Magnetic *Parkia pendula* seed gum as matrix for Concanavalin A lectin immobilization and its application in affinity purification

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ABSTRACT

The present work aimed to magnetize *Parkia pendula* seeds gum and use it as a matrix for Concanavalin A covalent immobilization. This composite was applied in affinity purification of glycoconjugates. *Parkia pendula* seeds were hydrated and the gum provenient from the supernatant was precipitated and washed with ethanol and dried. The gum was magnetized in co-precipitation using solutions of Fe^{+2} and Fe^{+3}. Matrix activation was accomplished with $NaIO_4$. Magnetized *Parkia pendula* seeds gum with covalently immobilized Concanavalin A was used as an affinity matrix for the recognition of bovine serum fetuin glycoprotein. Fetuin elution was carried out with a solution of glucose (300mM) and evaluated through SDS-PAGE. The efficiency of lectin immobilization and fetuin purification were 63% and 14%, respectively. These results indicate that the composite produced is a promising magnetic polysaccharide matrix for lectins immobilization. Thus, such system can be applied for affinity purification allowing an easy recovery by magnetic field.

Key words: immobilization, magnetization, *Parkia pendula* seed gum, Concanavalin A.

INTRODUCTION

Many biomolecules have been isolated using separation techniques based on the interaction of biospecific molecules. Among these molecules of particular interest there is a class named lectins that are sugar-specific and cell-agglutinating proteins of non-immune origin (Sharon 2007). This class of proteins functions as recognition molecules in cell-molecule and cell-cell interactions in a variety of biological systems (Ghazarian et al. 2010). Because of this, lectin-carbohydrate interactions are extensively studied, from basic to applied natural and clinical sciences. Such inter- and multi-disciplinary approaches corroborate the importance of developing new methodologies for the study of lectin-saccharide interactions and their potential in biotechnology (Gemeiner et al. 2009).

Thus, immobilized lectins have found applications in the purification and analysis of polysaccharides (Fraguas et al. 2003), glycoconjugates

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(Yang et al. 2012) and cells (Ribeiro et al. 2012). For instance, the lectin extracted from *Canavalia ensiformis*, named Concanavalin A (Con A) has been extensively used in the isolation, fractioning and structural characterization of glycoproteins (Bucur et al. 2004, Uygun et al. 2012) and other important glycoconjugates bearing glucose and/or mannose residues (Fraguas et al. 2004).

Among the matrices used in protein immo-bilization, those which contain carbohydrates have become the focus of intense interest in biotechnology (Fraguas et al. 2003, 2004, Mislovicová et al. 2004, Angeli et al. 2009). Furthermore, to increase their performance, matrices have been magnetized to decrease the processing time of samples, the utilization of chemicals as well as to facilitate separation and process automation (Pan et al. 2005, Angeli et al. 2009).

In our laboratory, several kinds of supports have been magnetized with Fe_3O_4 magnetite particles prepared by co-precipitating Fe^{2+} and Fe^{3+}. An example is a composite of the levan carbohydrate from *Zimomonas mobilis* that was easily ferromagnetized by Angeli et al. (2009) and subsequently recovered by a magnetic field. Glycoproteins recognized by lectins attached to the composite were recovered by washing the composite with a high ionic strength solution or with the lectin specific monosaccharide solution. Finally, these glycoproteins were collected from supernatant and the composite was reused. The washing procedures were facilitated by the magnetic field and all the process can be automated (Angeli et al. 2009).

Natural plant gums are another source of carbohydrates that can be exploited. *Parkia pendula* (Fabaceae) is a plant with pan tropical distribution found in the Atlantic Forest in the Northeast of Brazil and in the Brazilian Rainforest (Anderson and Pinto 1985). When hydrated with water, its seeds produce gum. In this way, the aim of this work was to magnetize the *P. pendula* seeds gum (PpeG) and use it as a matrix for Con A immobilization for affinity chromatography of glycoconjugates.

MATERIALS AND METHODS

GUM PURIFICATION

PpeG purification was carried out according to Rodrigues et al. (1993). Briefly, seeds of *P. pendula* were hydrated with distilled water for 24 h at 25°C. The supernatant (75 ml) was diluted in 300 ml of distilled water, filtered through cheesecloth, and the gum was precipitated with 750 ml of ethanol for 24 h at 4°C. Afterwards, the precipitate was filtered through cheesecloth, washed twice with 150 ml of ethanol at 4°C, and dried at 36°C overnight.

GUM MAGNETIZATION

Dried PpeG (500 mg) was added to 50 mL of 1% acetic acid solution and stirred for 4 h at 25°C. Solution (10 mL) containing 1.1M $FeCl_3.6H_2O$ and 0.6M $FeCl_2.4H_2O$ (1:1) was added to the mixture; the pH was adjusted to 11 with a 28% solution of NH_4OH and heated in water-bath for 30 min at 80°C. Finally, the magnetic particles were collected by centrifugation and washed with distilled water until the supernatant reached pH 7.0. PpeG magnetized ($_{mag}$PpeG) was then dried at 25°C, grounded and sieved (particle size ≤ 250 μm). From this time on the $_{mag}$PpeG particles were collected by a magnetic field (6,000 Oe). This procedure was performed according to Carneiro Leão et al. (1991), except for incubation time (30 min), temperature (80°C) and final pH of the mixture (11). Particle sizes produced by this method were previously determined by Maciel et al. (2012), and in our work the same sieve was used in the final step.

MATRIX ACTIVATION AND CON A IMMOBILIZATION

One milliliter of sodium meta-periodate solution (100 mg/mL in 0.01M sodium phosphate buffer pH 7.4 - from now on called buffer) was added to 50 mg of $_{mag}$PpeG. Activation reaction was developed in the dark, under stirring for 4 h at 25°C. The matrix was washed 10 times with buffer and incubated with 1 mL of a solution of Con A (400 μg/mL in

buffer) under stirring for 20 h at 4°C. Afterwards, $_{mag}$PpeG-Con A was washed with buffer (5 times) and incubated with 1 mL of a solution of 0.03M sodium borohydride for 2 h at 4°C. The matrix was washed 10 times with the buffer and kept at 4°C until use. Efficiency of Con A immobilization onto $_{mag}$PpeG was determined by the difference between the protein content of Con A offered and the one in the supernatant after immobilization process using Lowry et al. (1951). All experiments were carried out in quintuplicates.

AFFINITY BINDING WITH $_{mag}$PPEG-CON A

One milliliter of a solution of fetuin (400 μg/mL in buffer) was incubated with $_{mag}$PpeG-Con A (50mg) for 1 h under stirring at 25°C. Afterwards, the magnetic particles were collected and washed twice with the buffer. Protein determination was established for the supernatants according to Lowry et al. (1951). The washed magnetic particles were incubated with 0.3M glucose (1 mL) for 1 h at 25°C in order to disrupt the Con A-fetuin complex. Efficiency of fetuin recognition by Con A immobilized on the $_{mag}$PpeG was determined by the difference between the fetuin offered and the one in the supernatant. All experiments were carried out in quintuplicates.

SDS-PAGE

Samples were dialyzed, lyophilized and resuspended in distilled water prior to the gel running. SDS-PAGE (12.5%) was carried out according to Laemmli (1970) and visualized with silver staining according to Keshoven and Dernick (1985). Protein standards used were bovine serum albumin (66 kDa), fetuin (64 kDa) egg-albumin (46 kDa), gliceraldehyde-3-P enzyme (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and Con A (32 kDa), obtained from Sigma-Aldrich (St. Louis, MO, USA).

PHYSICAL-CHEMICAL PARTIAL CHARACTERIZATION

PpeG and $_{mag}$PpeG were analyzed for elemental content and infrared spectrometry in the Analytical Laboratory of the Chemistry Department at the Federal University of Pernambuco (UFPE), Brazil. Fourier transform infrared (FTIR) spectrum from the KBr pellet method in the range of 4000–400 cm^{-1} was recorded in a BRUKER instrument model IFS 66. Elemental analysis of samples was determined by a LECO Elemental Analyzer CHNS O932 and Unicam 929 AA spectrophotometer (USA).

RESULTS AND DISCUSSION

PHYSICO-CHEMICAL PARTIAL CHARACTERIZATION

The element's composition of the free and magnetized PpeG (Table I) showed the presence of carbon and hydrogen only. Nitrogen and sulfur detections were not observed in our preparation of PpeG. Furthermore, no amine and amide characteristic bands were visualized in the gum infrared analysis (Fig. 1). Anderson and Pinto (1985) evaluated the gum exudates from *Parkia bicolor* and *P. biglobosa,* and the gum extracted from the seed pods of *P. pendula.* According to their results all three gum polysaccharides were of high weight-average molecular weight and intrinsic viscosities. The major features revealed that there are close similarity of the exudate gums from *P. bicolor* and *P. biglobosa,* and the extent of their differences from *P. pendula* seed-pod gum. These differences were observed both in ratios of found carbohydrates and in nitrogen values. The presence of 0.92 (*P. bicolor*) and 0.95 % (*P. biglobosa*) N content indicates the presence of 6% of protein. However, the N content of *P. pendula* was only 0.35% which indicates a lower N content and revels that the gum polysaccharide from the seed pods of *P. pendula* must be regarded as a typical plant gum (Anderson and Pinto 1995). The lack of N content in PpeG confirms the existence of differences among gums extracted from different species of this plant, besides variation between the same species collected in distinct places. Elemental analysis has also demonstrated a decrease of C and H content after the magnetization procedure. The magnetite incorporation in the PpeG explained the reduction observed.

TABLE I
Element composition of free and magnetized *P. pendula* seed gum.

Sample	%			
	Nitrogen	Carbon	Hydrogen	Sulfur
PpeG[a] (0.500 mg/mL)	0	35.83	5.29	0
mag PpeG[a] (0.500 mg/mL)	0	9.24	1.98	0

[a]PpeG and mag PpeG stands for free and magnetized *P. pendula* seed gum, respectively.
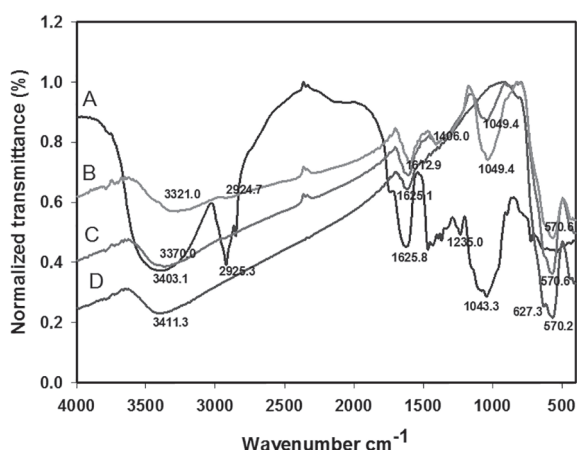

Fig. 1 - Infrared spectra of *P. pendula* gum (A), magnetized gum using 0.500 mg/mL (B) and 0.100 mg/mL (C) of the gum and magnetite (D).

Figure 1 presents the infrared spectra of the free PpeG (A), magnetic PpeG at different concentrations 0.500 mg/mL (B) and 0.100 mg/mL (C) and magnetite (D). Infrared spectroscopy showed that O–H groups are present in the PpeG polysaccharide, magnetite and magnetic PpeG near wavenumber of 3400 cm^{-1} with higher intensity for PpeG (3411.3). These O–H groups correspond to those present in organic compounds. The magnetic PpeG presented absorption bands in 2924.7 cm^{-1} due to stretching vibration of C–H bond band in 1049.4 cm^{-1} and due to stretching vibration of C–OH bond. These bonds are also present in the PpeG polysaccharide with bands in 2925.3 cm^{-1} (stretching vibration of C–H bond), band in 1043.3 cm^{-1} (stretching vibration of C–OH) indicative of the presence of polysaccharide in the magnetic

particles. Previous studies (Waldron 1955, Pan et al. 2005) reported that the characteristic absorption bands of the Fe–O bond of bulk magnetite were around 570 cm^{-1}. However, Ma et al. (2003) observed that these two bands shift of about 600 and 440 cm^{-1} respectively, and the band near 600 cm^{-1} is split into two peaks of 631.4 and 582.9 cm^{-1}. Here, a band near 600 cm^{-1} is also shown split in two peaks of 627.3 and 570.2 cm^{-1} for magnetite. Magnetic PpeG particles presented a similar band at 570.6 cm^{-1} with a lower peak at 627.3. This difference was also observed by Maciel et al. (2012) for magnetic levan. According to the authors, a difference in these bands can indicate that interactions between polysaccharide and magnetite had inter-molecular origins. The results confirm the success of the magnetization process of PpeG.

PpeG infrared spectrum is typical for polysaccharide (Fig. 1 A) as those reported for cellulose (Corti et al. 2004) and the cashew gum (Silva et al. 2004). The C=O axial deformation at the wavenumber 1736 cm^{-1} typical of glucuronic acid is present for the PpeG. Corti et al. (2004) observed that 1656, 1631 and 1558 cm^{-1} infrared bands corresponded to axial deformation of carbonyl in amide (in our case related to carbonyl of peptide bonds), N-H angular deformation of amine and N-H angular deformation of amide, respectively. Similar bands were observed by Pan et al. (2005). The absence of these bands in the spectrum of PpeG and the elemental analysis confirmed that no proteins were found in this structure.

IMMOBILIZATION AND ACTIVITY OF CON A

The partial oxidation of the gum by NaIO$_4$ aimed to randomly introduce aldehydes groups in the vicinal hydroxyls of the carbohydrates (Martinez-Barragan and Angel 2001, Hong et al. 2004). These aldehydes groups then react to amine group from amino acids chains such as lysine, sulfhydryl group from cysteine and imidazole group from histidine (Fraguas et al. 2004).

The immobilization of Con A on the PpeG retained about 62% of the offered protein (Table II). Kobayashi and Ichishima (1991) reported 40% of retention immobilizing bovine serum albumin on cellulose. Cavalcante et al. (2006) immobilized trypsin onto a membrane of a cellulosic exopolysaccharide produced by *Zoogloea sp.* in sugarcane molasses. Carbonyl groups were introduced into the matrix by sodium meta-periodate oxidation and the enzyme was immobilized either directly or through bovine serum albumin (BSA) as a spacer. The trypsin-membrane and trypsin–BSA-membrane retained 37.2% and 9.16%, respectively. It is worthwhile to notice that not all carbohydrate moieties are oxidized by the $NaIO_4$ (Silva et al. 2004). Furthermore, the PpeG structure is not completely identified yet and its linear and branched chains are not established.

TABLE II

Immobilization efficiency of Con A on

$_{mag}$PpeG and efficiency of fetuin recognition

by the immobilized Con A on $_{mag}$PpeG.

	Con A immobilization	Fetuin recognition
Offered (μg/mL)	400	400
Supernatant[a] (μg/mL)	147 ± 9.6	343.8 ± 11.23
Retained[a] (μg)	253 ± 9.7	56.2 ± 11.23
Efficiency[a] (%)	62.6 ± 2.30	13.8 ± 2.95

[a] All experiments were carried out in quintuplicates and the results expressed as mean ± Standard deviation.

The fetuin, composed of one polypeptide chain (Spiro 1963) containing 3 oligosaccharides N-linked (Spiro and Bhoyroo 1974) and 3 O-linked (Green et al. 1988), was used as a model. Table II summarizes the results of this purification and shows that about 14% of fetuin was complexed to about 63% of Con A. This relationship accounts for about 01 mole of fetuin per 08 mole of Con A considering that the molecular weights are respectively 64 kDa (Johnson and Heath 1986a) and 32 kDa (Fontaniella et al. 2004). The ratio of 1:1 mole: mole of fetuin and Con A was probably

not accomplished due to steric hindrance caused by the immobilization procedure.

Besides that, even with the higher quantity of oligosaccharides present in fetuin and its recognition by soluble Con A, Johnson and Heath (1986b), Green et al. (1988) observed that among the 23 N-types of oligosaccharides that could be found in fetuin (di- or tri-branched), only the di-branched ones would be recognized, which corresponds to 17%.

Figure 2 shows three bands (64, 58 e 55 kDa) for the fetuin (Lane B) in the SDS-PAGE. Nevertheless, only two bands (64 e 58 kDa) appear for the purified fetuin (Lane C) by affinity binding to the $_{mag}$PpeG-Con A. The Con A band (Lane D) is not present in the Lane C, which demonstrates that this lectin was covalently linked to the $_{mag}$PpeG, and was not detached during the washings.
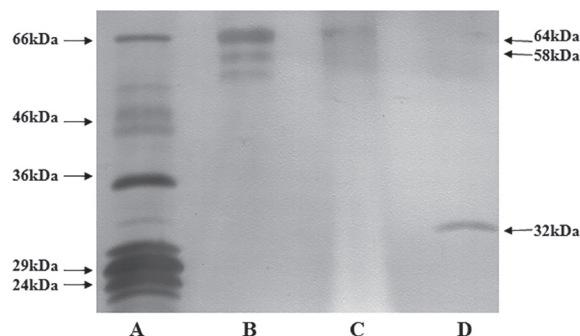

Fig. 2 - SDS-PAGE of eluted fetuin from the immobilized $_{mag}$PpeG-Con A. Standard proteins **(A)**, Fetuin **(B)**, Eluted Fetuin **(C)**, Con A **(D)**.

Johnson and Heath (1986a) observed that native fetuin, pre-fetuin and glycosilated fetuin (found in the rough endoplasmatic reticulum) presented molecular weight of 64, 49 and 58 kDa, respectively, in SDS-PAGE. Therefore, the observed 58kDa band in Figure 2 would be a fetuin with two N-type glycosylation.

According to Green et al. (1988) the recognition of Con A for di-branched fetuin justifies the absence of the 55kDa band in the lane C of the SDS-PAGE (Fig. 2). Authors observed that L-PHA

(*Leukoagglutinating Phytohemagglutinin*) and RCA-I (*Ricinus Communis* Agglutinin) interact with carbohydrates depending on the type of the bound between the saccharide residue and its position in the oligosaccharide chain. L-PHA has strong interaction if the neuraminic acid, present in fetuin, has one terminal α-2,3 bound, linked to the mannose's branching α-1-6. Otherwise, if the bound is α-2,6 on the same branching (α-1-6) the recognition of the sugar by L-PHA cannot be made. Furthermore, no significant interference, in recognition, was observed if the sugar was located on another branching (Green et al. 1988). So, the spatial changes caused by certain saccharides bearing N-type glycosylation in fetuin, would, possibly, impose a more or less stable interaction between Con A and fetuin.

Results from our groups have already demonstrated the use of other magnetized poly-saccharides such as levan as affinity matrix for direct lectin purification (Angeli et al. 2009) or as matrix for trypsin immobilization (Maciel et al. 2012) and cellulosic exopolysaccharide produced by *Zoogloea* sp. as a film support for trypsin immobilization (Cavalcante et al. 2006). Here, we demonstrated that Ppeg was efficiently magnetized and used as matrix for Con A immobilization. magPpeG-Con A was used as an affinity chromatography matrix for purification of fetuin under a magnetic field indicating that it is a promising matrix for biotechnology application.

ACKNOWLEDGMENTS

RESUMO

O presente trabalho objetivou magnetizar a goma de sementes de *Parkia pendula* e demonstrar seu uso como matriz para imobilização covalente da lectina Concanavalina A. O composto foi aplicado em puri-ficação de glicoconjugados por afinidade. Sementes de *Parkia pendula* foram hidratadas e a goma do sobrenadante foi precipitada, lavada com etanol e seca. A goma foi magnetizada pelo método de coprecipitação usando soluções de Fe^{+2} e Fe^{+3}. A ativação da matriz foi realizada com NaIO$_4$. Goma das sementes de *Parkia pendula* magnetizada com Concanavalina A imobilizada covalentemente foi usada como matriz de afinidade para o reconhecimento da glicoproteína fetuína sérica bovina. Eluição de fetuína foi realizada com uma solução de glicose (300mM) e avaliada por meio de SDS-PAGE. A eficiência de imobilização da lectina e purificação de fetuína foram 63% e 14%, respectivamente. Esses resultados indicam que o composto produzido é uma matriz polissacarídica magnética promissora para imobilização de lectinas. Esse sistema pode ser aplicado para purificação por afinidade que permite fácil recuperação por campo magnético.

Palavras-chave: imobilização, magnetização, goma da semente de *Parkia pendula*, Concanavalina A.

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