Cloning, expression, and purification of the N-terminal domain of the Flo1 flocculation protein from *Saccharomyces cerevisiae* in *Pichia pastoris*

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**A B S T R A C T**

*Saccharomyces cerevisiae* flocculation is governed by *FLO* genes, encoding Flo proteins (flocculins). Flo proteins are cell wall proteins consisting of three domains, sticking out of the cell wall and interacting with other yeast cells using their N-terminal mannose-binding domain. Until recently, flocculation research was focused on the genetic and cellular level. To extend the knowledge about flocculation to the protein level, we isolated the N-terminal domain of the Flo1p (N-Flo1p) that contains the mannose-binding domain, which is responsible for the strong interaction (flocculation) of *S. cerevisiae* cells. To obtain a high production yield and a more uniform and lower glycosylation of N-Flo1p, it was cloned in *Pichia pastoris*. The expression and the purification of N-Flo1p were optimised towards a one-step purification protocol. The activity of the protein, i.e. the binding of the purified protein to mannose using fluorescence spectroscopy, was demonstrated.

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**Introduction**

*Saccharomyces cerevisiae* flocculation is the phenomenon wherein yeast cells adhere in clumps, and sediment rapidly from the medium in which they are suspended [1]. Flocculation is governed by *FLO* genes, encoding Flo proteins [2–7]. Flo proteins are cell wall proteins consisting of three domains (the N-terminal, central and C-terminal domain). The proteins are covalently attached to the cell wall with their C-terminal domain and are interacting with other yeast cells using their exposed N-terminal mannose-binding domain. Cells expressing the Flo1 protein show a strong flocculation phenotype that can be reversibly inhibited by mannose [8–10].

The open reading frame of the *FLO1* gene from *S. cerevisiae* encodes a protein of 1537 amino acids, including the N-terminal mannose-binding domain of 240 residues (N-Flo1p) [8,11,12]. Recently, N-Flo1p was expressed in *S. cerevisiae*, purified, and its binding to mannose, dimannoses and mannann ligands were quantitatively characterised [13]. However, the protein yield was fairly low. Therefore, we assessed the expression of this protein in the yeast *Pichia pastoris* as an alternative eukaryotic expression organism. The purification protocol was reduced to a one step purification strategy and we found that the protein yield is 4 times higher compared to *S. cerevisiae* as expression organism. The purified N-Flo1p shows a lower and more uniform glycosylation and a similar mannose-binding activity.

**Materials and methods**

**Strains and growth conditions**

*S. cerevisiae* strain FY5 derived from S288C, was used for the preparation of genomic DNA. *Escherichia coli* DH5α was transformed with the pPICZa plasmid containing N-Flo1p to be expressed in *P. pastoris* strain X33, for the correct clone selection.

Bacteria were grown at 37 °C on low salt Luria–Bertani (LSLB) agar plates or LSLB broth, supplemented with Zeocin™ (25 mg/l), as a high ionic strength inhibits the activity of Zeocin™. LSLB contains 1% (w/v) peptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl.

*P. pastoris* was grown at 30 °C on YPD (yeast extract-peptone-dextrose), containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) d-glucose) plates or directly after transformation on YPDS (YPD with 1 M sorbitol) agar plates, supplemented with 1 M sorbitol; BMMG, buffered minimal glycerol medium; BMM, buffered minimal methanol medium; BMG, buffered complex glycerol medium; BMMY, buffered complex methanol medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMF, peptide mass fingerprinting; MALDI, matrix-assisted laser desorption ionisation.

[1] Abbreviations used: YPD, yeast extract-peptone-dextrose; YPDS, YPD with 1 M sorbitol; BMG, buffered minimal glycerol medium; BMM, buffered minimal methanol medium; BMG, buffered complex glycerol medium; BMMY, buffered complex methanol medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMF, peptide mass fingerprinting; MALDI, matrix-assisted laser desorption ionisation.
Zeocin™ (100–500 mg/l). Sorbitol is necessary to allow better recovery of the cells after electroporation. For cell culturing, yeast cells were grown at 30 °C in buffered minimal glycerol medium (BMG) containing 100 mM potassium phosphate at pH 6.0, 1.34% (w/v) yeast nitrogen base, 4 × 10⁻⁵% (w/v) biotin and 1% (w/v) glycerol. For induction, the cells were transferred to buffered minimal methanol medium (BMM) containing 100 mM potassium phosphate at pH 6.0, 1.34% (w/v) yeast nitrogen base, 4 × 10⁻⁵% (w/v) biotin and 0.5% (v/v) methanol.

**Extraction of genomic DNA from S. cerevisiae**

The isolation of genomic DNA from S. cerevisiae was accomplished using the DNeasy Tissue Kit (Qiagen, CA, USA). The procedure was adapted as follows: an overnight culture of S. cerevisiae strain S288C FY5 was grown until OD₆₀₀ of 2. The yeast cells were harvested by centrifugation for 12 min at 5000 rpm. Seventy mg cell pellet was resuspended in 530 μl buffer AP1 (DNeasy Tissue Kit, Qiagen, CA, USA) and 4 μl of RNase A stock solution (100 mg/ml). The mixture was incubated for 3 h at 65 °C to lyse the cells. Further steps were performed according to the manufacturer's protocol (Plant Tissue Mini Protocol, DNeasy Plant Handbook 07/2006, Qiagen, CA, USA). The elution of the DNA was done with 100 μl water, achieving an OD₂₆₀ of 2.5 and this DNA was used in the next experiments.

**Cloning of N-FLO1 into P. pastoris**

The N-terminal domain of Flo1p that contains the carbohydrate-binding sequence was determined based on alignments of the FLO1 gene and a homologous to FLO1 (Ig-FLO1). The coding sequence of the domain was amplified from the genomic DNA of S. cerevisiae using PCR with the oligonucleotides: Flo1_10 (5’-AGGGGTATCTCTCGAGAAAAGAGAGCTGAAGCTACAGAGGCCTGCT TACCAGCA-3’) and Flo1_11 (5’-GATGATGATGTCGACGAGGAGGTC AGGACAGTACAG-3’).

The amplified N-FLO1 gene was cloned into the XhoI and SalI-digested pPICzα vector using the In-Fusion™ method (Clontech, Mountain View, CA, USA). The correct construction of the expression plasmid was confirmed by PCR and DNA sequence analysis (Genetic Service Facility, VIB, Belgium). The pPICzα vector is used to express recombinant proteins in P. pastoris and contains the secretion sequence of the S. cerevisiae α-mating factor. Therefore, recombinant proteins are secreted into the medium. The vector has a Zeocin™ resistance gene for selection in both E. coli and Pichia. The vector provides at the 3’ side of the inserted gene, a sequence for 6 histidines, which leads to the addition of a His tag to the C-terminal end of the protein. The predicted molecular weight of the expressed protein is 26 kDa.

The transformation into CaCl₂-competent E. coli strain DH5α was carried out using a heat shock as described previously [14]. The P. pastoris strain X33 was transformed by electroporation.

**Expression analysis of N-Flo1p secreted from P. pastoris**

P. pastoris transformants were tested to select the clone with the highest expression level. Therefore, each colony was inoculated in 1 ml of BMG (buffered minimal glycerol) medium and allowed to grow for 24 h. The cells were pelleted by centrifugation (5000 rpm, 20 min, 4 °C) and resuspended in BMG (buffered minimal methanol) medium. After 24 h, the cells were discarded by centrifugation (5000 rpm, 20 min, 4 °C) and the supernatant was analysed on SDS–PAGE to detect the secreted protein.

The colony with the best expression level was analysed for the highest protein yield. Therefore, the transformed P. pastoris–cells were grown in both BMG and buffered complex glycerol medium (BMGY; contains BMG supplemented with 1% [w/v] yeast extract and 1% [w/v] peptone) to obtain a high cell density. To express the protein, the medium was changed into both BMG and buffered complex methanol medium (BMMY; contains BMM supplemented with 1% [w/v] yeast extract and 1% [w/v] peptone). The expression levels of all buffer combinations were compared. Also, the cells were harvested by centrifugation (8000 rpm, 10 min, 4 °C) at 8 h, 24 h, 36 h, 48 h and 72 h after induction to optimise the time of induction.

**Purification of N-Flo1p from P. pastoris**

The purification of secreted N-Flo1p from the culture supernatant was performed in one step as follows. The selected clone of P. pastoris that showed the highest expression of N-Flo1p was inoculated in 25 ml of BMG medium. The culture was shaken and incubated overnight at 30 °C. The next day, the culture was diluted in 1 l of BMG medium, divided in 4 shake flasks and allowed to grow to an optical density at 600 nm (OD₆₀₀) of 6 by incubation at 30 °C with shaking. Cells were harvested by centrifugation (12,000 × g, 10 min, room temperature) and resuspended in 1 l of BMM medium for induction. The protein is then secreted to the medium since it contains the secretion signal sequence of the S. cerevisiae α mating factor. Every 12 h, methanol (100% [v/v]) was added to the culture to a final concentration of 0.5% (v/v), to maintain induction. After 36 h, the cells were separated from the medium by centrifugation (12,000 × g, 20 min, 4 °C), and the resulting supernatant containing the protein was adjusted to pH 7.2 to protonate the histidines of the His tag. N-Flo1p was captured from the supernatant by affinity chromatography using a Ni–NTA Sepharose column (1 ml) and was eluted with 1 M imidazole in phosphate-buffered saline (PBS). The imidazole was then removed from the elution fractions by dialysis (Spectra/Por® Dialysis membrane, 3500 Da. Spectrum Laboratories, Inc., CA, USA) against 20 mM Tris pH 7.5, 150 mM NaCl. The purified protein was analysed with SDS–PAGE.

**Detection of (glyco)proteins**

After purification of the N-terminal domain of Flo1p, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, Biorad) was performed to visualise the protein and to estimate its molecular mass. The gel (12% [w/v) resolving gel) was stained with Coomassie stain (1 g/l Coomassie Brilliant Blue R250, 50% [v/v) methanol, 10% [v/v) acetic acid) for 30 min and destained with 50% [v/v) methanol and 10% [v/v) acetic acid. To visualise glycans, the Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain Kit (Invitrogen) was used.

**Maldi-ms pmf**

Peptide mass fingerprinting (PMF) was used to identify the purified protein after SDS–PAGE as described previously [13]. The protein bands of interest were excised from the SDS-gel and digested with trypsin (Promega). The peptides were extracted and then spotted on a matrix-assisted laser desorption ionisation (MALDI) target plate. The PMF was measured on a MALDI-tandem time of flight mass spectrometry (MALDI-TOF/TOF MS) system (model 4800 Proteomic Analyzer; Applied Biosystems) in MS mode and the resulting spectra were searched against the Swissprot database, using the Mascot platform to identify the protein.

**Fluorescence spectroscopy**

The binding of d-(-)-mannose to N-Flo1p was studied by fluorescence spectroscopy. The N-terminal domain of the Flo1 protein...
contains 3 tryptophan residues, and one of them is situated in the VSWGT binding motif [8]. This residue will be quenched upon binding of mannose [15]. The measurements were performed at 20 °C, using a luminescence spectrometer (model LS55, PerkinElmer, MA, USA). An excitation wavelength of 277 nm, a scan speed of 100 nm/min, and an excitation and emission slit width of 5 nm were used during the experiments. Emission spectra were collected from 300 to 420 nm and corrected relative to a buffer blank. The initial sample volume was 500 μL collected from 300 to 420 nm and corrected relative to a buffer blank. The initial sample volume was 500 μL.

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Activity of the purified N-Flo1p protein

The activity of the purified protein was evaluated by assessing the binding of 100 mM mannose to N-Flo1p using fluorescence spectroscopy (Fig. 2). Fig. 2 shows convincingly that N-Flo1p purified from \( P. \) \textit{pastoris} is binding to mannose. Moreover, the affinity between N-Flo1p and mannose was determined by adding increasing sugar concentrations to the protein. A titration curve was obtained by plotting the normalised fluorescence signal at 350 nm versus the sugar concentration (Fig. 3). The curve shows a biphasic nature, which implies the presence of two binding sites. The values for the dissociation constants at equilibrium are 113.0 ± 90.2 \( \mu \)M and 30.4 ± 2.0 mM, for the site with high affinity and low affinity, respectively. For N-Flo1p purified from \( S. \) \textit{cerevisiae}, those values are 57.77 ± 18.82 \( \mu \)M and 36.69 ± 6.27 mM, respectively [13]. This confirms that the activities of N-Flo1p produced by \( P. \) \textit{pastoris} and \( S. \) \textit{cerevisiae} are similar.

Discussion

The yeast flocculation phenomenon is of significant scientific and biotechnological interest because of its relevance for industrial yeast fermentation processes, e.g. the brewing industry [17–19]. A lot of research has been done to understand the underlying mechanisms of flocculation [4–7]. The main focus was on the behaviour of cells in varying conditions, however since the nineteen seventies, it was also aimed to study flocculation at the protein level.

Initially, the purification strategy was to isolate the flocculins from the surface of the yeast cells. Different methods – based on chemical extraction [1,20–24], enzymatic treatment [25] and shear forces [26,27] – have been tried and very divergent results were obtained. A more accurate and controlled way to purify flocculation proteins was needed. Therefore, recombinant expression and subsequent purification strategies have been developed.

The recombinant expression of a flocculin has been firstly reported for the expression of the N-terminal domain of \( Lg- \) Flo1p [28]. The protein was expressed in \( P. \) \textit{pastoris} and purified using a combination of ion-exchange chromatography and gel filtration. Also, the N-terminal domain of Flo11p was cloned and expressed in \( S. \) \textit{cerevisiae} and purified using a Ni–NTA-column [29,30]. The N-terminal domain of Flo5p was expressed in \( E. \) \textit{coli} and further purified using a combination of Ni–NTA affinity chromatography and gel filtration [31]. Recently, the N-terminal domain of Flo1p has been cloned and expressed in \( S. \) \textit{cerevisiae}, and purified [13]. However, the obtained yield for N-Flo1p was low.

Therefore, we decided to produce N-Flo1p in \( P. \) \textit{pastoris} to provide a more efficient way to purify this domain. Indeed, \( P. \) \textit{pastoris} is known to have a better secretory efficiency, a more uniform and minimal glycosylation and generally a higher product yield than \( S. \) \textit{cerevisiae} [32,33]. The \( P. \) \textit{pastoris} system has been successfully used to produce a wide variety of heterologous proteins, originating from viruses, bacteria, fungi, animals, plant and human beings [32,34–36]. However, for many years, \( S. \) \textit{cerevisiae} was preferred for the recombinant protein production as it is the best studied eukaryotic organism [37].

The expression of N-Flo1p in \( S. \) \textit{cerevisiae}, led to two populations with different molecular masses (approximately 36 and 100 kDa)
References


