Published in final edited form as:

Protein Expr Purif. 2010 August ; 72(2): 157–161. doi:10.1016/j.pep.2010.04.004.

Expression, Purification, and Characterization of Recombinant Human Pancreatic Duodenal Homeobox-1 Protein in *Pichia Pastoris*

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Abstract

Pancreatic duodenal homeodomain protein-1 (*PDX1*) is essential for the development of the embryonic pancreas and plays a key role in pancreatic β -cell differentiation, maturation, regeneration, and maintenance of normal pancreatic β -cell insulin-producing function. Purified recombinant *PDX1* (*rPDX1*) may be a useful tool for many research and clinical applications, however, using the *E. coli* expression system has several drawbacks for producing quality *PDX1* protein. To explore the yeast expression system for generating *rPDX1* protein, the cDNA coding for the full length human *PDX1* gene was cloned into the secreting expression organism *Pichia pastoris*. SDS-PAGE and western blotting analysis of culture medium from methanol-induced expression yeast clones demonstrated that the *rPDX1* was secreted into the culture medium, had a molecular weight by SDS-PAGE of 50 kDa, and was glycosylated. The predicted size of the mature unmodified *PDX1* polypeptide is 31 kDa, suggesting that eukaryotic post-translational modifications are the result of the increased molecular weight. The recombinant protein was purified to greater than 95% purity using a combined ammonium sulfate precipitation with heparin-agarose chromatography. Finally, 120 μ g of the protein was obtained in high purity from 1 L of the culture supernatant. Bioactivity of the *rPDX1* was confirmed by the ability to penetrate cell membranes and activation of an insulin-luciferase reporter gene. Our results suggest that the *P. pastoris* expression system can be used to produce a fully functional human *rPDX1* for both research and clinical application.

Introduction

The incidence of both Type 1 and especially Type 2 diabetes is increasing dramatically and diabetes now affects roughly 8% of the U.S. population [1]. Therefore, more than ever, there is an urgent need for new treatment modalities for diabetes. Although the two forms of diabetes have different pathophysiologic mechanisms, a shared feature is insulin insufficiency due to β -cell deficiency [2]. To cure diabetes, researchers are pursuing strategies for restoring β -cell mass by searching for factors that either promote endogenous pancreatic β -cell regeneration or reprogram nonpancreatic cells into insulin-producing cells

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(IPCs) [3]. Until recently, over-expression of key pancreatic transcription factor (PTF) genes by means of viral vectors was the most effective way to stimulate pancreatic cell differentiation into β -cells and to reprogram liver cells/liver stem cells into IPCs. Several key PTF proteins including *PDX1*, *Ngn3*, *NeuroD*, and *Pax4* are critical for pancreatic β -cell differentiation and maturation and they all contain a special amino acid sequence called a protein transduction domain (PTD) [4]. The most effective and well characterized PTDs are positively charged cationic sequences that allow proteins to rapidly enter living cells or whole organs [5]. It has been suggested that molecules containing these types of PTDs transduce cells by lipid raft-mediated macropinocytosis and activate the transcription of their target genes [6;7]. This strategy opens a new avenue for reactivating β -cell development or for directing stem-cell differentiation with protein therapy. *PDX1* contains a highly basic sequence of 16 amino acids, which constitutes an antennapedia-like PTD that facilitates penetration of plasma membranes [8]. This PTD allows *PDX1* to cross the cell and nuclear membranes, eliciting biological effects independent of endocytosis [8].

The *PDX1* gene consists of two exons coding for a protein of 283 amino acids with a predicted molecular mass of 31 kDa. *PDX1* is now widely regarded as a master transcriptional regulator in the pancreas and is critical for the development [9-11], regeneration [12;13], and maintenance of β -cell function [13;14]. During embryogenesis, the *PDX1* gene is expressed in all progenitor cells differentiating toward the exocrine, pancreatic ducts, and endocrine pancreas. In adults, *PDX1* expression is restricted mainly to β -cells and plays a key role in insulin gene expression [15;16].

Our recently published data offer a proof-of-principle demonstration that treatment of streptozotocin-induced diabetic mice with recombinant *PDX1* protein (*rPDX1*) promotes β -cell regeneration and transient liver-cell reprogramming, leading to restoration of normoglycemia [17]. Although *rPDX1* treatment of diabetic mice is a promising avenue, the *in vivo* biologic activity of bacterially-expressed *rPDX1* protein is much lower than transgene-expressed *rPDX1*. This is at least partially due to the limitations of the prokaryotic bacterial expression system since the *rPDX1* lacks post-translational modifications. In addition, an additional tag is needed for protein affinity purification, which may affect a protein's biological function, creating immunogenic epitopes that can stimulate antibody production.

The use of *Escherichia coli* as an expression system has drawbacks when used to manufacture *rPDX1* [18]. *PDX1* is a large protein that is glycosylated post-translationally. There is evidence that this modification plays a specific role in the binding of *PDX1* to DNA and it directly correlates with glucose-stimulated insulin secretion in β -cells [19]. Since the *E. coli* expression system lacks the ability to glycosylate proteins post-translationally [20], we explored the yeast expression system. *Pichia pastoris* is widely used as an expression system with the capacity to generate post-translationally modified proteins [21]. Moreover, *P. pastoris* can express proteins extracellularly, eliminating the need to use a His-tag for protein purification. Also, *P. pastoris* does not secrete many intrinsic proteins, simplifying the process of purification of the recombinant protein from the culture medium. Finally, *P. pastoris* is a methylotrophic organism that can be cultured to high cell densities at relatively low cost [21]. In the present study, our aim was to construct an efficient system for the expression and purification of *rPDX1* in *P. pastoris*.

Materials and Methods

Expression plasmid construction

Human *PDX1* cDNA (283 amino acids) was amplified by PCR on a plasmid encoding full-length human *PDX1* purchased from Origene technologies(USA). The sequence of the

amplified gene was analyzed and confirmed by an ABI 3130XL Genetic Analyzer. In order to express the native N-terminus of *PDX1*, an XhoI site was introduced to allow in-frame cloning behind the α -mating factor pre-secretion signal of pPICZ α A and a nucleotide sequence encoding the KeX2 gene cleavage site was placed upstream of the *PDX1* gene. The PCR products were cut with XhoI and XbaI and ligated into this site downstream of the alcohol oxidase 1 promoter (AOX1) in pPICZ α A (Invitrogen USA).

Transformation of *P. pastoris* and selection of transformants

Pichia pastoris X-33 was transformed with a linearized expression vector by digestion with SacI. The transformation was performed using the lithium chloride method following the kit manual (Pichia Easycomp Transformation Kit, Invitrogen, USA). The transformant cells were plated on YPDS (1% Yeast extract, 2% Peptone, 2% Dextrose, and 1M D-Sorbitol) plates containing 100 g/ml of Zeocin. Approximately thirty Zeocin-resistant colonies were replated on a YPD (1% Yeast extract, 2% Peptone, and 2% Dextrose) plate containing 500 g/ml of Zeocin. After incubation at 30°C for 2-3 days, several colonies appeared on the YPD plate and eight of the larger colonies were selected for protein expression.

Expression and purification of human PDX1

Eight transformants were screened for expression of secreted *rPDX1* by western blot analysis of culture medium, and the S1 clone was chosen for large scale expression and purification of *rPDX1*. S1 cells were grown in 500ml of BMGY (100 mM potassium phosphate, pH6.0, 1.34% YNB, 4×10^{-5} biotin, and 1% glycerol) for 48 h at 28°C. The cells were harvested by centrifugation (1000 rpm, 25 min at 4°C) and resuspended in 1.0 L of BMMY (100 mM potassium phosphate, pH6.0, 1.34% YNB, 4×10^{-5} biotin, and 0.5% methanol) in a 2 L flask for induction of AOX1. The yeast cells were grown in BMMY media for 96 h at 28°C with shaking. Every 24 h methanol (100%) was added to a final concentration of 0.5% to maintain induction. Cell culture supernatant was harvested by centrifugation and was precipitated with 60% saturated ammonium sulfate [22]. The precipitated proteins were collected and dissolved in buffer A (25 mM Hepes pH 7.9, 20% glycerol, 0.1M KCl, 0.2mM EDTA, and 0.5mM DTT), and dialyzed against the same buffer. The resulting sample was loaded onto a heparin-agarose column (Bio-Rad). The column was first washed with 0.2 M KCl in buffer A, and proteins were eluted with 1.5M KCl in buffer A. The elution fractions were analyzed using SDS-PAGE followed by Coomassie brilliant blue staining and western blotting. The purified *PDX1* was dialyzed against 10% glycerol in phosphate-buffered solution (PBS) and stored at -80 °C.

N-glycanase digestion and western blotting analysis

The *rPDX1* expression sample was digested for 5 h at 37°C with recombinant N-glycanase (PNGase F, New England BioLabs) according the manufacturer's instructions. The digested and undigested controls were subjected to SDS-PAGE on a 12% gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The blot was probed with rabbit anti-*PDX1* polyclonal antibodies and developed using the Amersham Pharmacia Biotech ECL detection system.

Insulin-reporter luciferase assay

The rat insulin II promoter luciferase reporter construct (pGL2-RIP2-Luc) was a gift from Dr. Carlotti [23]. Human hepatocellular carcinoma Huh-7 cells were seeded onto a 12-well plate and grown for 24 h in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS at 5% CO₂ and 37°C. One microgram of pGL2-RIP2-Luc reporter plasmid and 0.01 g of internal control vector HSV-TK-hRluc (renilla luciferase, pRL-TK, Promega) were used to co-transfect these cells using Lipofectamine 2000 reagent (Invitrogen, USA). Following

12 h transfection, the medium was replaced with medium containing *rPDX1* protein or PBS with 10% glycerol (protein storage solution) for negative controls. After 24 h protein treatment, cell lysates were collected and assayed for luciferase activities using Dual-Glo-Assay-System (Promega).

Results

Optimization of *rPDX1* expression conditions and strains

The resulting plasmid from the construction, pPICZα-*rPDX1*, was transformed into *P. pastoris*. Of the 15 single colonies that grew on YPD containing 500 μg zeocin/ml, 8 clones were selected for testing the capacity of *rPDX1* protein expression by western blot analysis (Fig. 1). All selected clones expressed *rPDX1* with a molecular weight of 50 kDa, which is slightly higher in yeast compared with native *PDX1* expressed in the rat insulinoma β-cell line INS-1 (46 kDa) (Fig. 1. Lane C), suggesting that the protein expressed in yeast might be glycosylated. There was no protein that reacted with anti-*PDX1* antibody in untransformed *Pichia pastoris* X33 (data not shown). Figure 1 shows that the S1 clone has the highest expression of the *rPDX1* and therefore was used for the subsequent time-course study.

To determine the optimal time for *rPDX1* expression, the S1 clone was seeded in the culture medium and samples were collected at 12h, 24h, 48h, 72h, and 96h. Figure 2A shows that the intensity of *rPDX1* protein increased over time, with the strongest signal intensity at 96 h. Therefore, a 96-hr incubation time was identified as optimal (Fig. 2A). To determine the secretory ability of the *rPDX1* in the yeast expression system, we examined the protein levels both in the culture medium and in the yeast cells by western blotting. Figure 2B reveals that the majority of the *rPDX1* protein was cell-associated whereas only 10% of *rPDX1* was secreted into the culture medium.

Large-scale fermentation and purification of *rPDX1*

To obtain the optimal fermentation conditions to scale-up protein production, we used the S1 clone after 96 hours incubation in the presence of 1% methanol. The *rPDX1* protein in the supernatant was purified by a two-step method [22]. First, *rPDX1* was precipitated with 60% saturated ammonium sulfate. The pellet containing *rPDX1* was dissolved in column buffer and subjected to Heparin-gel affinity chromatography. The *rPDX1* was eluted from the column with elution buffer containing 1.5M KCl. The purity of the *rPDX1* was assessed by SDS-PAGE (Fig. 4A). An average of about 120 μg of purified *rPDX1* was recovered from 1 L of culture medium. The purity of the purified *rPDX1* reached nearly 95% as confirmed by SDS-gel staining and western blotting with anti-*PDX1* antibodies (Fig 4B, Lanes 2 and 3).

Glycosylation analysis of purified *rPDX1*

To examine whether the *rPDX1* harvested from the yeast expression system underwent post-translational glycosylation, the purified *rPDX1* from supernatant was treated with PNGase F to remove N-linked glycosylation and subjected to SDS-PAGE and western blotting analysis. As shown in Figure 3, the pretreated *rPDX1* has a predicted molecular weight of approximately 50 kDa, whereas the treated *rPDX1* exhibited two distinct bands, one large band at 46 kDa and one faint band at 31 kDa (Fig.3). The shift in the band post-PNGase F treatment indicates that the *rPDX1* was indeed glycosylated in the yeast expression system. PNGase F is known to strictly cut N-linked glycans. As previously mentioned, protein expressed in yeast is glycosylated by both O- or N-glycosidic linkages. We interpret that the emergence of two bands is due to the ability of PNGase F to only remove the N-form of glycosylation from the *rPDX1* protein. It is also possible that the emergence of two bands

indicates that the PNGase F may have digested *rPDX1* only partially. Regardless, it is clear that *rPDX1* was secreted from the *P. pastoris* and was glycosylated post-translationally.

Biological activity of rPDX1

There are several direct target genes for *PDX1*, including the insulin gene [24;25]. To determine the biologic function of *rPDX1*, we transfected a rat insulin II promoter-luciferase reporter gene into human Huh-7 cells prior to treatment with different concentrations of purified *rPDX1*. Luciferase activity was detected after 24 h of incubation with *rPDX1*. Figure 5 shows that the cells treated with *rPDX1* showed an increase in expression of the insulin reporter gene (Fig.5). A previous report indicates that *rPDX1* protein produced in *E. coli* can also activate the insulin luciferase reporter gene, but at much lower levels when compared to our *rPDX1* protein produced in yeast [26].

Discussion

Our data show that *P. pastoris* yeast can successfully be used as an expression system for generating biologically functional *rPDX1* protein, serving as an alternative to prokaryotic expression systems. We demonstrate here that the *P. pastoris* yeast expression system has the ability to secrete glycosylated and biologically active *rPDX1* into the culture medium. Furthermore, we developed a purification strategy and protocol for *rPDX1* purification from the culture medium of *P. pastoris* yeast cells. Although *P. pastoris* cells expressed a large quantity of *rPDX1*, the quantity of secreted *rPDX1* protein was less than that we had hoped, posing a challenge for *rPDX1* generation using this system.

It may be possible to improve the expression level by varying the nature of the signal peptide [30], deleting the nuclear localization signal (NLS) [31], chaperone co-expression [27-29;32-35] or varying expression conditions [36], which have proven effective in other cases.

In addition to its roles in embryonic development of the pancreas and the maintenance of β -cells in the adult pancreas, *PDX1* over-expression induces the adoption of an insulin-producing cell phenotype in hepatocytes [8]. Its ability to direct β -cell differentiation and transdifferentiation to the β -cell phenotype gives it the potential for use in β -cell replacement therapy for Type 1 diabetes [37]. Inducing the over-expression of *PDX1* and other pancreatic transcription factors to study their effects typically requires the use of viral (e.g. lentiviral) vectors for deliver into cells and incorporation into the genome. This approach inevitably entails some risk of insertional mutagenesis [38;39] and raises issues for potential clinical application. *PDX1*'s PTD, therefore, makes it especially attractive for use as a differentiation reagent for generating patient-specific pluripotent stem cells to differentiate into pancreatic β -cells because it can translocate directly into cells without requiring the use of viral vectors.

Protein therapy using *PDX1* protein for diabetes provides hope for enhanced safety. Accumulating evidence shows that reactivating the developmental program for reprogramming non-pancreatic β -cells or for differentiating pancreatic stem/progenitor cells into insulin-secreting β -like cells has great clinical potential in diabetes. The ability to obtain biologically functional, near-native *rPDX1* protein in good yields is important for clinical application and for understanding the reprogramming process in pancreatic β -cells. Our current work provides a starting point for future efforts to optimize the expression, secretion, and purification of biologically active *rPDX1* for the therapy of diabetes.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health, NIDDK DK071831 (LJ Yang), from the University of Florida, OTL Research Fund, and Sponsored Research from Transgeneron Therapeutics, Inc.

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Fig. 1. Detection of secreted *rPDX1* protein from supernatants

Eight yeast clones were randomly selected from a 500 $\mu\text{g/ml}$ Zeocin YPD plate, seeded into 5ml of BMGY medium, and incubated for 24 h at 28°C. Cells were collected by centrifugation and resuspended to BMMY medium for continuing incubation for additional 72 h at 28 °C. Methanol was added to a final concentration of 0.5% every 24 h to maintain induction of *rPDX1* expression. The expressed *rPDX1* in the culture media was detected by western blotting with anti-*PDX1* antibodies (1:1000) following SDS-PAGE separation and protein transferring to the PVDF membrane. M- molecular weight markers; C-positive control for *PDX1* protein from INS-1 cell lysate; S1-S8 represent the *rPDX1*-producing yeast clones.

Fig. 2. Time-dependent *rPDX1* expression

(A) Western blot assay time-course of the *rPDX1* expression. Culture supernatant (20 μ l) from the S1 clone fermentation was collected at 12, 24, 48, 72 and 96 h, respectively. The supernatants were subjected to SDS-PAGE and blots were probed with anti-*PDX1* antibodies (1:1000). (B) Detection of *rPDX1* protein expression in two compartments. The 96 hr-culture supernatants and yeast cell lysates were subjected to protein SDS-PAGE, and the *rPDX1* proteins were probed by western blotting using anti-*PDX1* antibodies. Lane 1 is supernatant (30ul), lane 2 cell lysate for yeast cells (100ug), and lane 3 is rat insulinoma INS-1 cell lysate as positive control (100ug).

Fig 3. Confirmation of *rPDX1* glycosylation

The purified *rPDX1* from the yeast culture supernatants was treated without (–) and with (+) PNGase F for 5 h and the proteins were fractionated by SDS-PAGE and blotted by anti-*PDX1* antibodies. Arrows indicate the sizes of the *rPDX1* protein before or after the enzymatic digestion.

Fig 4. Purification (A) and confirmation (B) of *rPDX1* protein

The secreted *rPDX1* protein from culture supernatant, concentrated by ammonium sulfate precipitation, and purified by heparin-chromatography was separated by SDS-PAGE and either stained with Coomassie blue (A) or probed by western blotting (B) using anti-*PDX1* antibodies (1:1000) following transfer to the PVDF membrane. Lane 1 – Supernatants from the 96 h yeast cultures; Lane 2 - The precipitate from 60% saturated ammonium sulfate precipitation; Lane 3 - A fraction of the eluate from the heparin-agarose column.

Fig. 5. Biological function of *rPDX1*

Huh-7 cells transfected with the insulin promoter-luciferase reporter gene construct were treated with or without the *rPDX1* protein for 24 h. Cell lysates were collected for determination of luciferase activity. Renilla luciferase was used as an internal control for transfection efficiency. Bars represent means plus SD and this result is one of the three independent experiments. The difference between control and treated groups was significant ($p < 0.001$, *t*-test).