Original Research Article

Expression and Purification of Soluble, Biologically Active Recombinant Dipeptidyl Peptidase 4 (DPP4/CD26/ADAbp) Comprising the Extracellular Domain in the Yeast, *Pichia pastoris*

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Abstract

Purpose: To investigate Pichia pastoris expression system for producing clinically usable, high-quality dipeptidyl peptidase 4 recombinant protein.

Methods: The yeast, Pichia pastoris, expression system was used for the production of the human recombinant dipeptidyl peptidase 4 as a secreted form. The full-length human dipeptidyl peptidase 4 corresponding to the amino acid 31-766 was subcloned into a Pichia pastoris expression vector, pPICZ□, and transformed to Pichia pastoris X33 cells.

Results: The human recombinant dipeptidyl peptidase 4 protein was expressed enzymatically as active human $rDPP4_{(31-766)}$ as secreted form in the yeast P. pastoris, purified and monitored its biological activity. The test DPP4 recombinant protein induced a significant increase of DDP4 activity at 10, 20 and 30 min incubation time ($p < 0.05$) and at 40 min ($p < 0.001$). A similar pattern was found for the commercial (standard) DPP4 protein at 10, 20 and 30 min ($p < 0.05$) and at 40 min ($p < 0.001$). The high standard deviation (SD) associated with the mean value for the DPP4 activity is due to incubation time sometimes associated with high DPP4 values. The values were much higher than in other groups as expected.

Conclusion: Human recombinant dipeptidyl peptidase $4_{(31-766)}$ protein in the yeast Pichia pastoris, obtained using the technique employed in this study can further improve production efficiency and costs of human recombinant dipeptidyl peptidase 4 and other recombinant proteins.

Keywords: DPP4, Pichia pastoris, Recombinant protein, Expression, Purification

INTRODUCTION

Dipeptidyl peptidase 4 (DPP4/DPPIV/CD26 or ADAbp (adenosine deaminase binding protein)) is a 220 kDa homodimeric, type II transmembrane glycoprotein and a cell surface protease belonging to the prolyloligopeptidase family. A soluble form is also found in plasma [2]. DPP4/CD26 was originally characterized as a T-cell differentiation antigen and is expressed on various cell types [3,4]. It cleaves off N-terminal dipeptides from peptides with preferably proline or alanine at the penultimate position [5].

The yeast *Pichia pastoris* (*P. pastoris*) is one of the most widely used systems for expressing recombinant protein by heterologous expression due to its high production and secretion

efficiency[6, 7, 8]. This system uses strong promoters such as alcohol oxidase (AOX1) promoter [9]. It allows for the production of large amounts of the target protein with ease and relatively low cost. The *P. pastoris* expression system is its ability to produce high levels of functionally active proteins. It is thought that since mammalian native proteins are glycosylated, it must be necessary to have the correct glycosylation on recombinant proteins to ensure their biological activity. Thus, *P. pastoris* is a much more attractive host for the expression of recombinant proteins [10].

In this study, having established the suitability of *P. pastoris* for the expression of the human recombinant DPP4$_{(31-766)}$ (human rDPP4$_{(31-766)}$) as a secreted form, we set out to investigate the possibilities of producing the useful proteins having the correctly, post-translational modified properties for both basic laboratory research and industrial manufacture. Therefore, the optimization of the expression systems for the production of soluble recombinant proteins is very necessary to save time and decrease the cost to produce higher yield for further applications. In this study, we expressed enzymatically active human rDPP4$_{(31-766)}$ as secreted form in the yeast *P. pastoris*.

EXPERIMENTAL

Cells and media

E. coli transformants for pPICZα vector were selected on Luria-Bertani agar plates containing 25 μg/ml zeocin (Invitrogen, Carlsbad, CA, USA). *P. pastoris* transformants were selected on YPDS plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 1M sorbitol, 2 % agar, and 100 μg/ml zeocin), and then an YPD plates (the same as YPDS except that sorbitol was omitted) containing 500 μg/ml zeocin for isolation of single colonies. The BMGY medium contained 1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % yeast nitrogen broth, 0.4 μg/ml biotin, and 1 % glycerol. The BMMY medium was the same as the BMGY medium except that glycerol was replaced by methanol. The YPG medium contained 1 % yeast extract, 2 % peptone, and 1 % glycerol. All media components used in this study were purchased from Difco Laboratories (Detroit, MI, USA).

DPP4 activity assay

The activity of DPP4 was determined by direct photometric method according to the procedure described by Jarmolowska *et al* [11]. In sample and their blanks were added 50 μl of 0.3 M glycine/NaOH buffer (pH 8.7), 20 μl of 1.5 mM Gly-Pro-p-nitroanilide-p-toluensulfonate (Sigma-Aldrich, St. Louis, Mo, USA) and 50 μl of distilled water. Samples of standard contain 20 μl of 1.5 mM p-nitroaniline (Sigma-Aldrich) instead of substrate and their blanks contain 20 μl of distilled water. After 30 min incubation at 37 °C, 50 μl of ice-cold 1 M acetate buffer, pH 4.2, was added to blank to prevent the enzymatic reaction. Thereafter, 20 μl of sample was added to culture samples and their blanks. In standard samples and standard's blanks 20 μl of distilled water was added. Sample mixtures were incubated for another 30 min at 37 °C. Assay reaction was stopped by adding 50 μl of ice-cold 1 M acetate buffer, pH 4.2, in samples that contained either culture medium or the purified samples. Samples of standard and their blanks also contained the same volume of acetate buffer.

Construction plasmid and selection of transformants

For the expression of human DPP4$_{(31-766)}$ (Fig. 1A), full length of cDNA encoding for human DDP4$_{(31-766)}$ without the transmembrane domain (Fig. 1B) was obtained by RT-PCR and subcloned into pPICZα vector (Life Technologies, Grand Island, NY, USA) at *Xho*I/*Not*I site. The *Xho*I and *Not*I sites allow the direct insertion of the human DPP4$_{(31-766)}$ into pPICZα, resulting in a fusion to the α-mating factor secretion signal. This vector harboring the human DPP4$_{(31-766)}$ was designated pPICZα/DPP4$_{(31-766)}$. Fifteen microliter of the expression vector was linearized and transformed into 100 μl of *P. pastoris* X-33 strain by electroporation. Transfections were performed in 4 mm-gap cuvettes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a BTX ECM 830 electroporator (Harvard Apparatus Inc., Holliston, MA, USA) with a single pulse of 280 V and 12 ms [12]. *P. pastoris* transformants expressing the selectable marker (zeocin) were inoculated on YPDS plates containing 100 μg/ml zeocin. Resulting colonies were transferred to YPD plates (the same as YPDS except sorbitol omitted) containing 500 μg/ml zeocin. After incubation at 30 °C for 72 h, large colonies were isolated and shown to be capable of expressing human rDPP4$_{(31-766)}$. Stock seed cultures were prepared for protein expression.

Heterologous expression

Expression of the recombinant protein was performed by the method according to Kwon *et al* [30]. An YPD plate was streaked with the P. pastoris X-33 strain. The plate was then

incubated at 30 °C for 24 hr. A single colony was inoculated into BMGY in a flask. The cells were grown at 30 °C in a shaking culture until the A_{600} reached 2~6. This 25 ml culture was then used to inoculate 1 liter of BMGY (500 ml each in two 2 liter flasks) and a stock seed culture of *P. pastoris* was grown at 30 °C with shaking until the culture reached the log growth phase. The shake flask culture was harvested using the sterile centrifuge bottles by centrifuging at 2,500 x g for 5 min at room temperature. To induce expression, the supernatant was decanted and the cell pellet was resuspended to A_{600} of 2.0 in BMMY broth. The culture was aliquoted into several 2 liter flasks (600 ml in each flask). The flasks were then covered with cheesecloth or sterile gauze and returned to the incubator. The cells were allowed to continue to grow at 30 °C with shaking. Methanol as expression inducer was fed to the culture at a concentration of 0.5 % every 12 h until 72 h. After methanol induction, the cells were harvested by centrifuging at 4,000 x g for 10 min at room temperature. The supernatant was saved and filtered through 0.2 µm filter units. The supernatant was stored at -80 °C until used.

Purification of the recombinant DPP4(31-766) protein

At the post 72 h of induction with methanol, the culture supernatant was collected following centrifugation at 12,000 x g for 10 min. Then, the culture supernatant was precipitated byammonium sulfate precipitation at 25 % (w/v) saturation and followed by the 2nd precipitation with 75 % saturation at 4 °C, and then centrifuged at 15,000 x g and 4 °C for 30 min to collect the precipitate. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.4) and dialyzed against dialysis buffer (20 mM Tris-HCl buffer, pH 7.4) at 4 °C. The dialyzed sample was filtered through a 0.22 µm filter and loaded onto a Zn^{+2}-Chelate Sepharose Fast Flow column (Amersham-Pharmacia, Buckinghamshire, UK) that was equilibrated with 20 mM Tris-HCl (pH 7.4) using an HPLC assay and then eluted with imidazole at concentrations increasing from 0 to 50 mM. The pH was reduced to ~6 with acetic acid, and the sample were digested for 90 min using Endo H (1,000 U/ml, New England Biolabs, Ipswich, MA, USA) at room temperature. The pH was then increased to ~7.5 with 1 M Tris-HCl (pH 8.5). The Endo H digested sample was loaded onto a SP-Sepharose Fast Flow column (Amersham-Pharmacia), washed with 0.1 M NaCl and eluted with 0.25 M NaCl. The eluted solution containing the human rDPP4(31-766)

protein was concentrated, lyophilized, and stored at -80 °C.

Western blot analysis and antibody

Equal amounts of protein were loaded and resolved by 7 % SDS-PAGE. Resolved proteins were transferred to a nitrocellulose membrane (S&S, Dassel, Germany), blocked with 5 % non-fat dry milk in Tris-buffered saline, and probed with specific antibody against human DPP4 (DPPIV (Cell Signaling Technology, Beverly, MA, USA) followed by the secondary antibody coupled to horseradish peroxidase (Bio-Rad Laboratories, Inc). The immunoreactive proteins on the membrane were detected by chemiluminescence using the Western Blotting Detection Reagent Kit (AbSignal™, AbClonCo., Seoul, Korea).

Statistical analysis

All data are presented as mean ± SEM (n = 3). Statistical analysis was performed using SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Plasmid construction and selection of *P. pastoris* transformants

DPP4 gene was amplified by RT-PCT and then subcloned into the pPICZαvecter, where it is under the control of the *AOX1* promoter (Fig. 1B and 1C), at its N-terminus it has α–factor signal sequence for efficient secretion into the culture medium and it contains six histidine residues for detection and purificationat its C-terminus. The subcloned DPP4(31-766) gene was verified by digestion with the restriction endonuclease Xhol and Notl and by sequencing the subcloned vector (Fig 1C). The clones, designated by pPICZα-DPP4(Xhol/Notl), were used to transform into *P. pastoris*, and plated and selected on YPDS medium containing 100 µg/ml of zeocin (YPDS-zeocin plate). Colonies grown on the YPDS-zeocin plate were replated on YPD platescontaining 500 µg/ml of zeocin. At the post 72 h of incubation at 30 °C, each single colony was selected, amplified and confirmed with colony-PCR method according to the standard technique.
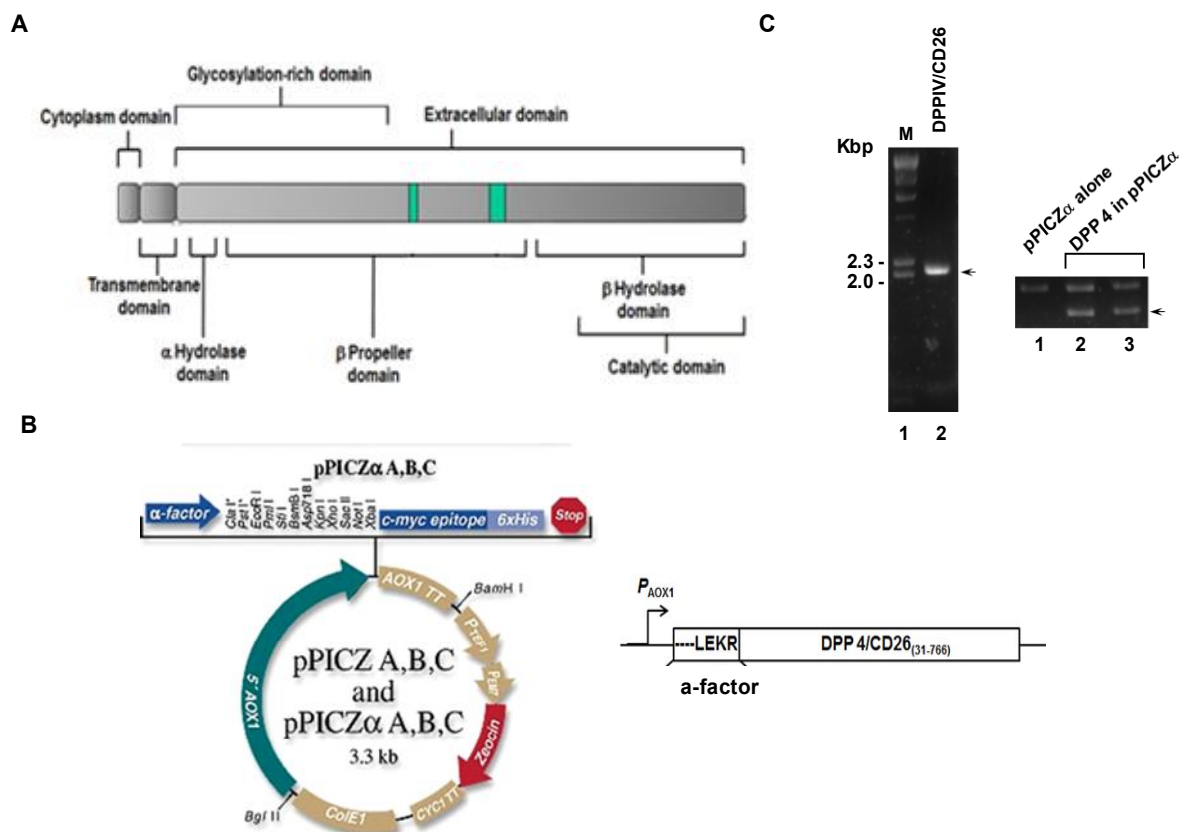
Fig 1: The schematic diagram of the human DDP4/CD26 molecule (A), cloning strategy for the human rDDP4$_{(31-766)}$ expression in pPICZα (B) and cloning of zeocin-based *P. pastoris* expression vector containing the human DPP4$_{(31-766)}$ gene. (A). Human DPP4/CD26 is a type II transmembrane glycoprotein composed of 766 amino acid (AA) residues and is anchored to the lipid bilayer by a single hydrophobic segment at residues 7-29. There is a short cytoplasmic tail (AA:1-6). The extracellular region contains the membrane anchor to glycosylated residues, adenosine deaminase (ADA)-binding domain (AA:340-343),fibronectin-binding domain (AA:469-479) and C-terminal catalytic domain. DPP4 contains three major activity domains including α-hydrolase (AA:39-51), β-propeller domain (AA:55-497) and β-hydrolase domain (AA:506-766) [1]. (B and C). Human DPP4$_{(31-766)}$ gene was inserted into pPICZα-vector anchoring the α-factor signal sequence at its N-terminus. The insertion of the genewas confirmed by double restriction enzyme digestion (*XhoI/NotI*) followed by resolution on anagarose gel. The arrow-head indicates the DNA fragment for the human rDPP4$_{(31-766)}$ insert

Expression of human rDPP4$_{(31-766)}$ protein in *P. pastoris* X-33 cells

Of the single selected, amplified colonies that grow on the selection media YPD plate containing 500 μg/ml of zeocin in the BMGY medium, ten clones were chosen to test human rDPP4$_{(31-766)}$ protein expression. Then the culture supernatants from each clone were subjected and elucidated their recombinant protein expression by Western blot analysis with antibody against human DPP4 (Fig 2A). Because the DPP4 gene was subcloned in frame with α-factor signal sequence for efficient secretion of the target protein at its N-terminus, we tested its expression with the culture supernatant. Eight clones expressed the human rDPP4$_{(31-766)}$ protein with apparent molecular weight of ~110kDa, which was larger in *P. pastoris* when compared with the calculated theoretical molecular weight of DPP4$_{(31-766)}$ (~85kDa). In addition, the Coomassie brilliant blue-stained SDS-PAGE gel with the ammonium sulfate precipitated sample and the immunoblotted protein bands showed blurry band (Fig 2).

Purification and biological activity of human rDPP4$_{(31-766)}$

Human rDPP4$_{(31-766)}$ secreted into the culture supernatant was purified using differential ammonium sulfate precipitation (25 – 75 %), a Zn^{+2}-chelate column chromatography and a SP-Sepharose column chromatography after the treatment of Endo H peptidase to remove the signal sequence. At the post 72 h induction, the 0.22 μm filtered culture supernatant of *P. pastoris* transformant harboring pPICZα-DPP 4$_{(31-766)}$ was centrifuged and 50 ml of supernatant was precipitated using 25 – 75 % differential ammonium sulfate precipitation. Under these conditions, 23.7 mg of protein was present in the
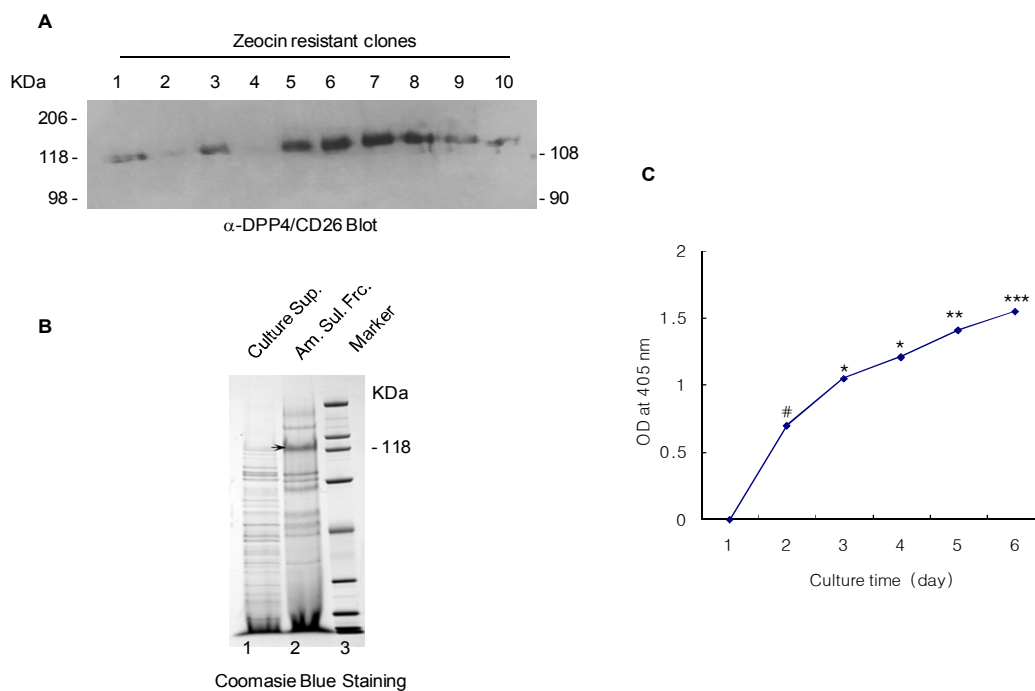
Fig 2: Human rDDP4$_{(31-766)}$ gene expressed in the yeast *P. pastoris*. (A). Ten zeocin-resistant clones were tested for the expression of human rDPP4$_{(31-766)}$ protein using Western blot analysis with antibody against DPP4. The culture supernatant was collected, performed to differential ammonium precipitation and subjected to Western blot analysis with anti-DPP4 antibody. (B). The human rDDP4$_{(31-766)}$ protein obtained from the yeast culture supernatant of clone #7 was precipitated without or with ammonium sulfate fractionation, resolved by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The arrow-head indicates human rDDP4$_{(31-766)}$ protein. (C). The expression of the human rDPP4$_{(31-766)}$ protein was monitored by its enzymatic assay determined by direct photometric method during cultivation with 20 µl of the culture supernatant of *P. pastoris* harboring the human DPP4$_{(31-766)}$ gene. Data are presented as the mean ± SEM (n = 3) for three independent experiments. #$p < 0.001$, when compared with control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with group treated with LPS only; comparison made by one-way analysis of variance, followed by Dunnett's multiple range tests

pellet. After dialysis, the solution containing the human rDPP4$_{(31-766)}$ protein was subjected onto Zn^{+2}-Chelate Sepharose column chromatography. The eluents with linear gradient of imidazole (0-50 mM) were subjected to SDS-PAGE gel to confirm the purity of the obtained human rDPP4$_{(31-766)}$ protein (Fig 3). In this step, 75.4 % of the human rDPP4 was recovered. After Endo H digestion, the solution was concentrated and then further purified onto a SP-Sepharose column chromatography. A total of 11.7 mg of the human rDPP4$_{(31-766)}$ protein was obtained. Recovery of the human rDPP4$_{(31-766)}$ protein with a purity of up to 95 % was about 49 % (Fig 3). The overall high purity obtained with differential ammonium sulfate precipitation and a Zn^{+2}-Chelate Sepharose column chromatography and renders these procedures the method of choice for reparative purification of human rDPP4$_{(31-766)}$.

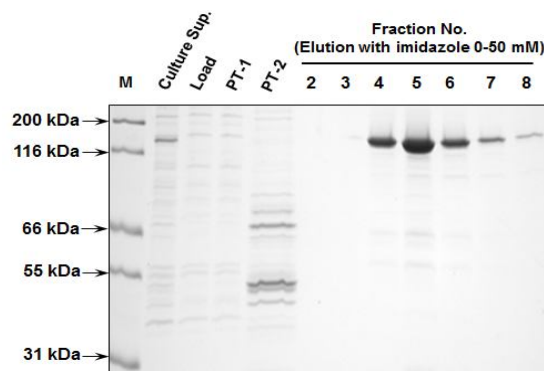**Fig 3:** Purification and SDS-PAGE analysis of the *P. pastoris*-expressed human rDPP4$_{(31-766)}$. The human rDPP4$_{(31-766)}$ protein, from *P. Pastoris* transformed with pPICZα/DPP4$_{(31-766)}$ vector, was purified using Zn^{+2}-Chelate Sepharose Fast Flow column, eluted linear gradient of imidazole with 0 to 50 mM, resolved by SDS-PAGE, and visualized with Coomassie brilliant blue R-250. In addition, we performed the comparison of biological activity for Gly-Pro-*p*-nitroanilide-*p*-hydrocholoride between the commercially available, purified human placenta DPP4 and out purified, human rDPP4$_{(31-776)}$
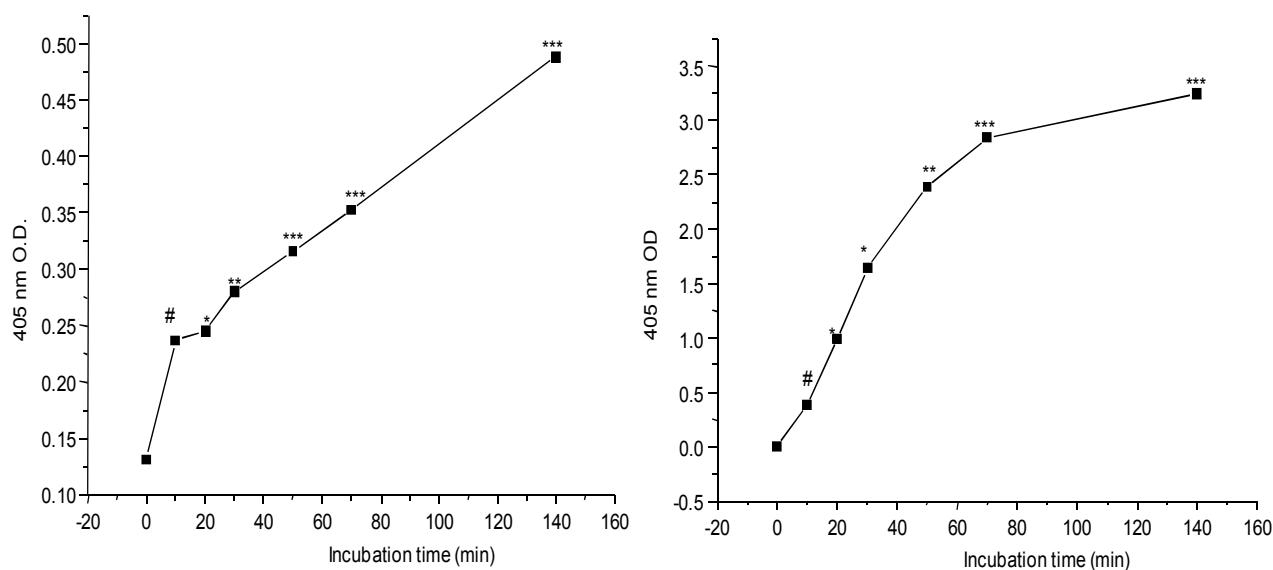
Fig 4: The enzymatic activity of the expressed human rDPP4$_{(31-766)}$ protein and the comparison between the activity of the commercially available, purified human placenta DPP4 (A; 0.2 μg) and the purified *P. pastoris*-expressed human rDPP4$_{(31-766)}$ (B; 0.05 μg) using a substrate, Gly-Pro-*p*-nitroanilide*p*-toluensulfonate. The enzyme activity of DPP4, depending on the incubation time, was monitored with the purified human placentaDPP4 (A) and the purified human rDPP4$_{(31-766)}$ through the Endo H digestion followed by SP-Sepharose Fast Flow column (B). Data are presented as the mean ± SEM (n = 3) for three independent experiments. #$p < 0.001$, when compared with control group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with group treated with LPS only; comparison made by one-way analysis of variance, followed by Dunnett's multiple range test

DISCUSSION

Specific inhibition of DPP4 dipeptidyl peptidase activity increases the half-life of the incretin hormones, glucagon-like peptide-1 and gastric inhibitory polypeptide, both involved in insulin secretion. Besides its enzymatic activity, DPP4 acts as receptor for plasminogen type 2 and adenosine deaminase (ADA), interacts with chemokine receptor CXCR4 and with mannose 6-phosphate/insulin-like growth factor II receptor. It is also a costimulatory molecule in T cell activation, associated marker of autoimmune diseases, adenosine deaminase-deficiency and HIV pathogenesis [13]. Thus, high surface levels of this protease are an indication of, at least, TH1 effector responses. Currently, DPP4 and its inhibitors are being investigated for their application both in animal studies and in clinical settings, and have been proposed as a diagnostic or prognostic marker for various tumors, hematological malignancies, immunological-, inflammatory-, psychoneuro-endocrine disorders, and viral infections [14]

This system offers the benefits of *E. coli* system combined with advantage of expression in a eukaryotic system such as post-translational modification. The real power of the *P. pastoris* expression system is its ability to produce high levels of functionally active proteins. Advantages of this yeast for expression include tightly regulated and efficient promoters and a strong tendency for respiratory growth. In addition, glycosylation is one of the most common post-translational modifications performed by *P. pastoris*.

The DPPIV 4 protein expressed in *P. pastoris* might be post-translationally modified, especially glycosylated. Recently, the three dimensional structure of DPP4 was determined [15]. DPP4 contains potential N-glycosylation sites mainly present in the β-propeller domain of the molecule. The type II transmembrane serine protease DPP4 is a major regulator of various physiological processes. It has been generally accepted that glycosylation of DPP4 and of other transmembrane dipeptidyl peptidase is a prerequisite for enzyme activity and correct protein folding (Fig. 1A; 16). Loch *et al* [17] demonstrated that inhibition of primary N-glycosylation of DPP4 using tunicamycin reduced the biological stability of the molecule dramatically. Although *P. pastoris* system cannot guarantee that the products' glycosylation pattern would be sufficiently, the human rDDP4$_{(31-766)}$ proteins produced in *P. pastoris* has the potential to obtain recombinant human proteins that have the desired carbohydrate structures. This finding increases the utility of *P. pastoris* as a host for the production of heterologous glycoproteins.

It was envisioned that the resulting purification scheme should be scalable (e.g. fermentation) and include as few steps as possible. Actually, several reports have described the fermentative production of recombinant proteins in *P. pastoris* [18]. Current study is directed towards the production of other glycosylated protein molecules with distinct functionalities.In addition, we performed the comparison of biological activity for Gly-Pro-*p*-nitroanilide-*p*-hydrocholoride between the commercially available, purified human placenta DPP4 and out purified, human rDPP4$_{(31-776)}$(Fig. 4). Although this comparison could not indicate the precise enzymatic activity, there was a similar activity pattern depending on the incubation time. Our results indicate that the human rDPP4$_{(31-766)}$ protein expressed in the *P. pastoris* expression system holds a promise for use in the clinical purpose.

The overall high purity obtained with differential ammonium sulfate precipitation and a Zn^{+2}-Chelate Sepharose column chromatography and renders these procedures the method of choice for reparative purification of human rDPP4$_{(31-766)}$.It was envisioned that the resulting purification scheme should be scalable (e.g. fermentation) and include as few steps as possible. Actually, several reports have described the fermentative production of recombinant proteins in *P. pastoris* [19]. There was a similar activity pattern depending on the incubation time. The findings of this study indicate that human rDPP4$_{(31-766)}$ protein expressed in *P. pastoris* expression system holds promise for clinical applications.

CONCLUSION

The present study reveals for the first time that human rDPP4$_{(31-766)}$ protein in *P. pastoris* improves the efficiency and lowers the cost of human rDPP4 production. This finding will aid efforts to enhance the expression and purification of clinically and industrially usable proteins.

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