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DEVELOPMENT OF AN INTERNAL CONTROL USING MUTAGENESIS BY OVERLAP EXTENTION TO MEASURE LDLR GENE EXPRESSION IN MONONUCLEAR CELLS USEFUL IN COMPETITIVE RT– PCR ASSAY

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Drug resistance remains a difficulty in the therapy of hypercholesterolemia. Considering the important role of the low-density lipoprotein receptor (LDLR) in cholesterol homeostasis, variations in LDLR gene expression may be implicated in this problem. In the present study, we report an original strategy to generate an internal control useful in competitive reverse transcription – polymerase chain reaction (RT-PCR) to measure LDLR mRNA levels in clinical specimens from patients with hypercholesterolemia. Total RNA was extracted using TRIzol® Reagent from 1 mL of mononuclear cells (7 x 10^6 cells/mL) isolated from blood by step gradient centrifugation on Histopaque. cDNA was produced from 1 µg of total RNA by RT using the SuperScript™ II RT RNAse- and random primers. We have deleted an 67-bp from 258-bp fragment of LDLR cDNA sequence using site-directed mutagenesis by overlap extention in PCR technique. Briefly, two segments (119-bp and 102-bp) of LDLR cDNA were amplified independently and then fused together in a subsequent reaction (206-bp). This fused fragment was inserted into *Eco*RI and *Bam*HI cloning sites of the pBluescriptII KS (-) vector (Stratagene, La Jolla, CA). The recombinant plasmid, pB-LDLR was used for transformation of *E.coli* DH5α. One colony of the transformands was isolated and analyzed to verify the presence of recombinant plasmid pB-LDLR. The presence of the insert into vector was confirmed by enzymatic restriction and PCR techniques. In summary, the procedure described here is rapid and simple, allowing application in RT-PCR assay to measure mRNA expression of any gene.

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Rec-hPRL production in *E. coli* periplasmic space with the use of a temperature sensible promoter.

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Human prolactin (hPRL) is a 199 amino acid protein, primarily produced by the anterior pituitary. Although this hormone mediates a variety of physiological processes, it is best known for its stimulatory effects on lactation. We have already described the synthesis of recombinant biologically active PRL (rec-hPRL) secreted into the periplasmic space of *Escherichia coli* (Morganti *et al.*, Biotechnol. Appl. Biochem., 1998; 27:63). This was obtained using an expression vector which had the PRL gene (preceded by a signal peptide sequence) under the control of the *tac* promoter. The expression of the rec-hPRL was induced by the addiction of 0.1 mM IPTG to the culture medium. Although this strategy was successful, with a production of up to 0.7 µg/ml per A_{600} unit, the induction with IPTG is expensive and therefore not useful for large-scale production. Moreover, IPTG is toxic and not recommended for industrial use and for the preparation of recombinant protein for injection. As an alternative, we have subcloned the hPRL gene in another vector under the control of the λP_L promoter. This promoter enables the induction of gene expression by a simple temperature increase from 30°C to 42°C, being cheaper for large-scale production and more proper for human use. In order to determine the best culturing conditions in erlenmeyer for this new strain, different studies on inoculum preparation and induction time were carried out. As a result, a production of 1 µg/ml per A_{600} unit was obtained. The recombinant hormone was analyzed in SDS-PAGE, Western Blotting and reversed phase HPLC. We intend to use this strategy to produce two analogs of hPRL, that are expected to act as antagonists of the hormone, i.e., to have an antiproliferative role on breast cancer development.

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Retrovirus-mediated transfer of growth hormone gene into primary human keratinocytes produces measurable circulatory levels of the hormone in immunodeficient-dwarf mice

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Transfer of exogenous DNA to keratinocytes has been demonstrated to be a suitable method for gene therapy applications. Keratinocytes are relatively easy to obtain and can easily be monitored for transgene expression. These cells can release a protein into the circulation and the systemic effects of the protein required for therapy can also be monitored.

In this study an *ex vivo* gene transfer protocol was utilized, in which primary human keratinocytes were transduced with an efficient retroviral vector (LXSN) encoding the human growth hormone gene. This construction and the utilization of a particular selection protocol yielded the highest *in vitro* secretion level ever described in the literature for this type of culture: 6.6 ± 0.6µg hGH/10^6 cells.day (n=6). When the grafting of the epithelial sheet made with these genetically modified keratinocytes was implanted into the *little/scid* mouse, an animal model that joins dwarfism to severe immunodeficiency, up to 1.5ng hGH/mL were detected in the serum, with a concomitant significant weight gain (0.060g/mouse; r=0.895, P<0.001).

A parallel strategy is being carried out using the mouse growth hormone gene for the purpose of studying a homologous system, which should provide a higher *in vivo* expression and a more evident phenotypic reversion.

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OPTIMIZATION APPROACH TO INCREASE THE SOLUBILIZATION OF RECOMBINANT FUSION PROTEIN COMPRISING THE EXTRACELLULAR DOMAIN OF SUBUNIT 2c OF THE HUMAN INTERFERON ALPHA RECEPTOR IN *E. COLI*

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Using system with high level of protein expression, the formation of inclusion bodies can be commum. Sometimes it is not desirable due to the arduous task of refolding the aggregated protein and the uncertainty of whether the refolded protein retained its biological activity. It was expressed the extracellular domain of subunit 2c of human interferon alpha receptor (rIFNAR2cEC) as fusion protein with glutathione S-transferase (GST) in *E. coli* DH5α. However, the recombinant fusion protein (rIFNAR2cEC-GST) induced the formation of inclusion bodies when it was expressed by 0.1 mM IPTG at 37 °C. We report here optimization approach to minimize the formation of inclusion bodies and to improve the solubilization of this recombinant fusion protein. The induction in the presence of 0.1 mM IPTG was tested at 25 °C and 30 °C. Three methods of cell lysis, sonication, French Press and the use of BugBuster Protein Extraction Reagent kit® (Novagen), were evaluated for extraction of the fusion protein. It was observed that the expression temperature of 25 °C and cell lysis by French Press method resulted in a high level of the soluble fusion protein. The cell lysis by sonication and by the use of BugBuster Protein Extraction Reagent kit® (Novagen) was inefficient to release the protein in soluble form although the protein expression was induced at 25 °C. Co-expression with bacterial chaperones GroES and GroEL was tested in order to increase the solubilization. of the fusion protein, but the result was not successful. The different pH (pH 7, pH 8, pH 9) of cell lysis buffer was studied and it was observed that the pH did not influence on the solubility of the fusion protein. In summary, the soluble recombinant protein rIFNAR2cEC-GST was obtained successfully by IPTG induction at 25 °C and by French Press cell lysis method. This soluble fusion protein was easily purified by glutathione sepharose affinity chromatography.

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HETEROLOGOUS EXPRESSION OF *Thermomyces* XYLANASE cDNA IN *Pichia pastoris*

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Xylanases are enzymes of great interest as catalysts in various biotechnological applications, such as in the bioconversion of xylan to fermentable sugars, the food industry and the biobleaching, an environmentally friendly technology. We have focused our research on the expression of the xylanase gene from the *Thermomyces lanuginosus* IOC-4145 in the methylotrophic yeast *Pichia pastoris* to try to improve enzyme production and to avoid the use of semi-solid media. Reverse-transcription polymerase chain reaction was performed to amplify the xylanase cDNA resulting in a fragment of 615 bp comprising the whole xylanase cDNA (585 bp) plus the NotI and EcoRI restriction sites to be subcloned into NotI/EcoRI pPIC9 vector (Invitrogen). The recombinant plasmid was linearized using SalI, inserted by electroporation into *P. pastoris* GS115 for expression of the interest protein and around 500 clones were obtained. The ability of 40 clones to produce xylanase was evaluated using the Congo red-polysaccharide interactions and all of them were positive. The enzyme produced by the best clones has been studied in the different conditions, and compared with the native enzyme profile. The specific activity of recombinant xylanase was 4 times higher than that obtained with the wild strain cultured on semi-solid media, with values of 1200U/mg of total protein even if using a very small scale of production.

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IMPROVED EXPRESSION OF PsaA FROM *Streptococcus pneumoniae* IN *E.coli* AND ITS PURIFICATION

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The actual vaccine against pneumococcus consists of polysaccharides from 23 strains of *S.pneumonie*, which is neither immunogenic nor protective in young children (the major risk group) and is very expensive. The substituition for conjugate vaccine is proposed by the World Heath Organization (WHO). This work aims at increasing the protective efficacy of the vaccine against pneumococcus and lowering the cost. We propose to express the conserved pneumococcal protein, PsaA (37 kDA), in *E.coli* and purify it for posterior conjugation. Therefore, it will be necessary to test different expression vectors and strains of *E.coli*. The gene for PsaA was cloned into the vector pRSETA-6xHis, between the Bam HI and Hind III restriction sites, and transformed into *E.coli* DH5α. Plasmids were purified and transformed into *E.coli* (BL21-DE3), leading to expression of the recombinant protein. In the attempt of purify recombinant PsaA, we have used the affinity chromatography in a nickel chelating resin. The recombinant protein was eluted from the Nickell-Sepharose column with a concentration of imidazol between 50 and 150 mM. We concentrated the recombinant PsaA in a filtration membrane (Centricon - 10, MWCO 10.000), and washed with 5 volumes of 50 mM Tris buffer pH 8.00, to eliminate some impurities as for example, the proteins under 37 kDa and also the imidazol. PsaA concentration was observed, however the low molecular proteins were not eliminated. A second Q-Sepharose column is being studied. Stability of the recombinant protein is being investigated. The purified proteins will be tested to verify the induction of humoral immune responses in mice and cross reactivity with different strains prevalent in Brazil.

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