Vol. 20, No. 1 (2021) 147-160
Revista Mexicana de Ingeniería Química

Partial recovery of MRJP1 protein expressed in *Pichia pastoris* using chromatographic techniques

Recuperación parcial de la proteína MRJP1 expresada en *Pichia pastoris* utilizando técnicas cromatográficas

A. Robles-Zamora, D. Enriquez-Ochoa, M. Ureña-Herrera, J.M. Aguilar-Yañez, M.E.G. Brunck, K. Mayolo-Deloisa*

Tecnologico de Monterrey, Escuela de Ingeniería y Ciencias, Centro de Biotecnología FEMSA, Av. Eugenio Garza Sada 2501 Sur, Tecnológico, 64849 Monterrey, N.L., México.

Received: May 13, 2020; Accepted: July 27, 2020

Abstract

Major Royal Jelly Protein 1 (MRJP1) is the main protein in the complex mixture royal jelly, which is the only nutrient source for queen bees. The protein promotes increased lifespan, body size and fertility. Recombinant production of MRJP1 represents an alternative to direct extraction from royal jelly. Production in *Pichia pastoris* results in high density biomass, and a complex supernatant containing high amount of impurities. Various chromatography methods have been applied to recover and/or purify MRJP1. In this work, exploiting the physicochemical properties of MRJP1, reverse phase chromatography (RPC), size exclusion chromatography (SEC) and ion-exchange chromatography (IEX) were investigated as alternative methods to recover MRJP1 directly from supernatant. All techniques showed a 57-kDa band in SDS-PAGE analysis, corresponding to the size of recombinant MRJP1, with contaminants attributed to culture media. However, the chromatogram from SEC coupled to IEX shown a single peak suggesting it may be a good protocol to recover recombinant MRJP1 from *P. pastoris* supernatant. This is the first report about partial recovery of recombinant MRJP1 produced in *Pichia pastoris* utilizing IEX without the use of histidine tags.
Keywords: MRJP1, *Pichia pastoris*, ion exchange chromatography, size exclusion chromatography.

Resumen

MRJP1 es la principal proteína de la mezcla compleja de jalea real, la cual es la única fuente de nutrientes de las abejas reinas. La proteína promueve un incremento en el tiempo de vida, el tamaño corporal y la fertilidad. La producción recombinante de MRJP1 es una alternativa a la extracción directa a partir de jalea real. La producción en *Pichia pastoris* resulta en una alta densidad de biomasa y un complejo sobrenadante con altos niveles de impurezas. Varios métodos cromatográicos han sido utilizados para recuperar y/o purificar MRJP1. En este trabajo, explotando las propiedades fisicoquímicas de MRJP1, se investigaron RPC, SEC e IEX como métodos alternativos de recuperación de MRJP1 directamente del sobrenadante. Todas las técnicas mostraron una banda de 57 kDa en SDS-PAGE, correspondiente al tamaño de MRJP1 recombinante, con contaminantes atribuidos al medio de cultivo. Sin embargo, el cromatograma de SEC acoplado a IEX mostró un pico único, sugiriendo que este podría ser un protocolo aceptable para recuperar MRJP1 del sobrenadante de *P. pastoris*. Este es el primer reporte sobre la recuperación parcial de MRJP1 producida en *Pichia pastoris* utilizando IEX sin el uso de etiquetas de histidina.
Palabras clave: MRJP1, *Pichia pastoris*, cromatografía de intercambio iónico, cromatografía de exclusión molecular.

1 Introduction

Major Royal Jelly Protein 1 (MRJP1), also called royalactin, is the major protein component of the bee-produced complex mixture royal jelly (Shen *et al*., 2015; Ibarra-Herrera *et al*., 2014, Buttstedt *et al*., 2014). It has been reported that MRJP1 promotes health benefits spanning various species including the mouse *Mus musculus*, the rat *Rattus norvegicus*, the fruit fly *Drosophila melanogaster* and the worm *Caenorhabditis elegans* (Kamakura[b] *et al*., 2001; Detienne *et al*., 2014; Shorter *et al*., 2015; Chen *et al*., 2016; Xin *et al*., 2016).

* *Corresponding author. E-mail*: kmayolo@tec.mx
https://doi.org/10.24275/rmiq/Bio1713
Tel. 81-83-58-20-00, Fax 81-83-58-20-00
ISSN:1665-2738, *issn-e*: 2395-8472

Mechanisms of actions suggest that Epidermal Growth Factor Receptor (EGFR) may be a receptor for MRJP1 (Kamakura[d], 2011). To further investigate MRJP1, without impacting natural bee populations, researchers have developed its recombinant production in model organisms, as well as strategies for its recuperation post-production (Ibarra-Herrera *et al*., 2014; Kamakura[c] *et al*., 2006; Júdová *et al*., 2004; Shen *et al*., 2010; Wan *et al*., 2018). In its native conformation, extracted directly from royal jelly, MRJP1 is a complex of four 55-57 kDa glycoproteins of 432 amino acids each one, bound in the center by two apisimin residues (Mandacaru *et al*., 2017; Tian *et al*., 2018). However, MRJP1 can be produced recombinantly as a 57 kDa monomer protein (Ibarra-Herrera *et al*., 2014).

Some research groups have worked towards recovery and concentration of MRJP1 post-recombinant expression using a variety of techniques (summarized in Table 1). Overall different types of chromatographies have been applied for the recovery of MRJP1. The production of MRJP1 in diverse expression systems affects recovery and purification processes. For example, *Escherichia coli* was used by Kamakura *et al*. to produce MRJP1 (Kamakura[c] *et al*., 2006).

In this approach, a glutathione S-transferase (GST) tag was used to improve solubility by avoiding aggregates in inclusion bodies and to enhance the protein purification. *Pichia pastoris* was considered as a suitable expression system for MRJP1 due to its ability to post-translationally modify proteins and its capability to secrete recombinant proteins to culture media, facilitating recovery (Ibarra-Herrera *et al*., 2014; Shen *et al*., 2010). Furthermore, culture conditions and derived expenses are less than in mammal expression systems (Maccani *et al*., 2014). In previous works, MRJP1 produced in *Pichia pastoris* has been tagged with histidine's to facilitate its recovery using HisTrap FF crude affinity column while SEC has been coupled with dialysis and ultrafiltration (Shen *et al*., 2010; Ibarra-Herrera *et al*., 2014). Different types of expression systems will contain different impurities. Therefore, they will imply different purification procedures, always trying to minimize the number of steps without compromising the quality of the final product. In addition, purification process will depend on the specific application in which the protein is intended to be use (GE Healthcare, 2004). In particular, the production of MRJP1 in *Pichia pastoris* results in a high-density biomass and viscosity, with complex culture supernatant containing impurities (Ibarra-Herrera *et al*., 2014).

To prevent possible hindrance from tags or reporter proteins added to MRJP1, for instance major differences in molecular weight (Shen *et al*., 2010), targeted chromatographic techniques can be selected to recovery untagged recombinant MRJP1, directly from a clarified supernatant, exploiting the physicochemical properties of MRJP1.

Table 1. Techniques applied in recovery of MRJP1.

Author	Production method	Expression host	Original sequence	Protein modification	Chromatographic method	Type of column
Kamakura[c] *et al*., 2006	Recombinant	*Escherichia coli*	*Apis mellifera*	Glutation-S-transferase tag	SEC	HiPrep 16/60 Sephacryl S-200
Kamakura[a] *et al*., 2001	Natural	N/A	N/A	N/A	IEX coupled to SEC	IEX: DEAE Toyopearl 650 M SEC: HiLoad Superdex 200
Kamakura[b] *et al*., 2001	Natural	N/A	N/A	N/A	SEC	HiLoad 16/10 Superdex 200
Wan *et al*., 2018	Recombinant	Chinese Hamster Ovary cells	*Apis mellifera*	Histidine tags	IMAC coupled to IEX	Not specified
Shen *et al*., 2010	Recombinant	*Pichia pastoris*	*Apis cerana*	Histidine tags	N/A	HisTrap FF crude affinity columns
Ibarra-Herrera *et al*., 2014	Recombinant	*Pichia pastoris*	*Apis mellifera*	α-factor secretion signal from *Saccharomyces cerevisiae*	SEC	HiPrep 26/60 Sephacryl S-300
This work	Recombinant	*Pichia pastoris*	*Apis mellifera*	α-factor secretion signal from *Saccharomyces cerevisiae*	1. SEC coupled to IEX 2. RPC	1. SEC: Superose® 12 10/300 GL IEX: Tricorn 50/50 column using Q Sepharose Fast Flow 2. Sep-Pak C18 cartridges

Reverse-Phase Chromatography (RPC) separates molecules with high resolution from a complex mixture, based on differences in their selective polarity. MRJP1 has an average hydropathy value of -0.44 ± 1, reflecting its low hydrophobicity (Mandacaru *et al*., 2017). As a result, MRJP1 requires strongly hydrophobic ligands to force sufficient binding to allow subsequent separation. Therefore, it was hypothesized C18 ligands allow recombinant untagged MRJP1 recovery through RPC.

The isoelectric point of MRJP1 ranges from 4.7 to 5.2 due to the varied isoforms of the protein as a result of post-translational modifications (or glycoforms) (Furusawa *et al*., 2016; Cruz *et al*., 2011; Mandacaru *et al*., 2017). Similarly, optimum pH is between 4.4 and 5.4 (Furusawa *et al*., 2016; Cruz *et al*., 2011). These characteristics allow the applicability of IEX to separate MRJP1 from culture contaminants. SEC emerges as an alternative because it exploits the differences in molecular weight among contaminants and MRJP1.

In the seeking of an alternative method to recovery *Apis mellifera* MRJP1 directly from *P. pastoris* fermentation cultures for identification and molecular weight determination, RPC, SEC and IEX were tested by taking advantage of the physicochemical properties of MRJP1. To the best of our knowledge, no prior reports characterize the recuperation of honeybee *Apis mellifera* MRJP1 expressed in *Pichia pastoris* using IEX without the use of histidine tags.

2 Materials and methods

2.1 *Production of recombinant Apis mellifera MRJP1 in Pichia pastoris*

Recombinant MRJP1 was produced using a modified strain of *Pichia pastoris* (Ibarra-Herrera *et al*., 2014). Briefly, cultivation was performed in flasks using BMGY media (buffered complex medium containing glycerol) for biomass generation and BMMY (buffered complex medium containing methanol) for protein production following PichiaPink™ Expression System instructions (Invitrogen/Life Technologies, Cat. A11150), with the addition of 0.5% methanol as inducer (the induction begins when optic density reaches 20 OD_{600nm} approximately at 30-34 hours, departing from a 0.05 OD_{600nm} in fed-batch). To monitor methanol in the medium, it was required a previous determination of μmax under culture conditions with methanol as inducer, to monitor the cell growth during the experiment and estimate the need of methanol. After 120 h of cultivation, the culture was centrifugated for cell removal, and supernatant was clarified with Whatman filter paper of 2 μm pore size. Clarified supernatant underwent tangential flow filtration, using a Pellicon®XL system with 10KDa NMWL membrane. The supernatant was washed through three diafiltration volumes (3DV). Finally, the concentrated protein (10 g/L) was filtered passing by 0.2 μm sterilized bottle filter system (Corning, Cat. 430186).

2.2 *Recovery of recombinant MRJP1 by reverse phase chromatography (RPC)*

Solvent A was prepared using 0.12% trifluoroacetic acid (Sigma, Cat. 302031) in milli Q H_2O. Solvent B was prepared using 700 mL of acetonitrile (Sigma, Cat. 271004) with 0.1% trifluoroacetic acid mixed with 300 mL of milli Q H_2O. Briefly, Sep-Pak C18 cartridges (Waters, Cat. WAT043395) were activated by pumping acetonitrile (2 mL per cartridge at a flow rate of 2 mL/min) using a 20-mL plastic syringe (BD, Cat. 309661), followed by solvent A (2 mL/cartridge). Then, 180 mL of clarified supernatant from MRJP1-producing *P. pastoris* (2 g/L) was pumped through the cartridge at an average flow rate of 1 mL/min using a 50-mL plastic syringe (BD, Cat. 309653). Solvent A (4 mL/cartridge) was pumped at a flow rate of 4 mL/min and the eluate discarded. Then, Solvent B (2 mL/cartridge) was used at a similar flow rate, followed by 1.5 mL of acetonitrile 100% at a flow rate of 1 mL/min and the eluate collected into tubes (Corning, Cat. CLS430055). The volume of eluate was reduced to 500 μL under pressure conditions in a Speed-Vac concentrator in approximately 60 min at room temperature. Samples were resuspended in phosphate buffered saline (PBS) pH 7.4 (NaCl, Sigma, Cat. S9888; KCl, Sigma, Cat. P9541; Na_2HPO_4, Sigma, Cat. 255793; KH_2PO_4, Sigma, Cat. P0662). Finally, the fractions were stored at -20 °C.

2.3 *Recovery of recombinant MRJP1 by size exclusion chromatography (SEC)*

Sodium phosphate monobasic (Cat. 02-004-215), sodium phosphate dibasic (Cat. 3828-01) and potassium chloride (Cat. 02003738) were obtained from J.T. Baker. Samples of clarified supernatant from MRJP1-producing *P. pastoris* were centrifuged 15 min at 3220 x g. After that, samples were diluted

1:3 with sodium phosphate buffer 10 mM pH 7.0, containing 150 mM potassium chloride. Mobile phases were filtered using 0.22 μm nylon membrane (Merck Millipore, Cat. GVWP04700) and the samples were filtered using 0.2 μm Acrodiscs Syringe Filters (Pall Corporation, Cat. 4554T) before loaded into Äkta Avant 150 system (GE Healthcare, Sweden). Superose 12 HR 10/300 GL column (10 mm inner diameter, 300 mm length, average particle size of 11 μm, also from GE Healthcare) was used with an isocratic mobile phase of 10 mM sodium phosphate buffer pH 7.0, containing 150 mM potassium chloride at a flow rate of 0.8 mL/min. Fractions were monitored at 280 nm, collected and concentrated using Amicon ultra-15 centrifugal filter units MWCO 3kDA (Merck Millipore, Cat. UFC900324). Samples were kept at 4 °C during all the process and stored at -20 °C after concentration.

2.4 *Recovery of recombinant MRJP1 by ion-exchange chromatography (IEX)*

In order to optimize the purification of recombinant MRJP1 using IEX, several operating conditions were tested (Table 2). Trizma base (Cat. T1503), hydrochloric acid (Cat. H1758) and sodium chloride (Cat. S9888) were purchased from Sigma. Ammonium sulfate (Cat. A702-500) was obtained from J.T. Baker. Samples of clarified supernatant from MRJP1-producing *P. pastoris* were centrifuged 15 min at 3220 x g and diluted 1:3 with buffer A. Mobile phases were filtered using 0.22 μm nylon membrane (Merck Millipore, Cat. GVWP04700) and the samples were filtered using 0.2 μm Acrodiscs Syringe Filters (Pall Corporation, Cat. 4554T) before loaded into Äkta Avant 150 system (GE Healthcare, Sweden). 1 mL Q Sepharose Fast Flow column (Tricorn 5/50 Column, 5 mm inner diameter, 50 mm length, particle size of 45-165 μm) was used with a 500 μL injection loop. The column was pre-equilibrated with Buffer A Tris-HCl 20 mM (pH 8.2) and eluted with a 10, 20, or 30 column volume (CV) linear gradient using Buffer B Tris-HCl 20 mM (pH 8.2) containing either ammonium sulfate or sodium chloride (0-0.4 M) at a flow rate of 0.8 mL/min. Fractions of 1 mL were monitored at 280 nm, collected and concentrated using Amicon ultra-15 centrifugal filter units MWCO 3kDA (Merck Millipore, Cat. UFC900324). Samples were kept at 4 °C during all the process and stored at -20 °C after concentration.

Table 2. IEX tested conditions.

pH effect	7.2 8.2
Column linear gradient effect	10 CV 20 CV 30 CV
Salt concentration effect*	0.4 M 0.2 M 0.1 M 0.07 M
Type of salt concentration effect	Ammonium sulfate Sodium chloride

*Buffer B was modified in gradient elution according to the tab

2.5 *Recovery of recombinant MRJP1 by size exclusion chromatography (SEC) coupled to ion-exchange chromatography (IEX)*

All chromatography methods were developed in Äkta Avant 150 system (GE Healthcare, Sweden) as described in sections 2.3 and 2.4.

In different runs, samples obtained from SEC (Fractions 3 and 4, separately) were diluted 1:1 with buffer A, syringe filtered and loaded into a 1 mL Q Sepharose Fast Flow column using a 1000 μL injection loop. The column was eluted with a linear gradient using buffer B Tris-HCl 20 mM (pH 8.2) containing ammonium sulfate (0-0.4 M) at a flow rate of 0.8 mL/min. Fractions of 1 mL were monitored at 280 nm, collected and concentrated using an Amicon chamber with a Diaflo ultrafiltration membrane of 3kDA (Merck Millipore, Cat. PLBC07610). Samples were kept at 4 °C during all the process and stored at -20 °C after concentration.

2.6 *Total protein quantification*

The crude extract and all fractions were quantified using a BCA Protein Assay Kit supplied by Thermo Scientific (Cat. 23225), following the manufacturer's protocol. Briefly, 25 μL of each standard (with a range from 25 to 2000 μg/mL of albumin standard in a calibration curve) or unknown sample was pipetted thrice, in a 96-well microplate (obtained from Corning, Cat. 3364). Next, 200 μL of the working reagent and mixture of reagents A and B (50:1) were added per well. The microplate was mixed for 30 seconds, covered, incubated for 30 minutes at 37 °C and measured on a Biotek Synergy HT microplate

reader (SynergyTM HT, BioTek Instruments, USA) at 562 nm.

2.7 SDS-PAGE

After quantification, all fractions, including a sample of clarified supernatant from MRJP1-producing *P. pastoris*, were diluted to obtain the same protein total concentration. Later, samples in Laemmli Buffer 6X (375 mM Tris-HCl pH 6.8, 6% SDS, 4.8% Glycerol, 9% β-Mercaptoethanol, 0.03% Bromophenol blue) were denatured at 100 °C for 10 min. After, the method described by Laemmli was followed (Laemmli, 1970). The gel was performed using 4% stacking and 10% resolving gels with 40% acrylamide solution (Bio-Rad, Cat. 1610140). The proteins were stained using a silver staining protocol (González-González *et al.*, 2012). The molecular weight was verified using a protein molecular weight marker (Precision Plus Protein Dual Xtra Standard, 2-250 kDa, Bio-Rad, Cat. 1610377). For Coomassie stain, the procedure in colloidal form was developed (Neuhoff *et al.*, 1988).

2.8 Deglycosylation of MRJP1

The deglycosylation of MRJP1 obtained from clarified supernatant from MRJP1-producing *P. pastoris* was performed using a PNGase F deglycosylation kit (New England Biolabs, Cat. P0704S), as per manufacturer's instructions. Deglycosylation of the protein was evaluated using SDS-PAGE and silver staining, following the methods described above.

2.9 Western blot

Identification of collected fractions was confirmed by Western blot analysis. Briefly, samples were diluted with PBS for loading in Laemmli Buffer. Samples were denaturalized for 7 min at 90 °C. SDS-PAGE was performed using a 10% acrylamide gel for 1.5 h at 100 V. Gel was then wet-transferred for 1.5 h at 100 V onto an Immun-Blot PVDF membrane (Bio-Rad, Cat. 162-0177) and blocked with 5% nonfat milk for 1 h. Membranes were incubated with anti-MRJP1 polyclonal antibodies (Epigentek, Cat. A53730) diluted 1:1000 in PBS-T over night, then with peroxidase-conjugated anti-rabbit IgG polyclonal antibodies diluted 1:3000 in PBS-T, for 1.5 h. Finally, membranes were incubated with Super signalTM West Dura Extended Duration Substrate (Thermo, Cat. 34075), according to manufacturer's instructions. Membranes were analyzed in the BioRad Molecular Image ChemiDoc XRS+, using the software Image Lab 5.2.1.

3 Results and discussion

MRJP1 is a protein with low hydrophobicity (Mandacaru *et al.*, 2017). More hydrophilic samples such as MRJP1 require strongly hydrophobic ligands to accomplish sufficient binding for correct separation. To enhance MRJP1 binding to a hydrophobic surface, the use of C18 columns was selected in RPC. Usually, C18 ligands are employed to separate synthetic peptides, shorter peptides and oligonucleotides. Using RPC, from a sample of clarified supernatant from MRJP1-producing *P. pastoris* containing 360 mg of total protein, it was possible to recover only 5.7 mg (1.58%) of MRJP1. SDS-PAGE showed more proteins in addition to MRJP1 (57 kDa) in the sample, as can be observed in Figure 1, where bands with molecular weight of 70, 47 and 30 kDa are revealed by Coomassie staining, probably as a result of contaminants attributed to culture media during fermentation. In this case, MRJP1 was desalted and concentrated in one single step. However, some contaminants were still observed after RPC. Importantly, complex enzymes and overall proteins, such as MRJP1, are likely to lose activity as a result of the loss of tertiary structure in the presence of the non-polar solvents employed in the RPC separation.

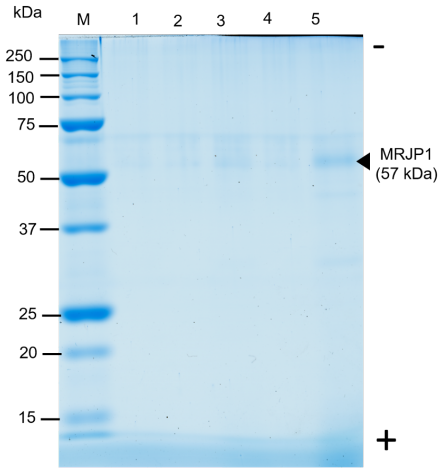

Fig. 1. SDS-PAGE with Coomassie blue staining of proteins from MRJP1-producing P. pastoris clarified supernatant, post reverse-phase chromatography. M: MW marker; lane 1 and 2: 1 mg; lane 3 and 4: 5 mg; lane 5: 10 mg.

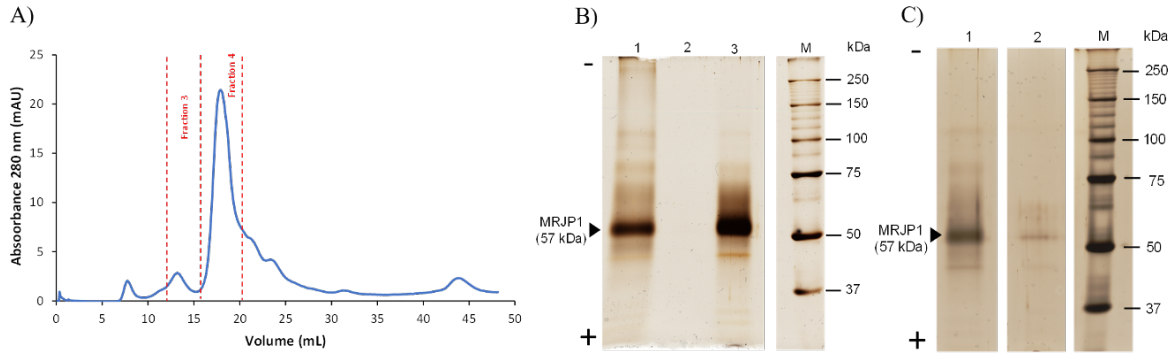

Fig. 2. Chromatographic profile from Size Exclusion Chromatography (SEC) using clarified supernatant from MRJP1-producing *P. pastoris*. (A) 20 mg clarified supernatant from MRJP1-producing *P. pastoris* were injected into the column (Superose 12 HR 10/300 GL). (B) SDS-PAGE stained with silver stain. Lane 1: 3.5 mg clarified supernatant from MRJP1-producing *P. pastoris*; lane 2: empty; lane 3: 3.5 mg fraction 3; M: MW marker. (C) SDS-PAGE stained with silver stain. Lane 1: 1 mg clarified supernatant from MRJP1-producing *P. pastoris*; lane 2: 270 ng fraction 4; M: MW marker.

However, the effect is reversible when the molecule is returned to the initial conditions, favoring the native structure (GE Healthcare, 2006). As a result of the high levels of MRJP1 lost in RPC, additional chromatographic techniques based on various relevant physicochemical properties of the protein were implemented. SEC has been employed to separate molecules with different molecular weight corresponding to contaminants from clarified supernatant of MRJP1-producing *P. pastoris*. The molecular weight reported for MRJP1 is 57 kDa approximately, which falls within the fractionation range of the matrix column used in this work (Superose 12: 1000 to 300000 Da) through steric interactions with the stationary phase (Kamakura[b] *et al*., 2001; Ibarra-Herrera *et al*., 2014; Carta *et al*., 2010). Superose 12, as showed in Figure 2. A, has a better resolution compared to HiPrep 16/60 Sephacryl S-200 column employed by Kamakura *et al*. to recover MRJP1 produced by *E. coli* and HiPrep 26/60 Sephacryl S-300 column used by Ibarra-Herrera *et al*. to separate MRJP1 produced by *Pichia pastoris*, whose resins are a cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide with an average particle size of 50 μm (Kamakura[c] *et al*., 2006; Ibarra-Herrera *et al*., 2014). However, HiLoad Superdex 200 column tested by Kamakura *et al*. to purify MRJP1 from royal jelly has a higher resolution as a result of the resin composition which combines cross-linked dextran with cross-linked agarose with a mean bead size of 13 μm, increasing physical and chemical stability to separate molecules with selectivity (Kamakura[a] *et al*., 2001; GE Healthcare Life Sciences, 2020).

The matrix of the SEC column employed in this work consists in highly cross-linked agarose, with an average particle size of 11 μm. Starting from a sample of clarified supernatant from MRJP1-producing *P. pastoris* containing 20 mg of total protein, a total of 9 fractions were separated. The first 2 fractions correspond to higher molecular weight proteins. Next fractions, 3 and 4, were smaller in order of decreasing molecular weight, with a 57 kDa band in both cases, and a low molecular weight band is revealed in both fractions (Figure 2. B, C). This adjacent band with lower molecular weight was also observed by other authors (Kamakura[c] *et al*., 2006; Ibarra-Herrera *et al*., 2014; Shen *et al*., 2010). Kamakura *et al*. explained that this could be the result of a degradation product of MRJP1 (Kamakura[c] *et al*., 2006). Evidence suggests that deglycosylation with endoglycosidases changes the molecular weight of MRJP1 from 57 kDa to approximately 47-48 kDa, corresponding to the adjacent band with lower molecular weight observed (Mandacaru *et al*., 2017). A 47 kDa molecular weight in MRJP1 is expected after deglycosylation according to the analysis of the amino acid sequence of the protein (Mandacaru *et al*., 2017). MRJP1 is a chain of 432 amino acids, and after a signal peptidase activity, there is a cleavage of a 19-residue segment with N-terminal resulting in a modified chain of 47 kDa. After protein maturation, MRJP1 undergoes post-translational modifications leading to a 57 kDa molecular weight (Mandacaru *et al*., 2017). As shown

in Figure 2. B and C, the fractions with a molecular weight similar to 57 kDa (MRJP1) were fractions 3 (concentration of 37.66 μg and recovery yield of 0.188%) and 4 (concentration of 2.31 μg and recovery yield of 0.011%). As mentioned before, fractions 1 and 2 were too big to be considered, while fractions 5 to 9 were too small and not related with the expected molecular weight of 57 kDa. In this regard, the matrix and particle size have an important role in the resolution of the peaks separated by SEC. The resin used in this work tends to separate with a convenient fractionation range and optimal resolution. Also, our flow rate was lower than the reported by other authors (Kamakura[c] *et al*., 2006; Kamakura[a] *et al*., 2001). This was achieved to increase the resolution between peaks, because the setting of a lower flow rate for the run allows molecules to diffuse in the column resin and improves resolution (GE Healthcare Life Sciences, 2020).

As a result of the contaminant bands observed in SEC, the next step was to employ IEX. The resin used for IEX was Q Sepharose Fast Flow. This matrix guarantees the reduction of non-specific binding, especially important to reduce levels of impurities derived from the host cell expression system in the elution and final recovery of MRJP1. The best separation performance was achieved using a buffer with a pH above the isoelectric point of the protein, in this case Tris-HCl 20 mM pH 8.2, and a linear gradient of ammonium sulfate (0-0.4M) to elute the protein, as showed in Figure 3. A. Starting from a sample of clarified supernatant from MRJP1-producing *P. pastoris* containing 20 mg of total protein, the chromatographic profile showed different fractions, from which only fraction number 1 (concentration of 33 μg and recovery yield of 0.165%) demonstrated a band corresponding to 57 kDa molecular weight (Figure 3. B). This peak eluted at around 39.50 mL at a conductivity of 17.72 mS/cm. Bands of a different molecular weight were also observed in the electrophoretic analysis of this peak, including the adjacent band with lower molecular weight observed in SEC, evidencing a correct protein characterization but not enough for a complete purification. In the IEX assay developed by Kamakura *et al*. a DEAE Toyopearl 650 M resin was employed, which is a weak anion exchanger, to bind the protein to the resin and sodium chloride (0-1.0M) as elution linear gradient (Kamakura[a] *et al*., 2001). Strong ion exchangers, like the one used in this work, perform a complete ionization in a wide pH range, contrary to weak ion exchangers, which just partially ionizes in a narrow pH range.

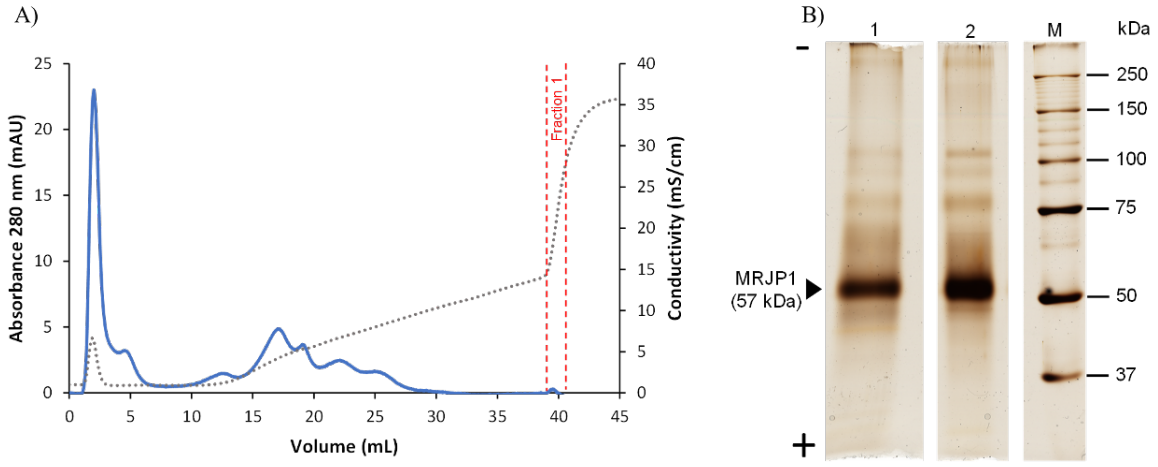

Fig. 3. Chromatographic profile from Ion-Exchange Chromatography (IEX), using clarified supernatant from MRJP1-producing *P. pastoris*. (A) 20 mg clarified supernatant from MRJP1-producing *P. pastoris* were injected using Q Sepharose Fast Flow column. Buffer A: Buffer Tris-HCl 20 mM pH 8.2. Buffer B: Buffer A + ammonium sulfate (0-0.4 M), resulting in a single fraction of the expected size of 57 kDa (fraction 1). Absorbance 280 nm (mAU) is represented by the blue line, while conductivity (mS/cm) is represented by the dotted gray line. (B) SDS-PAGE and silver staining of fraction 1. Lane 1: 3.5 mg clarified supernatant from MRJP1-producing *P. pastoris*; lane 2: 3.5 mg of IEX-eluted fraction 1; lane M: MW marker.

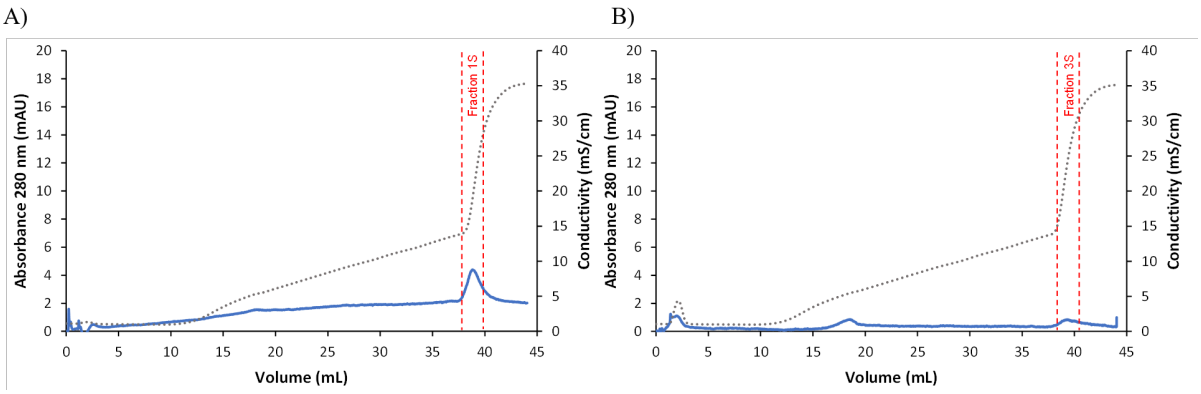

Fig. 4. Chromatographic profiles from Ion-Exchange Chromatography (IEX) elutions in multistep approach. Fractions 3 and 4 previously collected from SEC were submitted to IEX separately, yields new fractions with the expected 57 kDa size. Absorbance 280 nm (mAU) is represented by the blue line, while conductivity (mS/cm) is represented by the dotted gray line. (A) Fraction 3 (obtained from SEC), resulted in one single fraction (identified as "1S"). (B) Fraction 4 (obtained from SEC), resulted in three new fractions (only fraction "3S" is represented in the chromatogram because it was the only one showing a 57 kDa band in SDS-PAGE in IEX from Fig 2). Separation was made on Q Sepharose Fast Flow column pre-equilibrated with Buffer Tris-HCl 20 mM pH 8.2. Elution was performed using a linear gradient by increasing ammonium sulfate (0-0.4 M).

For this reason, strong ion exchangers are preferable on initial standardization of a purification procedure (Acikara, 2013). On the other side, by increasing the salt gradient using ammonium sulfate instead of sodium chloride, the ionic strength also increases modulating separation, and the proteins with attraction to the stationary phase dissociates depending on the affinity (GE Healthcare, 2004).

To achieve a high purity of recombinant proteins, it may be necessary to use multiple sequential chromatographic steps. Therefore, a multistep chromatographic approach was investigated, in which SEC was coupled to IEX. However, using several chromatography steps represents a higher cost in downstream processing. This approach may be justified if the cost of the product is high enough. SEC was selected as a first step because previous results showed a higher purity of the protein, in comparison to IEX. IEX was coupled as a polishing procedure. In these experiments, two fractions (fractions 3 and 4) were obtained after injecting a sample of clarified supernatant from MRJP1-producing *P. pastoris* containing 20 mg of total protein in SEC. Then, after performing IEX to each of the fractions obtained by SEC, chromatograms showed that fraction 3 resulted in one single fraction (named as fraction 1S, concentration of 3.62 μg and recovery yield of 0.018%), while fraction 4 resulted in three fractions (of which only fraction 3S is in position of 57 kDa according to fraction 1 from IEX performed separately). The fraction 1S eluted at around 38.88 mL at a conductivity of 19.65 mS/cm (Figure 4. A), while fraction 3S eluted at around 39.35 mL at a conductivity of 24.33 mS/cm (Figure 4. B). The elution conditions of proteins corresponding to fractions 1S and 3S obtained by SEC coupled to IEX are similar to the fraction 1 obtained by single step IEX (eluted at around 39.50 mL at a conductivity of 17.72 mS/cm), in which indeed a 57 kDa band was found in SDS-PAGE. However, it is still necessary to demonstrate the presence of a 57 kDa band by SDS-PAGE in both fractions obtained by SEC coupled to IEX.

In biopharmaceutical production, downstream processing represents the main expense comprising a 50-80% approximately of manufacturing costs. To simplify recovery and purification processes, it is suggested that an expression system capable of producing the protein of interest at high relative purity is selected (Maccani *et al*., 2014). In fact, it has been reported that *Pichia pastoris* is not capable of secreting many intrinsic proteins (Li *et al*., 2010). Therefore, the clarified supernatant from MRJP1-producing *P. pastoris* contains a low concentration of host cell proteins, compared with other expression systems, which in turn simplifies the purification process of recombinant proteins from the culture medium (Maccani *et al*., 2014; Li *et al*., 2010). On the contrary, Dong *et al*. established that the impurities in *Pichia*

pastoris culture supernatant are very complex, and may include proteins, polysaccharides, proteinases, fatty acids and coloring components (Dong *et al*., 2012). In their work, Martínez-Hernández *et al*. observed an increase in the protein content of *Pichia pastoris* culture supernatant, which is similar to the bands with different molecular weight observed in Figures 1; 2. B, C; 3. B; 5. C. They attribute this to the increase of their recombinant product, which was the main protein fraction observed in SDS-PAGE (Martínez-Hernández *et al*., 2020). López-Pérez *et al*. established that these bands are the result of several proteins released during *Pichia pastoris* lysis process (López-Pérez *et al*., 2017). It was found that the supernatant from wild type *Pichia pastoris* (X-33, Thermo Fisher Scientific, Cat. 18000) contains several proteins that were quantified with BCA as total protein and evaluated by SDS-PAGE. When chromatograms from X-33 strain supernatant were compared with chromatograms from MRJP1-producing *P. pastoris*, in both SEC and IEX assays, the peaks absent in X-33 supernatant were fractions 3 and 4 in the case of SEC (Figure 5. A), while in the case of fraction 1 from IEX the pattern observed suggests a coincidence of peaks between both *Pichia pastoris* strains (Figure 5. B). Regarding SDS-PAGE results, the same bands found in supernatant from X-33 strain can be observed in supernatant from MRJP1-producing *P. pastoris*, except for 57 and 47 kDa bands, corresponding to MRJP1 and deglycosylated MRJP1, respectively (Figure 5. C). This information suggests that fractions 3 and 4 in SEC, and fraction 1 in IEX correspond to MRJP1 protein, while the bands with different molecular weight observed both in SEC and IEX are the result of minimal culture media components secreted during *Pichia pastoris* growth.

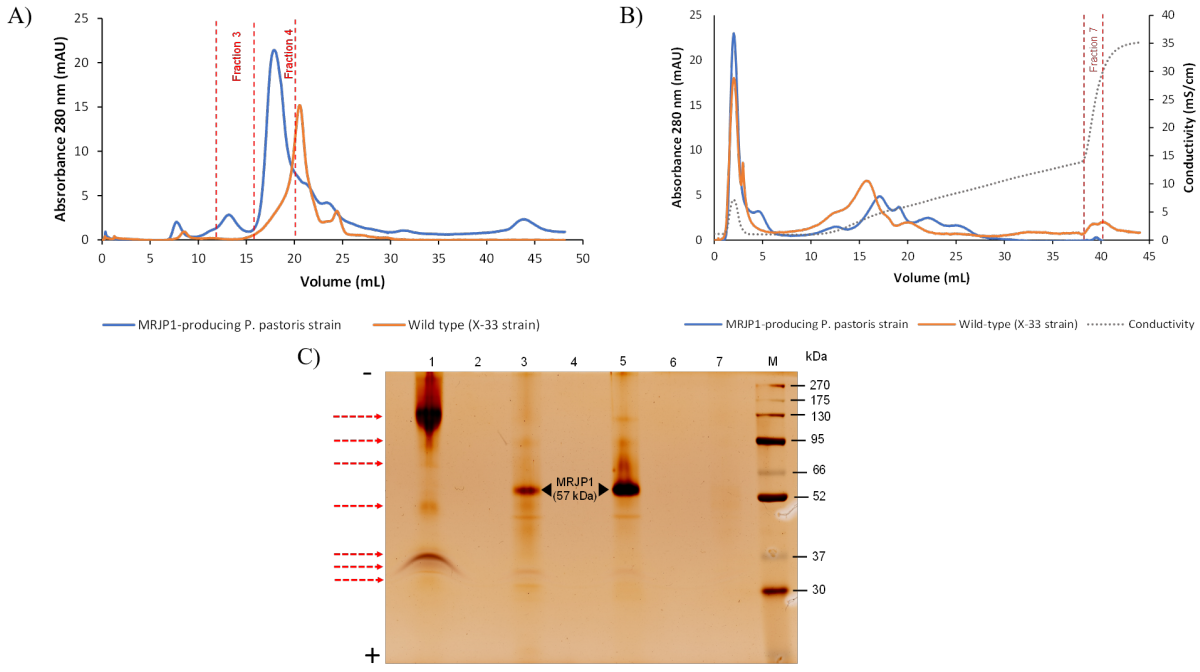

Fig. 5. Chromatographic profiles from SEC and IEX, comparing clarified supernatant from MRJP1-producing *P. pastoris* with supernatant from X-33 strain (wild-type). (A) Both supernatants were analyzed using Superose 12 HR 10/300 GL for SEC. (B) Both supernatants were injected on a Q Sepharose Fast Flow column for IEX. (C) SDS-PAGE stained with silver stain. Lane 1: 20 μg clarified supernatant from X-33 strain; lanes 2, 4, 6 and 7: empty; lanes 3 and 5: 20 μg clarified supernatant from MRJP1-producing *P. pastoris* from different batch; M: MW marker. The position of several bands in X-33 strain agrees with the position of the same bands in MRJP1-producing *P. pastoris* (except 57 and 47 kDa bands, for MRJP1 and deglycosylated MRJP1, respectively, which are present only in MRJP1-producing *P. pastoris*), as indicated by dotted lines.

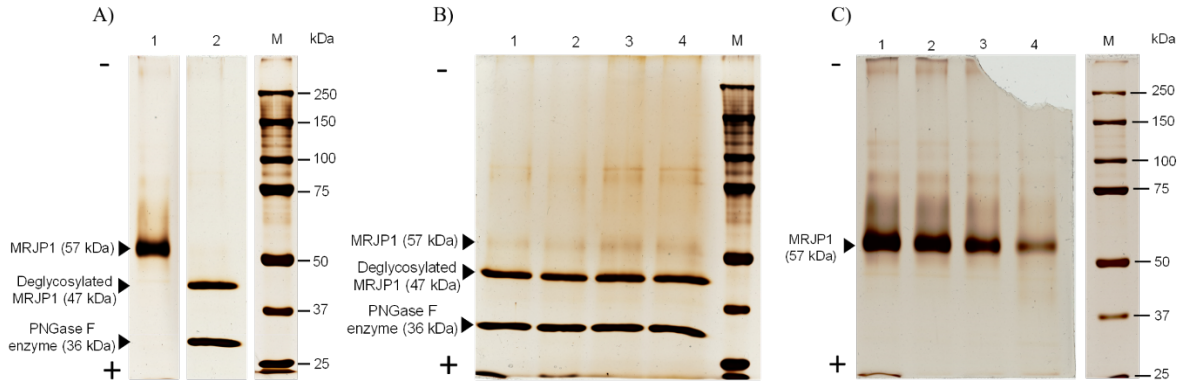

Fig. 6. Deglycosylation of MRJP1 using PNGase F. (A) Lane 1: A total of 20 mg clarified supernatant from MRJP1-producing *P. pastoris* denatured 10 min at 100°C; lane 2: enzymatic digestion for 60 min at 37°C; lane M: MW marker. (B) Enzymatic standardization of a total of 20 mg clarified supernatant from MRJP1-producing *P. pastoris* at 15, 30, 45 and 60 min at 37 °C; lane 1: enzymatic digestion for 60 min at 37°C; lane 2: enzymatic digestion for 45 min at 37°C; lane 3: enzymatic digestion for 30 min at 37°C; lane 4: enzymatic digestion for 15 min at 37°C; lane M: MW marker. (C) Analysis of degradation of a total of 40 mg clarified supernatant from MRJP1-producing *P. pastoris* at 37°C at 15, 30, 45 and 60 min; lane 1: 40 μg MRJP1 incubated for 15 min at 37°C; lane 2: 40 μg MRJP1 incubated for 30 min at 37°C; lane 3: 40 μg MRJP1 incubated for 45 min at 37°C; lane 4: 40 μg MRJP1 incubated for 60 min at 37°C; M: MW marker.

Various methods have been employed to produce MRJP1 (Table 1) leading to different recovery conditions and contaminants. In the case of Kamakura *et al*., they estimated that the molecular weight of MRJP1 was 47 kDa due to lack of post-translational modifications in *E. coli*, however the GST-tag used lead to 73 kDa (Kamakura[c] *et al*., 2006).

The authors detected MRJP1 as the major protein, but they also found a band with lower molecular weight adjacent to MRJP1 in SDS-PAGE. Shen *et al*. also employed a tag: MRJP1 (constructed using Chinese honeybee cDNA) tagged with histidine was purified from *Pichia pastoris* supernatant using HisTrap FF crude affinity columns, avoiding extra chromatographic techniques for purification (Shen *et al*., 2010). In the case of MRJP1 purification implemented by Kamakura *et al*., IEX followed by SEC was performed (Kamakura[a] *et al*., 2001). However, the chromatogram for IEX does not agree with the SDS-PAGE results, in which they observed a single band despite of having two peaks when they analyze it sequentially with SEC. MRJP1 purification performed also by Kamakura *et al*. a year later was accomplished only using SEC (Kamakura[b] *et al*., 2001). In both studies, the protein was purified from royal jelly produced by *Apis mellifera*. In this work, both techniques, SEC and IEX, were evaluated separately and a band in the 57 kDa position was found when performing SDS-PAGE in both cases. Also, an adjacent band of 47 kDa was observed. After, when SEC coupled to IEX was performed, it was possible to observe the same fraction (~57 kDa protein) eluting at around 38.88 mL at a conductivity of 19.65 mS/cm without the fractions observed in the chromatograms of SEC and IEX performed separately, attributed to culture media components of *Pichia pastoris* during fermentation. Gamboa-Suasnavart *et al*. deduced that it is not possible to elucidate if the change in some parameters of fluids during growth in bioreactors are the result of culture media composition, biomass, or morphology (Gamboa-Suasnavart *et al*., 2019). In order to study the N-glycosylation pattern of MRJP1, *Pichia pastoris* supernatant containing MRJP1 was deglycosylated using PNGase F. MRJP1 is a glycoprotein, with glycans at the N144 and N177 positions (Mandacaru *et al*., 2017), including a Galβ1-3Gal NAc unit. Deglycosylation with endoglycosidases as PNGase F changes the molecular mass from 57 kDa to an approximate 47 kDa molecular weight, as it can be observed in SDS-PAGE gel (Figure 6. A). It has been previously reported that MRJP1 is degraded in a proportion correlated with time of storage in samples kept at 40 °C (degradation is in proportion to both storage temperature and storage period) (Kamakura[a] *et al*., 2001).

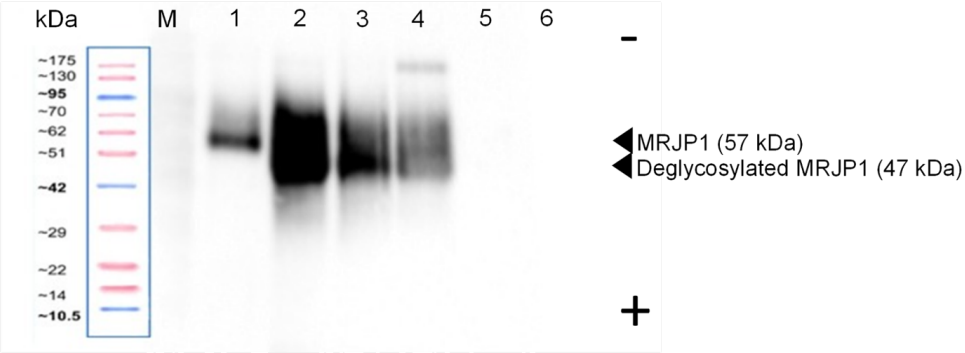

Fig. 7. Western blot analysis using anti-MRJP1 polyclonal antibody. M: MW marker; lane 1: 20 μg clarified supernatant from MRJP1-producing *P. pastoris*; lane 2: 20 μg fraction 3 from SEC; lane 3: 20 μg fraction 4 from SEC; lane 4: 20 μg fraction 1 from IEX; lane 5: 1 μg BSA; lane 6: 0.5 μg BSA.

In this work, MRJP1 was deglycosylated gradually over time when sample was incubated at 37 °C during 15, 30, 45 and 60 min while the deglycosylation reaction was taking place (Figure 6. B). Also, degradation is evident over time at 37 °C when MRJP1 is incubated without PNGase F (Figure 6. C). This suggests that the band with molecular weight of 47 kDa occurred due to a deglycosylation phenomenon probably during the chromatographic procedure or when stored after recovery. Another plausible explanation for the observed deglycosylation process is the metabolism of *Pichia pastoris*. Despite a previous report showing that the secretion of endogenous proteins by *Pichia pastoris* is scarce (Tachioka *et al*., 2016), it is usual to have proteases in the medium, causing protein degradation (Rosano & Ceccarelli, 2014). Niu *et al*. stated that glycosylated proteins are more resistant to proteases due to reduction of proteases accessibility to cleavage sites (Niu *et al*., 2015).

Validation with Western blot analysis evidenced the presence of MRJP1 (57 kDa position) in fractions 3 and 4 from SEC and fraction 1 from IEX, and in the supernatant from MRJP1-producing *P. pastoris* without chromatographic recovery. Also, the adjacent band with molecular weight of 47 kDa was identified in the same fractions, except for the direct clarified supernatant from MRJP1-producing *P. pastoris*, where deglycosylation appeared less prominent. All additional bands at different molecular weight than 57 kDa (except for 47 kDa band) were distinct proteins probably related to *Pichia pastoris* or fermentation culture media (Dong *et al*., 2012) (Figure 7). This agrees with the available literature, as Kamakura *et al*. evaluated the 57 kDa protein and the adjacent band with lower molecular weight (47 kDa), performing Western blot assay using an anti-MRJP1 polyclonal antibody, finding that the antibody recognized both proteins, suggesting that 47 kDa protein may be identical to MRJP1 (57 kDa) with a distinct glycosylation pattern (Kamakura[c] *et al*., 2006).

The results reported here demonstrate that MRJP1 from *Apis mellifera* produced in *Pichia pastoris* can be recovered by RPC, IEX and SEC from culture fermentation supernatant.

Conclusions

This work evidences that MRJP1 can be identified post RPC, SEC and IEX chromatography using supernatant from MRJP1-producing *P. pastoris*. RPC showed a band in 57 kDa, however the presence of contaminant bands in SDS-PAGE led to the exploration of other techniques. SEC and IEX resulted in a strong 57 kDa band and an adjacent band with molecular weight of 47 kDa, indicating a possible deglycosylation effect of these chromatography techniques on the protein. Furthermore, both techniques showed contaminants from *Pichia pastoris* attributed to culture media growth, evidencing a correct protein characterization but not enough for an actual purification. SEC coupled to IEX showed a single peak in the chromatogram at the elution position that showed a band of 57 kDa. The approach developed here serves as a procedure to identify and recover MRJP1 in fermentation culture of

Pichia pastoris for recombinant production of MRJP1. MRJP1 production and its recovery are affected by the regulatory metabolism of *Pichia pastoris*. Therefore, it may be relevant to improve the protein production processes by exploring alternatives such as optimization of the current expression system, change of culture condition variables, and development of culture media formulations.

Acknowledgements

Alejandro Robles-Zamora, Daniela Enriquez-Ochoa and Milena Ureña-Herrera thank the Consejo Nacional de Ciencia y Tecnología (CONACyT) for the respectively awarded scholarships 442482, 712327 and 1019579 as part of their graduate studies. We also thank M.Sc. Héctor Castañeda-Aponte for producing the MRJP1 recombinant protein.

References

Acikara, Ö.B. (2013). *Ion-Exchange Chromatography and Its Applications, Column Chromatography*, Dean F. Martin and Barbara B. Martin, *IntechOpen*, https://doi.10.5772/55744. Available from: https://www.intechopen.com/books/column-chromatography/ion-exchange-chromatography-and-its-applications. Accessed: February 12, 2020.

Buttstedt, A., Moritz, R.F.A. and Erler, S. (2014). Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. *Biological Reviews 49*, 255-269. https://doi.org/10.1111/brv.12052

Carta, G. and Jungbauer, A. (2010). *Protein Chromatography: Process Development and Scale-Up*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Chen, D., Xin, X.X., Qian, H.C., Yu, Z.Y. and Shen, L.R. (2016). Evaluation of the major royal jelly proteins as an alternative to fetal bovine serum in culturing human cell lines. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 17*, 476-483. https://doi.org/10.1631/jzus.B1500295

Cruz, G.C.N., Garcia, L., Silva, A.J., Barbosa, J.A.R.G., Ricart, C.A.O., Freitas, S.M. and Sousa, M.V. (2011). Calcium effect and pH-dependence on self-association and structural stability of the *Apis mellifera* major royal jelly protein 1. *Apidologie 42*, 252-269. https://doi.org/10.1007/s13592-011-0025-9

Detienne, G., De Haes, W., Ernst, U.R., Schoofs, L. and Temmerman, L. (2014). Royalactin extends lifespan of *Caenorhabditis elegans* through epidermal growth factor signaling. *Experimental Gerontology 60*, 129-135. https://doi.org/10.1016/j.exger.2014.09.021

Dong, Y., Zhang, F., Wang, Z., Du, L., Hao, A., Jiang, B., Tian, M., Li, Q., Jia, Q., Wang, S. and Xiu, Z. (2012). Extraction and purification of recombinant human serum albumin from *Pichia pastoris* broths using aqueous two-phase system combined with hydrophobic interaction chromatography. *Journal of Chromatography A 1245*, 143-149. https://doi.org/10.1016/j.chroma.2012.05.041

Furusawa, T., Arai, Y., Kato, K. and Ichihara, K. (2016). Quantitative analysis of apisin, a major protein unique to Royal Jelly. *Evidence-Based Complementary and Alternative Medicine 2016*, 1-9. https://doi.org/10.1155/2016/5040528

Gamboa-Suasnavart, R.A., Marín-Palacio, L.D., López-Griego, L., Córdova-Aguilar, M.S., Valdez-Cruz, N.A. and Trujillo-Roldán, M.A. (2019). Volumetric power imput as a reliable parameter for scale-up from shake flask to stirred-tank bioreactor: production of a recombinant glycoprotein by *Streptomyces lividans. Revista Mexicana de Ingeniería Química 18*, 1085-1099.

GE Healthcare Life Sciences (2020). Fundamentals of size exclusion chromatography. https://www.gelifesciences.com/en/us/solutions/protein-research/knowledge-center/protein-purification-methods/size-exclusion-chromatography. Accessed: February 13, 2020.

GE Healthcare. (2004). *Strategies for Protein Purification*. Handbook 28-9833-31, Uppsala, Sweden.

GE Healthcare. (2006). *Hydrophobic Interaction and Reversed Phase Chromatography. Principles*

and Methods. Handbook 11-0012-69, Uppsala, Sweden.

González-González, M., Mayolo-Deloisa, K. and Rito-Palomares, M. (2012). PEGylation, detection and chromatographic purification of site-specific PEGylated CD133-Biotin antibody in route to stem cell separation. *Journal of Chromatography B 893-894*, 182-186. https://doi.org/10.1016/j.jchromb.2012.03.002

Ibarra-Herrera, C.C., Torres-Acosta, M.A., Mendoza-Ochoa, G.I., Aguilar-Yañez, J.M. and Rito-Palomares, M. (2014). Recovery of major royal jelly protein 1 expressed in *Pichia pastoris* in aqueous two-phase systems. *Journal of Chemical Technology & Biotechnology 89*, 941-947. https://doi.org/10.1002/jctb.4342

Júdová, J., Šutka, R., Klaudiny, J., Lišková, D., Ow, D.W. and
uSimúth, J. (2004). Transformation of tobacco plants with cDNA encoding honeybee royal jelly MRJP1. *Biologia Plantarum 48*, 185-191. https://doi.org/10.1023/B:BIOP.0000033443.60872.f1

Kamakura[a], M., Fukuda, T., Fukushima, M. and Yonekura, M. (2001). Storage-dependent degradation of 57-kDa protein in royal jelly: a possible marker for freshness. *Bioscience, Biotechnology, and Biochemistry 65*, 277-284. https://doi.org/10.1271/bbb.65.277

Kamakura[b], M., Suenobu, N. and Fukushima, M. (2001). Fifty-seven-kDa protein in royal jelly enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. *Biochemical and Biophysical Research Communications 874*, 865-874. https://doi.org/10.1006/bbrc.2001.4656

Kamakura[c], M. and Sakaki, T. (2006). A hypopharyngeal gland protein of the worker honeybee *Apis mellifera* L. enhances proliferation of primary-cultured rat hepatocytes and suppresses apoptosis in the absence of serum. *Protein Expression and Purification 45*, 307-314. https://doi.org/10.1016/j.pep.2005.08.004

Kamakura[d], M. (2011). Royalactin induces queen differentiation in honeybees. *Nature 473*, 478-483. https://doi.org/10.1038/nature10093

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*, 680-5. https://doi.org/10.1038/227680a0

Li, S.W., Sun, Y., Donelan, W., Yu, H., Scian, J., Tang, D. and Yang, L.J. (2010). Expression, purification, and characterization of recombinant human pancreatic duodenal homeobox-1 protein in *Pichia pastoris*. *Protein Expression and Purification 72*, 157-161. https://doi.org/10.1016/j.pep.2010.04.004

López-Pérez, M. and Viniegra-González, G. (2017). Differential toxicity caused by methanol on the growth of *Pichia pastoris* cultured in solid-state and in submerged fermentation. *Revista Mexicana de Ingeniería Química 16*, 735-743.

Maccani, A., Landes, N., Stadlmayr, G., Maresch, D., Leitner, C., Maurer, M., Gasser, B., Ernst, W., Kunert, R. and Mattanovich, D. (2014). *Pichia pastoris* secretes recombinant proteins less efficiently than Chinese hamster ovary cells but allows higher space-time yields for less complex proteins. *Biotechnology Journal 9*, 526-37. https://doi.org/10.1002/biot.201300305

Mandacaru, S.C., do Vale, L.H.F., Vahidi, S., Xiao, Y., Skinner, O.S., Ricart, C.A.O., Kelleher, N.L., Valle de Sousa, M. and Konermann, L. (2017). Characterizing the structure and oligomerization of Major Royal Jelly protein 1 (MRJP1) by mass spectrometry and complementary biophysical tools. *Biochemistry 56*, 1645-1655. https://doi.org/10.1021/acs.biochem.7b00020

Martínez-Hernández, S.L., Marín-Muñoz, M.A., Ventura-Juárez, J. and Jáuregui-Rincón, J. (2020). Fed-batch cultivation and operational conditions for the production of a recombinant anti-amoebic vaccine in *Pichia pastoris* system. *Revista Mexicana de Ingeniería Química 19*, 691-705.

Neuhoff, V., Arold, N., Taube, D. and Ehrhardt, W. (1988). Improved staining of proteins

in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis 9*, 255-62. https://doi.org/10.1002/elps.1150090603

Niu, C., Luo, H., Shi, P., Huang, H., Wang, Y., Yang, P., and Yao, B. (2015). N-Glycosylation improves the pepsin resistance of histidine acid phosphatase phytases by enhancing their stability at acidic phs and reducing pepsin's accessibility to its cleavage sites. *Applied and environmental microbiology 82*, 1004-1014. https://doi.org/10.1128/AEM.02881-15

Rosano, G. L., and Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in microbiology 5*, 172. https://doi.org/10.3389/fmicb.2014.00172

Shen, L., Wang, Y., Zhai,, L. and Zhou, W. (2015). Determination of royal jelly freshness by ELISA with a highly specific anti-apalbumin 1, major royal jelly protein 1 antibody. *Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 16*, 155-166. https://doi.org/10.1631/jzus.B1400223

Shen, L., Zhang, W., Jin, F., Zhang, L., Chen, Z., Liu, L., Parnell, L.D., Lai, C-Q and LI, D. (2010). Expression of recombinant AccMRJP1 protein from royal jelly of chinese honeybee in *Pichia pastoris* and its proliferation activity in an insect cell line. *Journal of Agricultural and Food Chemistry 2010*, 9190-9197. https://doi.org/10.1021/jf1007133

Shorter, J.R., Geisz, M., Özsoy, E. and Magwire, M.M. (2015). The effects of royal jelly on fitness traits and gene expression in *Drosophila melanogaster*. *PLoS ONE 10*, 1-10. https://doi.org/10.1371/journal.pone.0134612

Tachioka, M., Sugimoto, N., Nakamura, A., Sunagawa, N., Ishida, T., Uchiyama, T., Igarashi, K., and Samejima, M. (2016). Development of simple random mutagenesis protocol for the protein expression system in *Pichia pastoris*. *Biotechnology for biofuels 9*, 199. https://doi.org/10.1186/s13068-016-0613-z

Wan, D.C., Morgan, S.L., Spencley, A.L., Mariano, N., Chang, E.Y., Shankar, G., Luo, Y., Li, T.H., Huh, D., Huynh, S.K., Garcia, J.M., Dovey, C.M., Lumb, J., Liu, L., Brown, K.V., Bermudez, A., Luong, R., Zeng, H., Mascetti, V.L., Pitteri, S.J., Wang, J., Tu, H., Quarta, M., Sebastiano, V., Nusse, R., Rando, T.A., Carette, J.E., Bazan, J.F. and Wang, K.C. (2018). Honey bee Royalactin unlocks conserved pluripotency pathway in mammals. *Nature Communications 9*. https://doi.org/10.1038/s41467-018-06256-4

Tian, W., Li, M., Guo, H., Peng, W., Xue, X., Hu, Y., Liu, Y., Zhao, Y., Fang, X., Wang, K., Li, X., Tong, Y., Conlon, M., Wu, W., Ren, F. and Chen, Z. (2018). Architecture of the native major royal jelly protein 1 oligomer. *Nature Communications 9*, 3373. https://doi.10.1038/s41467-018-05619-1

Xin, X.X., Chen, Y., Chen, D., Xiao, F., Parnell, L.D., Zhao, J., Liu, L., Ordovas, J.M., Lai, C.Q. and Shen, L.R. (2016). Supplementation with major royal-jelly proteins increases lifespan, feeding, and fecundity in *Drosophila*. *Journal of Agricultural and Food Chemistry 64*, 5803-12. https://doi.10.1021/acs.jafc.6b00514