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Production of Human Pancreatitis-Associated Protein (hPAP) in *Komagataella phaffii (Pichia pastoris)*

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Abstract

Pancreatitis-associated protein (PAP) is a pancreatic stress protein that is not produced in a healthy pancreas but is highly synthesized in pancreatic acinar cells in response to acute and chronic pancreatitis, hypoxia, toxins, diabetes, lipopolysaccharides hypotransferrinemia and organ transplantation. Changes in the PAP levels in serum are an important biological marker in the early stage of pancreatic diseases. In this study, the recombinant human PAP protein, which has the potential to be used as a diagnostic marker and as research material in proliferation, apoptosis, cell migration, cell invasion, and immunoassay studies, was expressed efficiently under the control of the AOX1 gene promoter in the *Komagataella phaffii (Pichia pastoris) (K. phaffii)* X33 strain. We describe the conditions required for the efficient production of PAP protein by methanol induction and its use without purification. The produced unpurified protein was tested in sandwich ELISA and showed consistent results with the commercial product. These results are encouraging that the protein produced can be used as a biomarker standard in ELISA tests without the cost and labor of purification.

Introduction

Human PAP / REG3A (mouse PAP / REG3B) or pancreatitis associated protein is a type C lectin-like secretion protein which is discovered because of its association with pancreatic diseases such as acute pancreatitis (Fu et al., 2012), diabetes (Zechner et al., 2012) and cystic fibrosis (Thomas & Ren, 2020).

The PAP gene contains 2748 base pairs and the coding sequence spreads over six exons. The first three exons, respectively; encode the 5' non-coding region, signal peptide, and 39 amino acids of the mRNA of the NH2 terminal end of the mature protein (Dusetti et al., 1993). The gene has a promoter containing TATA and CCAAT boxes (-28 and -52 positions) (Dusetti et al., 1994). The other three exons encode a protein domain with significant homology to the carbohydrate-recognition domain of animal lectins. (Dusetti et al., 1993).

PAP is a small protein with a molecular weight of 18.4 kDa, which is not normally secreted by the healthy pancreas but is secreted in pathological conditions, and its overexpression has been shown to be closely related to many diseases (Closa et al., 2007; Q. Li et al., 2016; Zenilman et al., 2000). Although it is secreted by the acinar cells into the pancreatic fluid with zymogenic granules in the pancreas, its transition to serum is also observed.

PAP protein is thought to play a role in hepatocytic and cholangiolar differentiation and proliferation (Christa et al., 2000). PAP is known to be a new macrophage chemoattractant affecting Schwann cell-macrophage interactions involved in peripheral nerve regeneration and provides new insights into therapeutic interventions (Namikawa et al., 2006). Exogenous PAP may alter the adhesion and motility of normal and transformed melanocytes, suggesting a potential interaction with melanoma invasiveness (Valery et al., 2001).

Although various functions have been proposed for PAP, the physiological significance of PAP upregulation in inflammatory diseases is unknown. Recent observations suggest that PAP may have a protective effect against inflammatory damage in pancreatitis (Ho et al., 2006) and extrapancreatic inflammatory conditions (Gironella et al., 2005; Zhang et al., 2004). PAP was analyzed as a diagnostic and prognostic marker of acute pancreatitis (Kemppainen et al., 1996). In acute pancreatitis, serum levels of PAP have been used as a biomarker to determine the course of the disease and treatment of the patient (Barthellemy et al., 2001; Iovanna et al., 1994; Sarles et al., 1999, 2005; Sommerburg et al., 2010). In addition, PAP is a diagnostic marker in the early diagnosis of Cystic Fibrosis (CF) disease in newborn screening (Sommerburg et al., 2015). CF disease, which is an autosomal recessive and deadly genetic disease, increases the amount of PAP when the baby is in the womb due to the stress state of the pancreas. It is common to use Immune Reactive Trypsinogen (IRT) as an important marker in immune tests for the diagnosis of CF, and the PAP protein as a second confirmatory marker (Iovanna et al., 1994). Studies to clarify the function of PAP have been limited due to the problems in isolation and production of PAP isoforms. This is largely due to the low solubility of PAP isoforms in the liquid phase (Viterbo et al., 2010).

With this study, the production of recombinant PAP protein, which has many uses, has been described using the *K. phaffii* X33 yeast strain. Proteins can show structural differences depending on the host due to differences in post-translational modifications. Studies show that there are different isoforms of PAP called lithostatin that can vary from 16 to 19 kD in the glycosylation state (De Reggi et al., 1995). There may be functional differences in PAP expression in the prokaryotic host. The host used to maintain protein structure and function is crucial. Therefore, we used *K. phaffii* X-33 strain to obtain the closest glycosylation pattern to human. In addition, its activity was tested and compared with commercial human PAP (Reg3A) protein (Sino Biological) protein with sandwich ELISA.

In this study, we demonstrated that the soluble Fc labeled form of human PAP protein is successfully expressed in *K. phaffii*. Fc tag is one of the most common fusion tag used by molecular biologists. They are generally used for the efficient purification of recombinant proteins by affinity chromatography. Also, Fc tag provide an

advantage to recombinant protein studies due to the solubility. Biophysically, the Fc region folds independently and can improve the solubility and stability of the co-molecule both *in vitro* and *in vivo* (Carter, 2011). Previous studies show that the solubility of PAP is limited (Viterbo et al., 2010). Therefore, the recombinant PAP protein was produced as fused to the Fc-tag both to facilitate the purification of the PAP protein and to increase its solubility. Fusion of the Fc region to the PAP protein to increase the stability and half-life of the Fc fusion protein may be useful in research and even immunotherapeutic applications (Breikers et al., 2006).

Material and methods

Design of the plasmid

To produce the recombinant PAP protein in *K. phaffii* X33 yeast strain, the sequence was designed as shown in Figure 1B. It contains the Fc Tag, -Linker sequence, -Enterokinase (EK) cut site, -and the PAP gene (Figure 1(B)). It was synthesized by ATUM in the pD912 plasmid (Figure 1(A)). The plasmid vector was sent absorbed by the filter paper and isolated from the filter paper according to the manufacturer's instructions.

It was transformed into *E.coli* Top10 cells treated with $CaCl_2$ (Inoue et al., 1990). Transformed bacteria were selected on the Low Salt-LB (LS-LB) agar containing 25 µg/ml of *Zeocin*™ (Invitrogen), and grown in the liquid LS-LB containing Zeocin. Subsequently, plasmids to be used for transformation to *K. phaffii* were isolated from the liquid culture using the Plasmid Isolation Kit (GeneJET Thermo Scientific™). The plasmid DNA was linearized with SacI for transformation into the yeast cell. The linearized plasmid was cleaned by using a DNA Clean-Up kit (Sigma-Aldrich GenElute™ PCR Clean-Up Kit) and prepared for transformation.

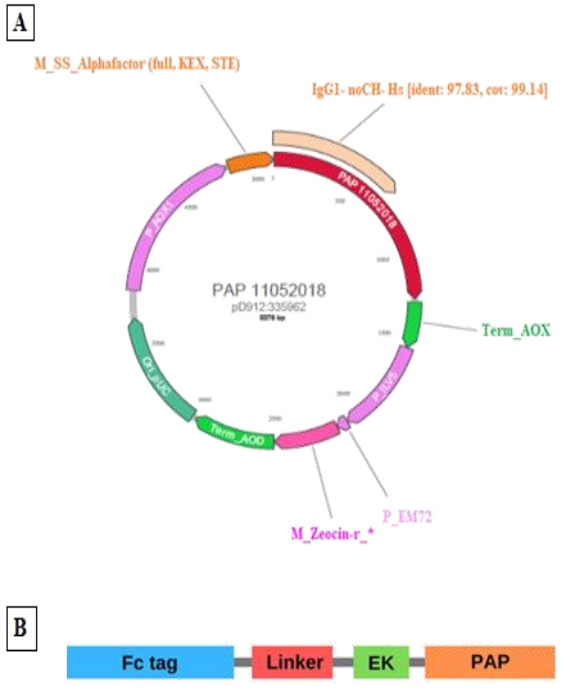

Figure 1. (A) - PAP - pD912 plasmid map (5078bp). (B) - Design of the PAP construct.

K. phaffii Transformation

Yeast expression plasmids containing alpha-factor secretion signal, IgG1 Fc region, EK cut site and PAP gene was transformed into *K. Phaffii* by electroporation (Divyapicigil et al., 2020).

K. phaffii was inoculated into 10 ml YPD (Yeast Extract Peptone Dextrose) Broth and grown at 30°C for 1 night. 200 µl from this culture was taken and inoculated into a 200 ml liquid YPD medium and was left to grow overnight at 30°C. The next day, the A_{600} value of the culture was measured as 1.211. The number of the cells was adjusted as $8x10^8$ cells per transformation.

Cultures were evenly distributed into 50 ml tubes and precipitated at 4°C and 4000 rpm for 7 min.

40 ml of dH_2O was added to the pellet and the centrifugation process was repeated with the same conditions. A suspension with 2 ml 100mM LiCl, 10mM DTT, 0.6 M sorbitol, and 10 mM Tris-HCL

at pH 7,5 was prepared for each tube and incubated for 30 min at room temperature. Then, the cells underwent the same precipitation and supernatant removal procedures. The pellet was washed in 50 ml 1M sorbitol which was stored at +4°C. Finally, the cells were re-washed with 25 ml, 10 ml, and 5 ml of 1M sorbitol, respectively, and resuspended to a final concentration of 10^{10} cells/ml. A_{600} value of the cells was taken at 1/1000 dilution. The A_{600} value of the cells was measured as 0.423 at 1/1000 dilution.

100 µl of the prepared *K. phaffii* cells were taken, mixed with 10 µl of linearized DNA, and kept in a 0,2cm electroporation cuvette on ice for 5 min. Then, the cells were electroporated at 2kV. Following the electroporation, 1M 1 ml of sorbitol stored in ice was added to the cuvette. This mixture was stirred for 1 hour in a shaking incubator at 30˚C. Then 1X 1 ml of YPD was added and again mixed for 1 hour in a shaking incubator at 30˚C. The entire mixture was seeded into YPD agar Petri dishes containing 100µg/ml Zeocin and 20% glucose and allowed to grow at 30˚C for 4 days.

Then, three types of YPD agar containing Zeocin at 0.5 mg/ml, 1 mg/ml, and 5 mg/ml concentrations to be able to select colonies that could produce higher amounts of protein.

50 µl/well of sterile dH_2O was added to a sterile 96-well plate. The growing colonies were taken with a sterile pipette tip and pipetted in the wells with dH_2O. 5 µl were taken from the wells and planted in the numbered areas. Petri dishes were incubated at 30˚C. At the end of the day, colony selection was made from Petri dishes containing 5 mg/ml Zeocin for PAP on YPDS (YPD with Sorbitol) agars.

Protein Expression in K. phaffii

The colony selected from YPDS agar petri was inoculated into 25 ml BMGY (Buffered Glycerol Complex Medium) medium in a sterile 250 ml flask. The cultures were incubated in a shaking incubator at 30°C until the OD_{600} reached 2-6. The 25 ml culture was transferred to a 50 ml tube and centrifuged at 3800 rpm for 12 min. Tubes were placed upside down on the sterile surface to remove supernatant and BMGY medium because cell expression can be inhibited if glycerol remains in the environment. The medium was resuspended with 20 ml BMMY (Buffered Methanol-Complex Medium) to wash the cell aggregates and remove the remaining medium. After this procedure, precipitation was carried out at 3800 rpm for 12 min at room temperature and the supernatant was removed. The cell aggregate was suspended in a 200 ml BMMY medium in the 1-liter flask to induce expression and grow until OD600 reached 1.0. The flask was closed with tinned cotton and the culture was allowed to grow in a shaking incubator at 30°C.

To maintain induction, 0.5% sterile methanol was added every 24 hours. Also, 1 ml of the culture was transferred to a 1.5 ml microcentrifuge tube at 24 hours intervals for four days. The aliquot was centrifuged at room temperature for 7 min at 13200 rpm. The supernatants were transferred to clean 1.5 ml microcentrifuge tubes.

Protein expression was increased by inducing AOX promoter with the addition of methanol every 24 hours for a total of 96 hours. The produced proteins which were secreted into the liquid medium were checked with Western blot and sandwich ELISA.

Expression Control

Samples taken at different time points were analyzed by ELISA, SDS-PAGE, and Western blot to determine the optimal period for protein expression after induction. For sandwich ELISA, 100 µl 1µg/ml anti PAP rabbit monoclonal antibody (Sino Biological) was used as the capture antibody. The plate was blocked with 1% Hammerstein casein and 1.5 % polyvinylpyrrolidone (PVP) in PBS and then wells were washed 3 times with 1X PBS-T (0.05% Tween 20 in PBS). After washing, Yeast culture

supernatant was added to the wells and incubated for 1 hour at 37 ° C. At this stage, human PAP (Reg3A, Sino Biological) was used as the positive control and PBS was used as the negative control. Then, 100 µl of 3µg/ml, anti-PAP monoclonal antibody (9B7) labeled with HRP developed in the TÜBİTAK Marmara Research Center was used. After washing microtiter plates were incubated with TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution at room temperature in the dark until the desired color intensity is reached. After adding 2M sulfuric acid as stop solution the absorbance was read at 450nm on a Biotech Synergy HT microtiter plate reader. 9B7 was also used in Western blot analysis.

Results and Discussion

The human PAP gene was successfully transformed into *K. phaffii* X-33. The production of PAP protein by 8 transformant *K. phaffii* colonies was tested with sandwich ELISA. The test has confirmed that all the transformed produce PAP, and the highest expression occurs in the colony labeled 2 (Figure 2). That colony was selected for subsequent analyses.

The cultivation procedure and 24-hrs interval subsampling was repeated for colony 2 under the same conditions. The PAP protein levels were identified by Sandwich ELISA (Figure 3).

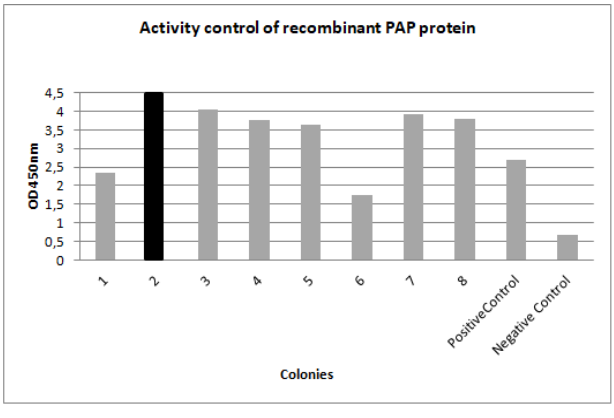

Figure 2. PAP analysis by sandwich ELISA in *K. phaffii* supernatants that are cultured after transformation. PBS was used as a negative control

In addition to ELISA, SDS-PAGE followed by Western blot was performed (Figure 4(A) and Figure 4(B)).The results indicate the highest production of recPAP 48-hrs following the induction. The recombinant PAP protein was observed to be both stable and active at the end of the 48th hour (Figure 3, Figure 4(A), and Figure 4(B)). When we have compared the quality of unpurified FC fusion PAP with commercially available purified human PAP (Reg3A) protein (Sino Biological). It was determined that it is compatible with the existing protein structure and properties specified in the literature.

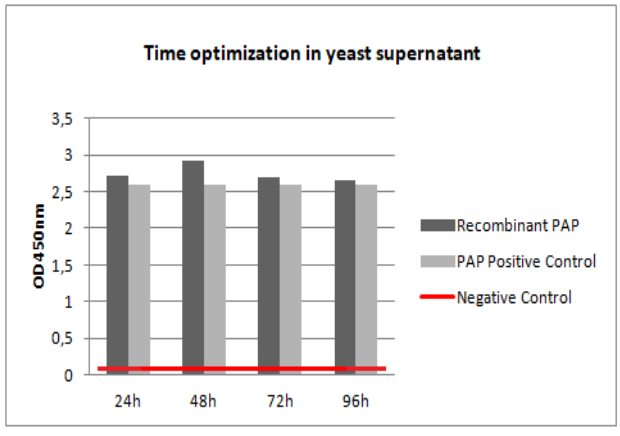

Figure 3. Sandwich ELISA results of the *K. phaffii* supernatants were collected at 24, 48, 72, and 96th hours

It is aimed to use the PAP protein produced in this study for commercial purposes. Advances in protein purification techniques reasonably show that any protein with a certain degree of stability can be purified according to homogeneity standards. However, the cost of the protein purification process is extremely high and constitutes an important part of the total cost. Therefore, it is necessary to evaluate the steps towards saving cost and time. For this purpose, *K. phaffii* culture supernatants were directly collected without any purification, diluted with PBS at different ratios, and tested with the sandwich ELISA method (Figure 5). In this test, PAP protein found in yeast supernatant was also compared with the commercial human PAP protein (Sino Biological) to evaluate its potential to be used as a

test standard without purification. As evident from the graph, the unprocessed recombinant yeast culture supernatant and commercial PAP showed consistent results. The experiment was carried out in 3 repetitions. The dilution rates were adjusted to be 2x. Based on these results, 2.5 ng of commercial PAP corresponds to recombinant PAP at 1/160 supernatant dilution. The amount of protein produced was calculated as 4mg/l.

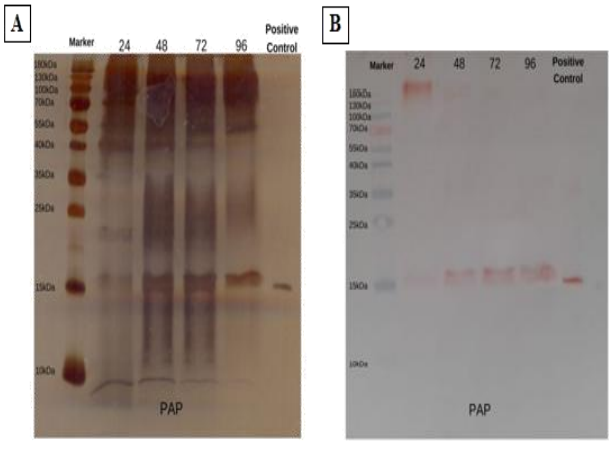

Figure 4. (A) – SDS-PAGE gel silver staining image of the recombinant PAP antigen and positive control purified human PAP (Reg3A). (B) - Western blotting image of the recombinant PAP antigen.

The max reagent dilution rate was determined as 1/160. Saturation was observed at a dilution ratio of 1/320.

The role of PAP protein isoforms in the occurrence of acute pancreatitis has attracted great attention from researchers. PAP protein is used in the investigation of many important diseases such as cancer, cystic fibrosis, hepatic fibrosis, active inflammatory bowel diseases, and diabetes. The PAP protein has the potential to be used in many areas, but there are many aspects of this protein that have not been elucidated, and this valuable protein needs further research. For this reason, the recombinant PAP protein we produce has a high potential for use. In previous studies, the recombinant production of different isoforms of PAP protein has been performed (A. Li et al., 2003; Loonen, 2013; Schiesser et al., 2001), but unprocessed recombinant PAP (Reg3A) protein has been successfully studied in this study for the first time. Although all Reg proteins containing PAP isoforms are often homologous in sequence and structure, it is understood that each protein is unique and requires individual purification protocols (Christa et al., 2000). It is clear that this family of proteins (Reg, PAP) requires special treatment and precise purification methods to maintain and protect their optimal biological function. In the protein purification process, completing the process in as few steps as possible and in a short time is very important in terms of the cost of the process. Otherwise, product loss may occur during the process. Therefore, many problems can be encountered in the purification process. In this study, it has been demonstrated that recombinant PAP protein produced by transformed *K. phaffii* can be used effortlessly, without purification, especially in immunoassay studies. Thus, we gained an advantage in terms of time and cost.

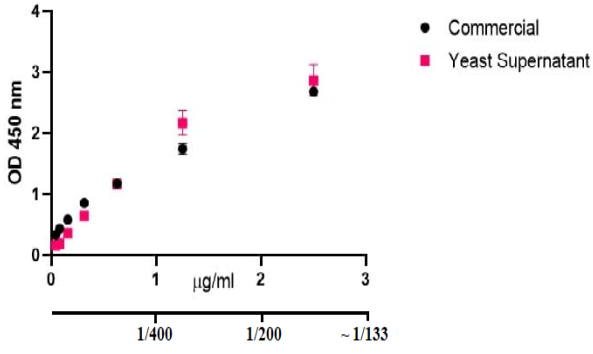

Figure 5. Quantitative comparison of commercial PAP and recombinant PAP

Sandwich ELISA, SDS PAGE, and Western blot experiments prove that the recombinant PAP protein can be detected by the antibody developed against the commercial PAP protein and exhibits similar antigenic properties. The recombinant protein has been proven to give positive results by generating parallel responses with commercial PAP even at high dilution rates without purification. In future studies, the produced PAP protein can be used

for antibody development studies or as PAP antigen standard. In addition to acute pancreatitis, recombinant PAP protein proteins can be used in many areas such as the diagnosis of important diseases such as Cystic Fibrosis and cancer.

Conclusion

This study aims to produce recombinant human PAP protein. The recombinant protein we produced worked effectively without being purified, giving us a huge advantage in terms of time and cost. The antigenic properties of the recombinant human PAP protein were tested by ELISA and verified by SDS-PAGE and Western blot. Recombinant PAP has proven to have antigenic properties similar to commercial PAP. A quantitative comparison between recombinant and commercial PAP was made by ELISA test. As a result, recombinant human PAP protein, which will find a use for therapeutic and diagnostic purposes in many areas, especially as an immunization molecule or antigen standard in tests to detect PAP protein, has been successfully produced.

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Conflict of interest

Authors declare no conflict of interest.

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