate vertebrates exhibit very disparate profiles of relaxin genomics, proteomics and functional biology. The expression of the hormone in an active form is a tough task as the hormone relaxin is synthesized as a single-chain precursor, preprorelaxin that is processed by cleavage of the signal peptide and by internal cleavage of a connecting peptide to form two disulfide-linked chains to form the active hormone. Further, there are no reports of expression of canine relaxin in any of the hosts. Expression of relaxin in heterologous system offers several advantages as with any recombinant protein. Here we have described the expression and characterization of the recombinant canine relaxin in *Pichia pastoris*, a heterologous host.

Materials and Methods

Sample Collection

The placental samples were collected from bitches after whelping from the Polyclinic of the institute and stored at –80°C until further use.
Isolation of Total RNA and cDNA Synthesis

Total RNA was isolated from 100 mg of testis samples using RNAGents (Promega, USA) following the method described by the manufacturer. The absorbance of RNA was measured at 260 nm/280 nm in Nanodrop spectrophotometer (Nanodrop®, USA) to check the purity. The cDNA was synthesized using Revert Aid™ H-Minus First Strand cDNA synthesis kit (Fermentas, USA) using oligo(dT) primer.

Primer Design

Primers were designed using Primer-BLAST online tool provided by the NCBI (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/index.cgi?LINK_LOC=BlastHome) using the relaxin sequence available in the GenBank database of NCBI (Acc.No. AF233687) excluding the signal sequence. Forward primer (ExRLXF2 5’-GCATGTCCGCGGACTTAAGGCATGGGTCGTGA-3’) having SacII site at its 5’ end and reverse primer (ExRLXR2 5’-TCAGCATCTAGA GCAAGCTCTTT CTGTTACAAC-3’) having the XbaI site at its 5’ end were used for directional cloning.

PCR Amplification, Cloning and Sequencing of Relaxin Gene

The PCR was standardized using 20 mM Tris-HCl, pH 8.8, 10 mM (NH₄)₂SO₄, 10 mM KCl, 0.1 mg/mL BSA, 0.1% Triton X-100 and 2.0 mM MgSO₄, 200 µmoles of each dNTP, 25 pmoles of each primer and 2.5 µL of Pfu DNA polymerase in a final volume of 25 µL. The thermal cycling conditions were: initial denaturation at 94°C for 5 min, followed by cycle denaturation at 94°C for 30 sec; annealing at 56°C for 30 sec; extension at 72°C for 30 sec for 35 cycles, and final extension at 72°C for 10 min, using cDNA synthesized from canine placental total RNA. The amplified PCR product was confirmed for their size with 1.0% agarose gel. The relaxin gene and pPICZαC/RGN1 were incubated on ice for 5 min in an ice-cold sterile tube. Cells were centrifuged and the cell pellet was resuspended in 0.5 mL of ice-cold sterile water. The above step was repeated once again and the resulting cell pellet was finally resuspended in 10 mL of ice-cold 1 M sorbitol. Finally, the cells were again centrifuged and the cell pellet was resuspended in 0.5 mL of 1 M sorbitol. The cells were kept on ice until transformation.

Electroporation of P. pastoris GS115 Competent Cells

An 80 µL of electrocompetent P. pastoris cells along with 10 µg of linearized recombinant pPICZαC/RGN1 were incubated on ice for 5 min in an ice-cold 0.2 cm electroporation cuvette. Cells were pulsed in an electroporator according to the manufacturer’s instructions (Biorad, Micropulser™, USA.) One mL of ice-cold 1 M sorbitol was added to the cuvette immediately after electroporation. The cuvette contents were transferred to a sterile 15 mL tube and incubated at 30°C without shaking for 2 h. And later 1 mL of YPD medium was added and further incubated at 30°C for 3 h in a shaker incubator. A 200 µL of this culture was spread on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) plates containing different concentrations of zeocin (100, 500, 1000 µg/mL). The plates were incubated at 30°C for 4 d. Ten colonies were picked from 1000 µg/mL zeocin plate and inoculated to YPD broth for further studies.

Colony PCR for Screening of P. pastoris Integrants

Ten clones were picked up from zeocin YPDS plate and suspended in 10 µL of nuclease free water with 25 units of lyticase (Sigma, USA), incubated at 30°C for 10 min and then frozen at –80°C for 10 min and thawed. The supernatant was used as a template for setting PCR with AOX1 primer. The reaction was complete linearization of the plasmid was checked by loading a small aliquot of the digested plasmid on 1% agarose gel. The rest of the digested plasmid was subjected to phenol:chloroform extraction. The resulting DNA pellet was reconstituted in 10 µL of nuclease free water and stored at –80°C until further use.

Preparation of Electro Competent P. pastoris GS115 Cells

A 5 mL of the P. pastoris GS115 strain was grown in YPD medium at 30°C/250 rpm in a shaker incubator. This culture was inoculated into 250 mL of fresh YPD medium in a 1 L flask and allowed to grow till the OD₆₀₀ reached 1.5. All the centrifugation steps were carried out at 1500× g for 5 min at 4°C. The culture was centrifuged and the resulting pellet was resuspended in 250 mL of ice-cold sterile water. The cells were centrifuged and the cell pellet was resuspended in 125 mL of ice-cold sterile water. The above step was repeated once again and the resulting cell pellet was finally resuspended in 10 mL of ice-cold 1 M sorbitol. The cells were kept on ice until transformation.
set up using 10 mM TrisHCl, pH 8.8, 50 mM KCl, 0.08% Non-ident P40 and 1 mM MgCl$_2$, 200 µmoles of each dNTP, 5 pmoles of each primer in a volume of 24.5 µL. The reaction mixture was incubated in the thermal cycler at 95°C for 5 min and then 0.5 units of TaqDNA polymerase (Fermentas, USA) was added to make the final volume to 25 µL. The thermal cycling conditions used were similar to those used initially for relaxin gene amplification. In addition, vector control as well as no template control was also run. The PCR products were electrophoresed on 1% agarose gel electrophoresis.

**Expression of Recombinant Relaxin Protein in *P. pastoris* GS115 Strain**

For initial screening of clones from YPD broth containing zeocin (1 mg/mL), five clones were inoculated to a 10 mL of YPD broth and incubated in a shaker incubator at 30°C for overnight at 250 rpm. A 500 µL overnight grown culture was sub-cultured to 50 mL of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.04 mg% biotin & 1% glycerol) and incubated at 30°C at 250 rpm until culture reached an OD$_{600}$ 2-6. The cells were harvested and resuspended in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.04 mg% biotin & 0.5% methanol) till the OD$_{600}$ became 1.0 and returned for incubation at 30°C for overnight at 250 rpm. The culture was induced with methanol every 24 h to the final concentration of 0.5% till 48 h. The culture supernatant containing the protein was precipitated by pelleting and was precipitated using 50% ammonium sulfate. The protein precipitate was resuspended in 1× PBS, dialyzed against the same and analyzed on 12% denaturing SDS-PAGE.

**Confirmation of Relaxin Expression by Western Blot**

The electrophoresed recombinant relaxin was transferred onto PVDF membrane (Hybond-P, UK) at 100 V for 1 h 30 min as per the method described by Towbin and co-workers. After transfer, the membrane was blocked with 5% skimmed milk powder in PBS and kept overnight at 4°C. The membrane was washed thrice with 1× PBST (0.05% Tween 20). The proteins were probed with anti-His(C-terminal)-HRP conjugate (1:2000, Invitrogen). Following washing the unbound antibodies, the blot was developed using DAB substrate solution (6 mg DAB, 50 µL 8% NiCl$_2$, 10 µL H$_2$O$_2$ in 10 mL PBS). The reaction was stopped by washing with distilled water.

**Results and Discussion**

The need to measure relaxin in body fluids has spurred the development of radio immunoassay and enzyme-linked immunoassays$^{9-10}$. Although a few of the successful assays employ antibodies that cross-react with relaxins of several species. The relaxin molecules exhibit remarkable species variation. The structural differences can be so large that some relaxins have minimal or no cross-reactivity with antisera generated against other proteins. It is generally advised to develop homologous assays for each individual species under investigation in order to obtain more reliable quantitative data.

Relaxin has been purified and characterized from the ovary and placenta of different species$^{11-15}$. However, there are not many reports on expression of recombinant canine relaxin that may be partly because of patents$^{16-17}$ limiting its utility for clinical applications. In dogs, both the placenta and ovary are reported to secrete relaxin, among which placenta being the major contributor to the serum relaxin levels during pregnancy$^{3,14}$. The expression of the hormone in an active form is a tough task as the hormone relaxin is synthesized as a single-chain precursor, preprorelaxin that is processed by cleavage of the signal peptide and by internal cleavage of a connecting peptide to form two disulfide-linked chains to form the active hormone. In the present study, we have expressed and characterized the recombinant canine relaxin precursor in *P. Pastoris* that would be used for raising polyclonal antiserum.

The RNA isolated from the canine placenta was quantified using Nanodrop (Spectrophotometer, ND 1000) at A$_{260/280}$. The concentration of the RNA was 570 ng/µL and the ratio at A$_{260/280}$ was 1.90. It was intact when checked on 1.2% agarose gel. The total RNA isolated from canine placenta showed good purity. The PCR amplification of the relaxin gene from the cDNA of placenta showed good purity. The PCR amplification of the relaxin gene from the cDNA of placenta showed good purity. The PCR amplification of the relaxin gene from the cDNA of placenta showed good purity. The PCR amplification of the relaxin gene from the cDNA of placenta showed good purity.
which a product of ~430 bp was confirmative of the presence of relaxin gene (Fig. 2).

**Sequence Analysis of Relaxin**

Relaxin gene was sequenced and the nucleotide sequence was analyzed in Megalign software of DNASTAR Lasergene 6.0. The sequence was submitted to NCBI GenBank database and was assigned with Acc. No. KC977307. The nucleotide sequence analyzed for homology using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed 100% homology with earlier reported canine relaxin (Acc. No. NM_001003132). The amplified gene coded for 143 amino acids and apparent mol mass of this protein was 16.54 kDa, theoretical pI was 8.93; and consisted of 21 negatively and 25 positively charged amino acid residues as predicted by ExPASy (http://web.expasy.org/cgi-bin/protparam/protparam) program.

**Expression and Confirmation of Recombinant Regucalcin**

Number of colonies observed on YPDS zeocin agar was proportional to the concentration of the zeocin used. There were around 10 colonies in 1 mg/mL of zeocin YPDS agar plates. The Mut+ phenotype recombinant clones from 1 mg/mL of zeocin YPDS plate were induced with 0.5% methanol for up to 96 h. The expression kinetic study showed a higher level of expression of recombinant protein 48 h of post-induction. The culture supernatant collected at 48 h post-induction showed protein bands of ~24 kDa on SDS-PAGE (Fig. 3). The Western blot studies probed with anti-His(C-terminal)-HRP conjugate also showed a protein band of 24kDa (Fig. 4), indicating that the expressed recombinant protein is relaxin.
The pPICZα series of vector express the cloned gene as a secretory protein into the culture medium, which is a fusion protein having α-factor secretory signal peptide at the N-terminal, and C-myc epitope and His6 tag at the C-terminal of the recombinant protein. The α-factor and the C-terminal tag will add an approx 9.3 kDa and 2.5 kDa to the size of the recombinant protein, respectively. The predicted mol wt of the recombinant relaxin should be around 16.5 kDa and secreted recombinant protein should ideally show a mol wt of ~28.3 kDa, but the SDS-PAGE and Western blot had shown a protein of mol wt of 24 kDa (Figs 3 & 4).

The α-factor secretory signal peptide undergoes proteolytic cleavage in the culture medium by the Kex2 and Ste13 protease, which are secreted from the P. pastoris GS115 host into the culture medium. After cleavage of 9.3 kDa α-factor secretory signal peptide, the net mol wt of the recombinant relaxin would be around 19 kDa. The higher mol wt of the recombinant relaxin precursor could be due to post-translational modifications. There are reports of an increase in the mol wt of the protein up to 40 kDa over the actual mol wt of the expressed protein due to glycosylation in P. pastoris\textsuperscript{18}.

The culture supernatants of more than 48 h of post-induction were showing a reactive band on Western blot having a mol wt of 15 kDa (Fig. 5). It has recently been reported that those proteins having the internal accessible dibasic amino acids, such as, KR and RR, in the coding region of the protein are also cleaved by Kex2 protease\textsuperscript{19}. The deduced amino acid sequence showed the presence of RR (31\textsuperscript{st} & 32\textsuperscript{nd} residue; 140\textsuperscript{th} & 141\textsuperscript{st} residues) and KR (122\textsuperscript{nd} & 123\textsuperscript{rd} residue) (Fig. 6). The peptide sequence between 33\textsuperscript{rd}-121\textsuperscript{st} residues and 33\textsuperscript{rd}-139\textsuperscript{th} residues when analyzed using ExPASy server (http://web.expasy.org/protparam/) predicted a mol wt of 10 kDa and 12.2 kDa. It is interesting to note that the expressed protein when probed with anti-His(C-terminal)-HRP conjugate was reacting to the C-terminal His tag upon Western blotting. This clearly indicates that the Kex2 cleavage sites at RR (140\textsuperscript{th} & 141\textsuperscript{st} residues) and KR (122\textsuperscript{nd} & 123\textsuperscript{rd} residue) are inaccessible for cleavage. The only internal Kex2 site that probably cleaved is the RR (31\textsuperscript{st} & 32\textsuperscript{nd} residue) and the resulting peptide would be of 13.1 kDa. This predicted peptide along with the post-translational modifications could result in protein of ~15 kDa. The recombinant relaxin of 24 kDa expressed at 48 h post-induction was getting cleaved to 15 kDa with intact His tag at its C-terminal after 48 h.

The relaxin exerts numerous effects in a variety of tissues across a broad range of species. The hormone has a variety of actions associated with reproduction, including remodeling of the reproductive tract during pregnancy and stimulates human sperm motility. Attention has been directed recently to the cardiovascular effects of relaxin and vascular adaptations to pregnancy in the rat. It is also reported that the locally produced relaxin has a strong correlation in canine mammary tumor pathogenesis as an inducer of connective tissue remodeling\textsuperscript{20} and the metastatic potential of canine mammary tumors\textsuperscript{21}. Recently, the analysis of gene expression profiles of canine mammary carcinoma of various grades of malignancy showed significant difference in the expression of sehrl, zfp37, mipep, relaxin, and magi3 at mRNA level as well as at protein level\textsuperscript{22}. The mono-specific antiserum can be used for purification of native relaxin for its various functional studies. This is the first report describing the expression of relaxin precursor in P. pastoris. The antiserum against canine relaxin would be useful for detection of pregnancy in bitches and also in assessing the prognosis of canine mammary tumors.

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