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**CONCAVALIN A-AGAROSE REMOVES MANNAN IMPURITIES
FROM AN EXTRACELLULARLY EXPRESSED *PICHIA PASTORIS*
RECOMBINANT PROTEIN**

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Abstract: *Pichia pastoris* secretes few native proteins. However, the more than 1 g l⁻¹ of extracellularly expressed mannan interfered with the purification of our extracellularly expressed, non-glycosylated recombinant protein. Concanavalin A-agarose removed more than 95% of the unwanted mannan as monitored by phenol reaction. A ¹³C-based NMR assay confirmed this improvement. Concanavalin A-agarose can assist the purification of extracellular expressed, non-glycosylated proteins from yeasts.

Key Words: Concanavalin A-Agarose, Extracellular Expressed Protein, Mannan, *Pichia pastoris*

INTRODUCTION

Pichia pastoris provides a yeast expression system for recombinant protein production on the flask and fermenter scale [1]. Commercial kits based on *P. pastoris* provide a choice of extracellular or intracellular protein expression. Extracellular expression provides an initial purification step as *P. pastoris* secretes very few proteins at high concentrations [2]. However, *P. pastoris* also secretes yeast polysaccharides (mannans). There are few specific reports related to the purification of protein samples from mannan impurities. If the expressed protein is intended for NMR structural studies [3], immunological [4] or pharmaceutical purposes [5] then the protein should be free of mannan. Attention is rarely given to the degree of residual mannan in protein samples.

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Concanavalin A is a readily available lectin that specifically binds mannose-containing oligosaccharides. It is commonly attached to an immobile phase and used in the affinity chromatography and analysis of glycoproteins [for example, see 6, 7]. We investigated whether concanavalin A-agarose could be used as a first step in removing unwanted mannan from extracellularly expressed, non-glycosylated protein. We used a previously developed *P. pastoris* extracellular expression system for the N-terminal domain of calretinin, CR I-II, to test this novel idea [8].

MATERIALS AND METHODS

Protein expression

Calretinin domain I-II (CR I-II) was expressed, as described in [8]. Briefly *P. pastoris* strain KM71 (Invitrogen), transformed with pPEXCRI-II vector, was grown for 48 hours on BMG and the cells recovered by centrifugation and transferred to a 1/5 starting volume of BMM media, containing 0.5% methanol. The cells were grown for six days on this media with a daily supplement of 100% methanol to 0.5%. Protein labeled with ^{15}N and ^{13}C was produced using a similar protocol described in [3]. In this case, a modified BMG broth was used containing 0.5% ^{15}N -ammonium sulfate (instead of 1%) and 0.2% ^{13}C -glucose (instead of 1% glycerol). ^{15}N -ammonium sulfate and ^{13}C -glucose were obtained from Martek (Columbia, USA) and ^{13}C -methanol from Eurisotope (France). Cells were cultured in flasks, in a tabletop shaker at 250-300 rpm, 30°C. Polyacrylamide gel electrophoresis was carried out on 10% acrylamide gels in Tris-tricine buffer [9]. Purification was performed on DEAE-cellulose (ion exchange chromatography), after dialysis of the expression medium, in 50 mM Tris, 20 mM NaCl, 1 mM EGTA pH 8 buffer according to Kuźnicki *et al.* [10], and eluted in the same buffer substituting 2.5 mM CaCl_2 for EGTA.

Concavalin A-agarose treatment

Con A-agarose was purchased from Sigma (C-7555, Type VI concanavalin A immobilized on 4% cross-linked beaded agarose with 5-15 mg yeast mannan per ml binding capacity). After five days of recombinant protein expression, the cells were spun down at 3 500g for 10 min and the supernatant (250 ml) dialyzed (3.5 kDa MWCO (Spectrum, USA)) against 3 changes of a 10x excess of Milli-Q grade water for 12 to 24 hours. Then, the protein solution was adjusted to 0.1

Abbreviations used: con A - concanavalin A; con A-agarose - agarose attached concanavalin A; BMG - growth medium – 100 mM potassium phosphate, pH 6.0, 4×10^{-5} mg ml⁻¹ biotin, 1.34% YNB, 1% glycerol; BMM - expression medium – 100 mM potassium phosphate, pH 6.0, 4×10^{-5} mg ml⁻¹ biotin, 1.34% YNB, 0.5% methanol; CR I-II - rat calretinin residues 1-100 consisting of EF-hands I and II; GST - glutathione S-transferase; HMQC - heteronuclear multiple quantum correlation NMR spectroscopy; MWCO - molecular weight cut-off; YNB - yeast nitrogen base.

M Tris pH 8.0, 0.15 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM MnCl₂. Con A-agarose suspension was added to the protein solution (1:10, v:v; 35 ml of 50% suspension to 350 ml protein solution) and the batch rolled at 4 °C for 30 mins. Con A-agarose was separated by centrifugation at 3 000g for 10 min. The supernatant was retained. The incubation was repeated (i.e. 35 ml addition of 50% con A-agarose suspension) until the mannan content approached zero (or 5 rounds of purification). Finally, the solution was filtered through a 0.22 µm membrane filter (Millipore) to remove any remaining con A-agarose. Volumes are given for typical preparations but small preparations (e.g. 100 ml protein solution) used the same ratio of adducts (e.g. 10 ml additions of 50% con A-agarose suspension). The con A-agarose was regenerated after packing into a column and elution with 1 M α-D-glucopyranoside in 0.1 M Tris pH 8.0, 0.15 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM MnCl₂, and further equilibration in the same buffer and absence of α-D-glucopyranoside. The con A-agarose was added in this buffer and the buffer it was supplied in (1.0M NaCl, 0.1 M KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, pH6.0). DEAE-cellulose chromatography was performed as described above.

Polysaccharide and protein analyses

Mannan concentrations were determined by adding 1 ml concentrated H₂SO₄ (95-97%, Sigma) to 0.2 ml of sample/matched buffer (0.1 M Tris pH 8.0, 0.15 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM MnCl₂ (in variable proportions)) and 0.2 ml 5% phenol [11]. The resulting samples, and references prepared from known concentrations of sucrose (Sigma), were analyzed by UV spectroscopy at a wavelength of 490 nm (Shimadzu 160A). Protein concentrations were determined by the Bradford method [12] using bovine serum albumin as a reference and concentrated reagent purchased from BioRad.

NMR spectroscopy

CR I-II samples were dissolved in 50 mM Tris, 20 mM NaCl, 50 mM sodium acetate, pH 7.7 buffer. ¹H, ¹³C HSQC spectra of CR I-II samples were collected and processed on a Bruker DRX 500 instrument, using standard pulse sequences.

RESULTS AND DISCUSSION

CR I-II is a stable, independent domain derived from calretinin, a calcium-binding protein of undetermined structure [8, 10, 13, 14]. Fig. 1 illustrates the extracellular expression of CRI-II from *P. pastoris* in a non-glycosylated form (12.5 kDa) before any purification was initiated [8]. The band attributable to CR I-II is clearly observed and there are no other visible protein bands, Fig. 1, lane 1. The gel does not reveal other impurities originating from the medium, such as salts, metabolites and oligosaccharides. Our original protocol employed dialysis to exchange the expression medium for a buffer suitable for a single polishing step of ion-exchange chromatography (using the method developed to remove

thrombin from *Escherichia coli* produced CR I-II, after cleavage of GST-CR I-II fusion protein on a glutathione column [10]). However, unlabeled *P. pastoris* expressed CR I-II produced from this protocol revealed characteristic anomeric sugar signals that could be assigned to *P. pastoris* mannan on the basis of the assignment given by Vinogradov *et al.* [15], Fig. 2A. Previously reported difficulties in removing yeast mannan suggested that the source of the impurities in the NMR sample originated from the expression media [16].

Analysis of dialyzed expression medium revealed that the solution contained the equivalent of 1 g/L of mannan (referenced to sucrose) and 50-200 mg/L of protein (referenced to BSA). The protein yield was lower at higher degrees of isotope labeling. Clearly, the uncharged mannan (of undetermined size) did not pass through the 3.5 kDa MWCO dialysis tubing despite extensive dialysis. Larger MWCO tubing did not retain our protein. Controls indicated that high levels of sucrose did not affect the Bradford assay and that BSA did not affect the phenol assay. We decided to explore the possibility of using concanavalin A-agarose to selectively remove the mannan from our protein solution.

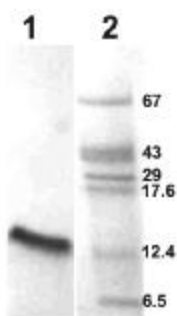


Fig. 1. Polyacrylamide gel analysis of recombinant calretinin I-II expressed from *P. pastoris*. Lane 1: 20 μ l of *P. pastoris* expression media, after 6 days and removal of cells, containing CR I-II before initiation of purification. Lane 2. Protein standards in kDa (Promega).

Successive rounds of con A-agarose treatment lowered the mannan concentration of the sample, as determined by the phenol reaction, Fig. 3. This was achieved without compromising the protein level, as determined by Bradford, Fig. 3. After five rounds of treatment, 95% of the mannan had been removed with the loss of 18% of the protein. Part of the protein loss may represent minor glycosylated protein impurities. The remaining mannan may originate from heavily crosslinked mannan that has a weak affinity for con A. Therefore, the data presented in Fig. 3 indicates that con A-agarose treatment removes most of the mannan impurity without affecting the recovery of the target protein.

Fig. 2A illustrates a HMQC spectra of unlabeled CR I-II produced from the *P. pastoris* expression system and purified by the original dialysis and ion-exchange chromatography protocol. Peaks with distinctive polypeptide character are below the threshold. The region containing non-anomeric mannan signals partly overlaps with a region expected to contain protein signals. The mannan anomeric signals are still present in the con A-agarose treated sample, Fig. 2B but the protein signals can now be observed at similar intensity. This is in agreement with the 95% removal of mannan suggested by Fig. 3. A reference sample of CR I-II produced from an *E. coli* expression system contains only the protein signals, Fig. 2C.

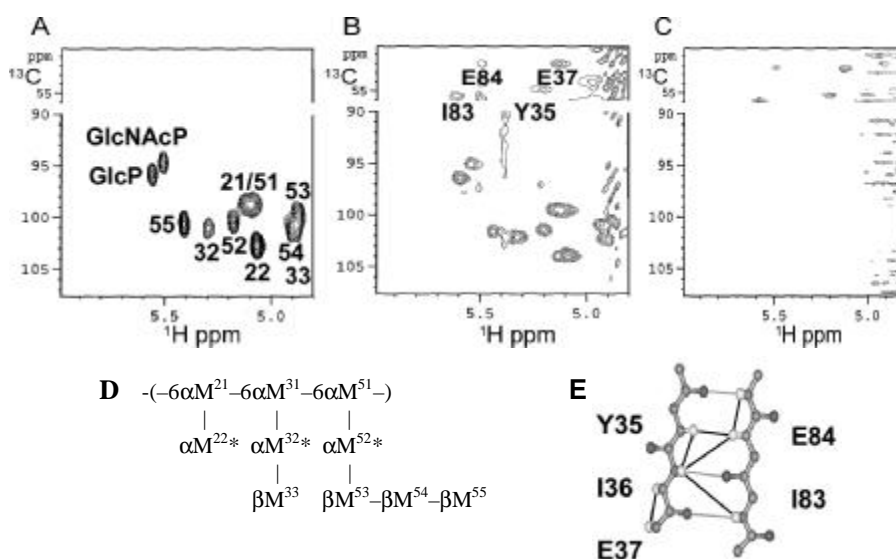


Fig. 2. HMQC spectra of CR I-II. (A) Unlabeled CR I-II purified from *P. pastoris* after dialysis and ion-exchange chromatography. (B) ^{13}C , ^{15}N -labeled CR I-II purified from *P. pastoris* after dialysis and treatment with con A-agarose and ion-exchange chromatography. (C) ^{13}C , ^{15}N -labeled CR I-II produced from an *E. coli* expression system [10]. (D) Structure of *P. pastoris* mannan determined by Vinogradov *et al.* [15] where the asterisks represent links to either H or the phosphodiester-linked α -D-Glc-P or α -D-GlcNAc-P groups. (E) The short, interacting extended structures of the calcium-binding loops of CR I-II, as defined in [14], involve residues K82-E84 and Y35-E37. The mutual interaction of the protein backbone is defined by NMR-derived interactions (black lines) and hydrogen bonds (gray lines). Numbered labels refer to the anomeric positions of the mannan structure given in panel D and observed in panels A and B. Other labels refer to the asterisked groups of the mannan (panels A and B) and C-H correlations of amino acids (panels B and C). Unlabeled peaks in the spectra are due to noise from the water signal (panels B and C).

About 1 g l^{-1} of mannan remains after dialysis compared to a protein yield of 0.1 g l^{-1} , depending on the degree of isotope labeling. The failure of ion-exchange

chromatography in our hands is unsurprising as this method, and size exclusion chromatography, are popular choices for the oligosaccharide purification [15, 17]. These methods can be expected to potentially co-purify mannan and protein, as appears to be the case with CR I-II. Denton *et al.* first noted the problem of mannan in obtaining pure protein from extracellularly expressed protein from a *P. pastoris* host system. They recovered only 13 % of their protein after two rounds of reverse phase chromatography [16]. Therefore, we decided to exploit the ability of con A-agarose to selectively remove mannans from our solutions containing CR I-II. An important point for the general application of this protocol is that the target protein is non-glycosylated and is not expected to bind to con A.

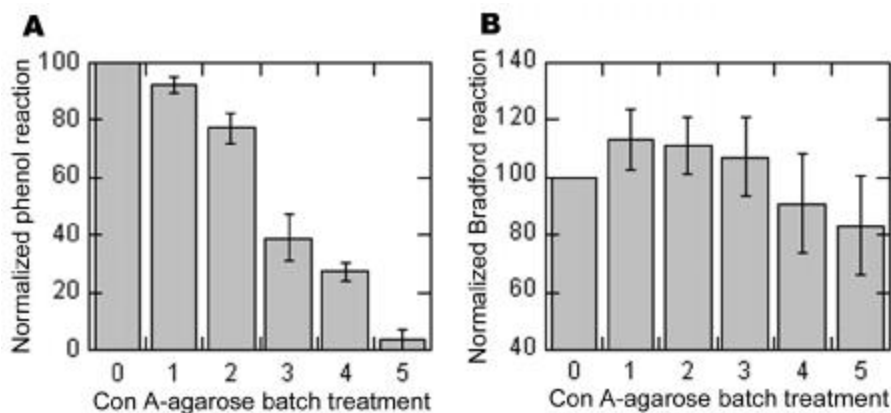


Fig. 3. Analysis of CR I-II solutions produced from *P. pastoris* as a function of con A-agarose batch treatments. (A) Mannan content determined by phenol reaction. (B) Protein content determined by the method of Bradford. All data are normalized to starting values. The means of five different batches of expressed protein with standard deviations are plotted.

After 400-fold concentration of the con A-agarose and ion-exchange treated CR I-II for NMR purposes, traces of mannan were still detected, Fig. 2B. This suggests that ion-exchange chromatography is a poor choice for the purification of proteins extracellularly expressed from *P. pastoris*. The rolled-batch method of con A-agarose treatment was developed as gravity-feed chromatography proved time-consuming and FPLC unavailable. We observed that the performance of con A-agarose resin degraded after 2-3 regenerations with methyl α -D-glucopyranoside, most likely due to the physical stress of the protocol. A lower capacity to chelate mannan was noted and a protein band at ~25 kDa on polyacrylamide gels, corresponding in mass with concanavalin A and absent in protein solutions not treated with con A-agarose, was a further sign of resin degradation. We believe that the protocol could be improved through the use of FPLC where the dialyzed expression media is pumped through a con A-

agarose column. This should provide a more efficient removal of mannan from protein solutions and a milder treatment of the con A-agarose with less contamination of the target protein with con A.

^{15}N -based experiments, similar to the ^{13}C -based experiments of Fig. 2, have been used to monitor the level of ^{15}N -labeled protein extracellularly expressed by *P. pastoris* [18]. The spectra included signals corresponding to the purified protein product and a number of impurities. The level of impurities appeared to be low [18]. Our ^{15}N -based NMR experiments on purified ^{15}N -labeled CR I-II also suggested a pure protein product. However, yeast mannan contains few amide moieties capable of providing ^1H , ^{15}N correlations. It is only when observing specific correlations present in mannan, i.e. ^1H , ^{13}C correlations, that mannan can be unambiguously detected. General methods of protein analysis – polyacrylamide gels, Bradford analysis, Circular Dichroism spectroscopy, Western blotting, ELISA and chromatography (using detection wavelengths of 280 nm to minimize protein degradation) – will fail to detect contaminating mannan. ^1H NMR and mass spectroscopy may detect mannan but not unambiguously and only if the analyses are performed in mind of the possible mannan contamination. In contrast, the phenol reaction provides a simple protocol for a protein laboratory to identify potential mannan impurities.

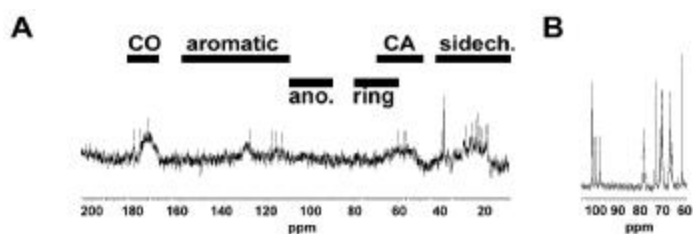


Fig. 4. 1D ^{13}C NMR spectra. A. Protein mixture (0.5 mM aprotinin, 0.5 mM α -lactalbumin, 0.2 mM ovalbumin and 0.2 mM BSA; 32 mg ml $^{-1}$ protein) and B. Mannan sample (20 mg ml $^{-1}$) derived from *Saccharomyces cerevisiae*. All compounds were purchased from Sigma and run in D_2O on a Bruker 500 MHz instrument. Expected chemical shifts for proteins (CO, carbonyl; aromatic carbons; CA, α -carbons; sidech., sidechain carbons) and carbohydrates (ano., anomeric carbons; ring carbons) are indicated by bars in panel A.

The use of ^{13}C NMR as a tool to identify mannan impurities, even in unlabeled samples, is novel. The 2D experiment illustrated in Fig. 2A required 48 hours of acquisition time and clearly identifies the *P. pastoris* mannan. However, for routine analysis, a 1D ^{13}C spectrum (requiring about 2 hours on the same sample) would also clearly identify the presence of polysaccharide. A clean ^{13}C spectrum of only protein is expected to contain signals in the 10-80 ppm (protein aliphatic sidechain potentially overlapped with ring carbons of sugar moieties), 110-160 ppm (protein aromatics) and 170-185 ppm (protein amide carbonyl) region and none in the 90-110 ppm (anomeric carbons of carbohydrate) region,

Fig. 4. Concentration of the sample to >1 mM is required for analysis at the natural abundance of ^{13}C . As such samples are typically employed to check the protein properties prior to ^{15}N - and ^{13}C - labeling for NMR structural studies, it is suggested that such spectra are routinely collected on yeast-derived proteins in order to check the quality of the purification protocol.

Analysis of protein samples for contaminating mannan is rare [16] and it is more often the case that no such analysis is reported [3, 8, 18]. Overlapping mannan and protein signals have prevented the sidechain assignment of ^{13}C , ^{15}N -labeled CR I-II [14] – a prerequisite to the determination of a high resolution structure. Successful structural studies of *P. pastoris* extracellularly expressed proteins involve a combination of at least two chromatographic steps [19, 20] or the application of protein-specific affinity chromatography [21, 22]. Only in one case was a single-step purification achieved [23]. Here, phenyl-Sepharose chromatography was used – hydrophobic interaction chromatography appears to be rarely used to purify oligosaccharides [17]. Despite the promise that one purification step should be sufficient (e.g. polyacrylamide gels – Fig. 1), in most cases at least two purification steps will be required for extracellularly expressed proteins from *P. pastoris*. One of those steps can include con A-agarose as it appears to selectively remove contaminants (mannan, glycosylated proteins) without affecting protein levels. In addition to interfering with the NMR analyses, mannan has also the potential to interfere with crystallization processes, which utilizes unlabeled protein. The presence of mannan in pharmaceutical preparations could also be problematic. There are few *P. pastoris* derived structures in the Protein DataBank [19-23] and this may reflect problems in purifying large amounts of protein to sufficient homogeneity.

To conclude, mannan is the major contaminant of *P. pastoris* extracellularly expressed proteins. Protein purification protocols should focus on the removal of mannan from protein, preferably using methods that favor protein retention over oligosaccharides. Con A-agarose, with further developments, may help in reducing mannan levels and allow the use of purification methods that otherwise co-purify mannan and protein. ^{13}C NMR spectroscopy provides a method for the unambiguous detection of mannan in protein samples and the suitability of protein samples to be used in NMR studies.

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