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Expression, Purification and Characterization of Human Recombinant Galectin3 in *Pichia pastoris*

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Background: Over the past century, the areas of genomics, proteomics and lipidomics have captured the attention of investigators worldwide. Carbohydrates, have recently received increased attention through the expanding field of glycobiology; probably because they are very complex and not encoded in the genome.
Objectives: The purpose of this study was to express and purify recombinant human galectin 3 via the *Pichia pastoris* expression system.
Materials and Methods: cDNA of human galectin3 gene was amplified with specific primers and cloned into a pcDNA3.1 vector with His-tag for easier purification using Ni2 and chromatography. Furthermore, galectin3 was purified to homogeneity and confirmed using SDS-PAGE and western blotting.
Results: The protein band corresponding to 29 kDa was excised from the gels, digested with trypsin and processed for mass spectrometric analysis by Matrix Assisted Laser Desorption/Ionization- Time of Flight Mass Spectroscopy (MALDI-TOF MS), using a Reflex III instrument.
Conclusions: Tryptic digest analysis clearly revealed that the purified protein was indeed galectin3. Similarly, the biological activity of recombinant galectin3 was confirmed using the hemagglutination inhibition assay.

*Keywords:*Pichia pastoris; Galectin3; Lgals3; Glycomics; Hemagglutination

1. Background

Barondes et al. initially proposed the general name 'galectins' in 1994 for all the S-type lectins (1). Galectins are found in multiple intracellular compartments and are secreted into the extracelluar space. There has been an explosion of information on these fascinating proteins in pathological states, particularly inflammation, fibrosis and cancer (2). Galectin3 is capable of binding different types of molecules and exhibits several autocrine and paracrine functions. It can affect cell adhesion, activation, motility, chemoattraction and apoptosis (3-7). Galectin3 expression in cancer has been widely studied; however, there are conflicting results, which make it difficult to come to a general conclusion about the expression profiles of galectin3 in cancer (8). Furthermore, some progress has been made in developing galectin3 inhibitors as anti-cancer agents (9, 10).

The use of *P. pastoris* as a cellular host for recombinant protein production has steadily increased. This species can be easily cultured, genetically manipulated, and can reach high cell densities (> 130 g L^{-1} dry cell weight) on methanol and glucose (11). Other benefits of the *P. pastoris* system are its strong induciblity and constitutive promoter systems. The methylotrophic yeast *Pichia pastoris* is widely used as a host system for recombinant protein production (12). Furthermore, it has come into focus for the production of glycol-proteins (with human-like N-glycan structures (13), as well as several metabolites and recombinant proteins.

2. Objectives

The purpose of this study was to express and purify recombinant human galectin3 in the *Pichiap astoris* expression system.

3. Materials and Methods

3.1. Strains and Plasmids

E.coli strain JM109 was used as a host for cloning. *E.coli* was grown at 37°C in Luria Broth (LB) media for cloning and *Pichia pastoris* at 37°C in Yeast-Peptone-Dextrose (YPD) media for expression; neomycin (25 $\mu g.mL^{-1}$) was added during growth of strains, containing plasmid. The high-

Implication for health policy/practice/research/medical education:
Lack of adherence to optimized protocols of recombinant galectin-3 production, this original paper is recommended for the scientists working in the field of glycobiology.

density fermentation media was prepared and vector pcDNA3.1 was used for expression of the target genes. All recombinant DNA manipulation was performed as per the manufacturer instructions (Fermentas).

3.2. Cloning Process

Human galectin3 transcript variant 1 gene cDNA clone/ open reading frame (ORF) clone was procured from Sino biological Inc. on Whatman FTA elute card (Cat. No.: WB120410) and the plasmid was prepared as described by the manufacturer's instructions. A full-length cDNA was amplified with PCR, utilizing P1/P2 (Table 1) as a primer pair in which pGEM-T gene construct served as a template. The 0.8 kb human galectin3 cDNA was subcloned into a yeast vector (pcDNA3.1) and digested with *EcoRI* andSpeI (Figure 1). The proper orientation of the cDNA insertion was confirmed by restriction enzyme analysis.

3.3. Primer Sequences

Primers were designed using Primer Express (Applied Biosystems) and, oligonucleotides were synthesized by Bioserve (100 pMol.μL^{-1}). The P1/P2primers were used in standard polymerase chain reactions (PCR) to verify the presence of the galectin3 gene in the recombinant *Pichia pastoris* DNA (underlined sections of the primer sequence indicate restriction sites, Hu = human).

3.4. Transformation of P. pastoris by Electroporation

P. pastoris X-33 competent cells were obtained (Invitrogen) and transformed, using the EasySelect Pichia expression kit as per the manufacturer's instructions; 0.5μg of pcDNA3.1 vector, with a His-tagged galectin3 insert was added to 100μL of *Pichia pastoris* X-33 competent cells. Fifteen minutes prior to each transformation, 100 μL aliquot of electrocompetent *P. pastoris* was kept on ice. The cells were transferred to a pre-cooled electroporation cuvette with a 2 mm interval. Transformation was performed using a BioRad Micropulser, a charging voltage of 2kV and a pulse length of 4ms. Transformation efficiency was recorded and several colonies were chosen for further analysis.

3.5. Galectin3 Purification

Recombinant galectin3 was purified from *Pichia pastoris* cultures (5mL) by binding to a HisBind Resin (Qiagen) affinity column. The column was washed with PBS to remove any unbound proteins until the absorbance reached background levels. The bound fraction was subsequently eluted isocratically and the eluted protein fractions containing recombinant galectin3 were pooled and analyzed by SDS-PAGE. Protein concentrations were determined using Bio-Rad protein assay (Bio-Rad) and bovine serum albumin as the standard, and western blots were used to verify the presence of recombinant galectin3.

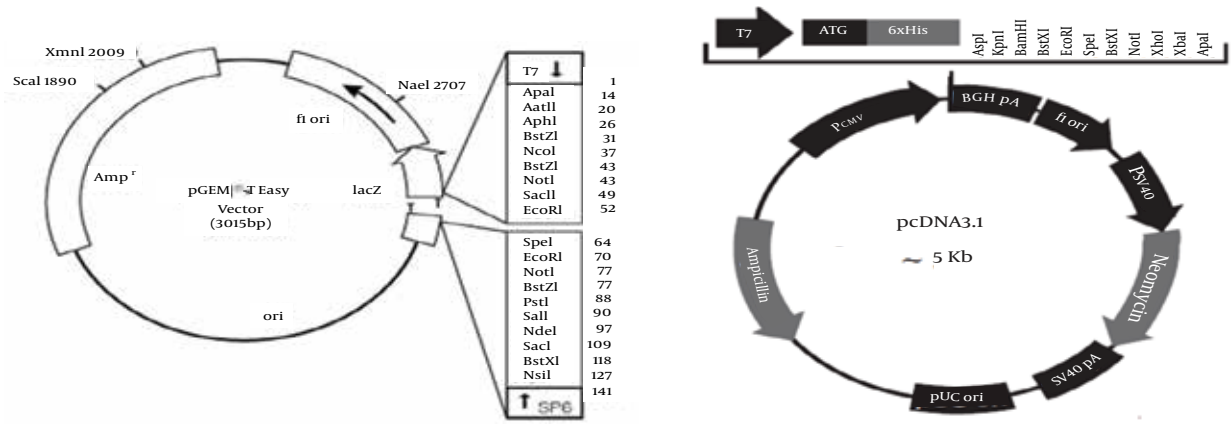

Figure 1. Vector Maps. Main restriction sites as well as genes and coding regionsare indicated. A: pGEM-T easy vector with LGALS3 cDNA gene. B: pcDNA3.1 vector with leader and recombinant protein region.

Table 1. Overview of Forward and Backward Oligonucleotides with Integration Site for pcDNA3.1 Shuttle Vector

Primer	Sequence 5' - 3'	Restriction Site
P1 (F)	ACGGAATTCATGGCAGACAATTTTTCG	*EcoRI*
P2 (R)	CGCACTAGTTTATATCATGGTATATGA	SpeI

3.6. Analysis of Recombinant Human Galectin3

This was performed as described previously (14), using human peripheral erythrocytes treated with glutaraldehyde. To prepare the suspension of red blood cells (RBCs) for the hemagglutination assay, heparin-treated human peripheral blood (10 mL) was first centrifuged at 2000×g for 5 minutes. Buffy coat was removed as much as possible and RBCs were washed three times with 50 mL PBS. Next, RBCs were diluted with PBS to obtain 100 mL of an 8% cell suspension. RBC suspension was treated with glutaraldehyde (at a final concentration of 3%) under rotation for one hour at room temperature, followed by washing with 0.0025% NaN_3 in PBS. Fixed RBCs were resuspended at 3–4% in PBS–NaN_3. Calibration of RBCs was required to obtain the appropriate concentration for lectin-mediated hemagglutination. Serial dilutions of galectins (from 0 to 10 mM) were mixed with the appropriate quantities of RBCs in the wells of a 96-well plate and incubated at 370°C for 30 minutes. When RBCs aggregate (hemagglutination), they spread out like a sheet covering the entire surface of the well. However, RBCs formed very tight button-like precipitations at the bottom of the well, which signifies that there was no aggregation.

3.7. Immunoblotting

Electrophoretic transfer of proteins from the protein gel to the nitrocellulose membrane (BioRad pure nitrocellulose membrane 0.2 μm) was carried out by the semi-dry blotter (Biorad Trans-Blot). For immunodetection, the membrane was incubated in phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 per liter) pH 7.4, containing 5% non-fat milk powder and 1% Tween 20 at room temperature for one hour with gentle shaking. Polyclonal rabbit anti-galectin3, or mouse anti-galectin3 (Abcam) were diluted (1:5000 or 1:1000) with PBS containing 5% nonfat milk powder and 0.1% Tween 20. The membrane was incubated in primary antibody solution at room temperature for 1.5 hours with gentle shaking. The membrane was then washed in antibody dilution buffer for 3 × 5 minutes at room temperature. Goat anti-rabbit or mouse (for anti-galectin detection) IgG horseradish peroxidase conjugate (Abcam) was used as the secondary antibody (1:1000 dilution) to treat the membrane at room temperature for 1.5 hours followed by 3 × 5 minutes washes in antibody dilution buffer and a rinse in distilled water. Bands were visualized by exposure to UVI-Tech (Gel Imager, UK).

3.8. Identification of p29

The protein band corresponding to 29 kDa was excised from the gels, digested with trypsin (15) and processed for mass spectrometric fingerprinting as described previously (16). In brief, peptide mixtures were partially fractionated on Poros 50 R2 Reverse Phase (RP) microtips and the resulting peptide pools were analyzed by matrix assisted laser desorption ionization-reflectron time of flight mass spectrometry (MALDI-reTOF MS) using a Reflex III instrument (BrukerFranzen). Selected mass values were used to search a protein non-redundant database (NR; National Center for Biotechnology Information) using the Peptide Search (17) algorithm.

4. Results

4.1. Plasmid and Cloning Process

Plasmid DNA was isolated by the plasmid Mini-Prep (GeneJET Plasmid, fermentas) as per the manufacturer's instructions. The quantity and quality of isolated DNA was evaluated spectrophotometrically andagarose gel electrophoresis, respectively. The isolated plasmid DNA showed an A260/A280 ratio of 1.8±0.2, indicating relative purity, and 10 μL of the plasmid DNA was used for detection viaethidium bromide stained agarose gels (Figure 2).

The cDNA for human galectin3 of size 753 bp was obtained from Sinobiologicals (HG 10289-G). PCR amplification of the cDNA was carried out using specific forward and reverse primers (Figure 3). The amplified cDNA was then inserted into the pcDNA3.1 vector using restriction enzymes, *EcoRI* and *SpeI*.

4.2. Analysis of Recombinant Proteins

4.2.1. HemagglutinationInhibition Assay of Galectin3

In order to test the bioactivity of galectin3, hemagglutination assay was performed using human peripheral RBCs. As expected, wells containing RBCs incubated with carbohydrate specific antibodies and 1-10 mM galectin3 showed sheet like agglutination, where as wells containing RBCs incubated with carbohydrate specific antibodies and 0mM galectin3 (PBS control), and RBCs incubated with carbohydrate specific antibodies alone (negative control) showed button like sedimentation (Figure 4).

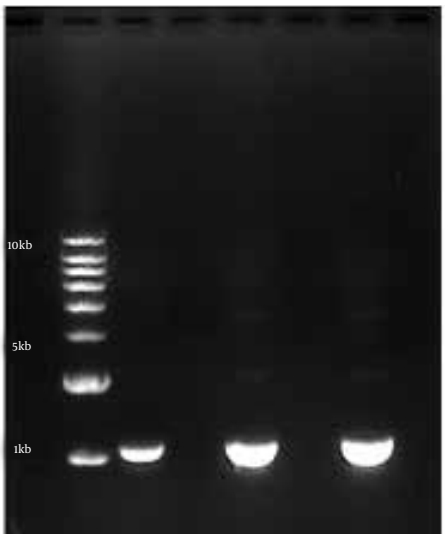

Figure 2. Plasmid DNA was isolated from an overnight bacterial culture and purified plasmid DNA was analyzed by agarose (1%) electrophoresis along a 1kb DNA ladder.

Figure 3. PCR Amplicon of Human Galectin3. Samples of the PCR products were analyzed on a 1.5% agarose gel and stained with ethidium bromide. Lane M shows size markers of a 100bp DNA ladder, lane one is the control reaction (no DNA) and lane 3 and 4 show the position of migration of the galectin3 product (predicted size, 800 bp).

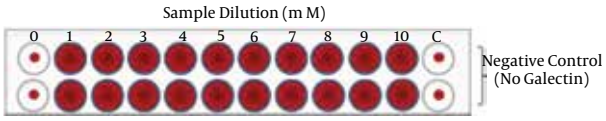

Figure 4. Hemagglutination Inhibition Assay Performed in a Microtitre-Plate Using Human Peripheral Blood

Well 1-10: when galectin was present, RBC spread out like a sheet covering the entire surface of the well. Well 0 and Control (C): when galectinwas absent, RBC showed button like sedimentation.

4.3. SDS-PAGE Analysis

Purified recombinant proteins were suspended in a sample buffer (4% SDS, 150 mMTris-HCl (pH 6.8), 20% glycerol, 0.1% bromphenol blue, 1% beta-mercaptoethanol) and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE). Various fractions (wash, elution) collected before, during and after protein elution were diluted (1/2) with milliQ ultrapure water before SDS-PAGE analysis. The molecular weight of the galectin protein was shown to be approximately 29 KD, as expected. Gel bands were visualized after staining with Coomassie Blue, as shown by Figure 5.

4.4. Western Blotting

In order to assess the immunological relationship between purified galectin3, the material eluted from the affinity column was submitted to SDS-PAGE and blotted onto nitrocellulose membranes. The 29 kDa protein band strongly reacted with this antibody, indicating that the purified protein is galectin3. A representative western blot, showing the reactivity of the purified galectin3 from two different extracts with the A3A12 anti-galectin3 antibody, is shown in Figure 6.

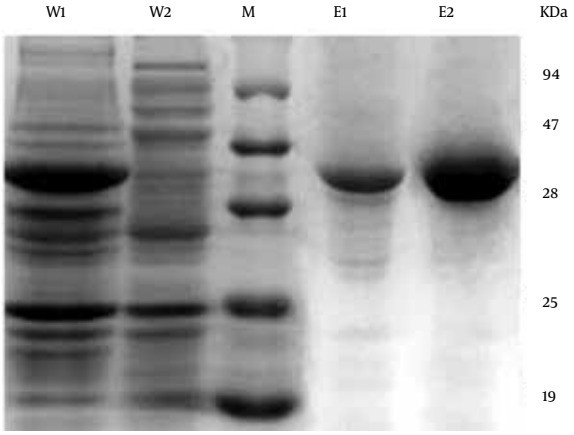

Figure 5. SDS-PAGE Analysis of His-tagged Recombinant Galectin3. Aliquots of washes (W1&W2) and elutions (E1&E2) of human galectin3 along with low range molecular weight markers (Bioexpress) were visualized after Coomassie Blue staining.

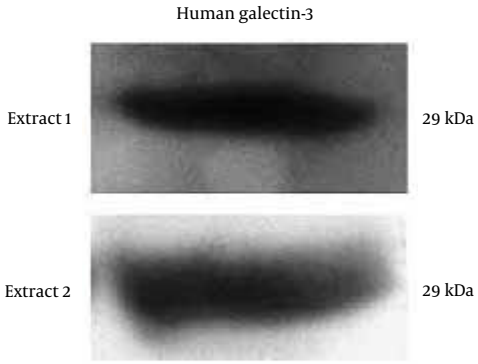

Figure 6. Western Blot Analysis of Recombinant Human Galectin3 From Two Separate Purification Runs. Galectin3 bands of 29kDawere detected with an A3A12 antibody.

4.5. Identification of p29

The nine most prominent peaks are labeled in Figure 7; the corresponding m/z values were taken to query the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database (NR; 512000 entries) for pattern matches, using the Peptide Search program. The following criteria were used: six matches out of nine, a mass accuracy of 40 ppm and a maximum of one missed cleavage sites per peptide. Only one protein of less than 100 kDa was retrieved, galectin3 (matches, 1273, 1324, 1429, 1539, 1626 and 1649; total sequence coverage, 27%). Under these search restrictions, random matches occurred at or below three out of nine. The tryptic digest analysis clearly revealed that the purified protein was indeed galectin3.

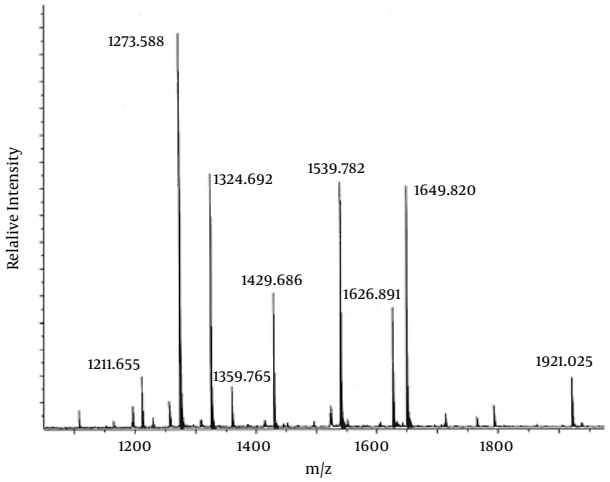

Figure 7. Identification of p29 by MALDI-reTOF. MALDI-reTOF mass spectrum of the 29 kDa band cut out from the gel was subjected to tryptic digestion. The tryptic digest mixture was loaded onto an RP microtip and the peptides were eluted with 30% acetonitrile, then analyzed by MALDI reflectron-TOF MS. Spectra were obtained by averaging 150 scans under constant irradiance; only the relevant portion is shown.

5. Discussion

Even though it is of great interest to find reliable, potent and easily available purification methods for galectin3, the quest has mostly been unsuccessful. Nevertheless, finding quick and efficient methods is of interest, yet the search could benefit from looking past galectin3. In this study, we constructed a novel shuttle vector in *Pichia pastoris* and used it for the secretion of human galectin recombinant protein. The galectin was expressed in *Pichia pastoris* X-33 by growing the yeast culture in YPD medium and purified by nickel-based affinity chromatography due to its His6 tag. In order to elucidate whether galectin3 expression was differentially regulated throughout the development of yeast cells, western blot analysis was performed. After cell lysis the protein was identified as a single 29 kDa band by 12% SDS-PAGE. Further, Protein bands were excised from the gels, digested with trypsin and processed for mass spectrometric fingerprinting. In brief, peptide mixtures were partially fractionated on Poros 50 R2 RP microtips and the resulting peptide pools were analyzed by matrix assisted laser desorption ionization-reflectron time of flight mass spectrometry (MALDI-reTOF MS). Selected mass values were then taken to search a protein non-redundant database using the Peptide Search algorithm. Mass spectrometric fingerprinting of the purified p29 identified it as galectin3. This indigenously produced recombinant human galectin3 was evaluated for its biological activity using hemagglutination inhibition assay. In summary, we concluded that, our studies have established an important role for galectin3 production in *Pichia pastoris*. In view of the well-established methods available for production, our studies suggest that our method could be easy and reproducible.

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Authors' Contributions

All authors participated equally in this study.

Financial Disclosure

There was no conflict of interest.

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