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Isolation from *Gluconacetobacter diazotrophicus* cell walls of specific receptors for sugarcane glycoproteins, which act as recognition factors☆

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Abstract

Glycoproteins from sugarcane stalks have been isolated from plants field-grown by size-exclusion chromatography. Some of these glycoproteins, previously labelled with fluorescein isothiocyanate, are able to bind to the cell wall of the sugarcane endophyte, N_2-fixing *Gluconacetobacter diazotrophicus*, and largely removed after washing the bacterial cells with sucrose. This implies that sugarcane glycoproteins use β-(1 → 2)-fructofuranosyl fructose domains in their glycosidic moiety to bind to specific receptors in the bacterial cell walls. These receptors have been isolated by affinity chromatography on a sugarcane glycoprotein-agarose matrix, desorbed with sucrose and characterized by sodium dodecyl sulfate polyacrylamide gel electrophresisand capillary electrophoresis (CE).

Keywords: Capillary electrophoresis; Gluconacetobarter; Glycoproteins; Receptors; Sugarcane

1. Introduction

Sucrose is accumulated in stalks of sugarcane, reaching the maximum concentration at a phase of the vegetative growth called "industrial ripeness" [1]. Other carbohydrates, different from sucrose, can also be produced and accumulated, reaching generally their maximum during post-ripening phase. These soluble carbohydrates are starch-like polymers [2,3], β-1,4-glucans [4], arabino-galactans [5] and heterofructans [6].

Two different heterofructans regarding their molecular mass and composed by fructose and galactitol, have been found in sugarcane juices [7]. One of them, high molecular weight carbohydrates, has a molecular mass higher than 10 kDa whereas the second one, mid-molecular weight carbohydrates, has a molecular mass varying from 0.7 to 10 kDa [8]. Later, the natural fluorescence of both polymers was studied and revealed that they are glycoproteins. They can clearly be recognized by the degree of tryptophan exposure to its chemical microenvironment [9].

These glycoproteins are produced from structural polymers of cell wall of parenchymatous cells [10]. Structural sugarcane polymers are partially hydrolysed by a glycosidase to liberate mainly high-molecular-mass glycoproteins (HMMGSs) to the cytosol, where mid-molecular-mass glycoproteins (MMMGSs) are produced later [11]. Both HMMGSs and MMMGSs, which can be recovered in sugarcane juice, also occur as response to the cut of stalks since a linear relationship between the number of cuts and the amounts of these glycoproteins produced has been found [12]. These heterofructans also occur as a consequence of upright and post-collection impairments [8].

On the other hand, a primary response of sugarcane plants to infections seems to be the production of these two classes of glycoproteins [13]. Smut is a major disease of sugarcane caused by *Ustilago scitaminea*. Fontaniella et al. [13] studied the production of glycoproteins after infection with

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smut teliospores of three cultivars of sugarcane defined by their resistance against smut. Germination decreases about 50% following 5 h of teliospore contact with HMMGSs and MMMGSs [13]. This may be related to the ability of glycoproteins to bind teliospore cell wall and to produce cytoagglutination [12].

An invading agent generally enhances the production of released exopolysaccharides [14]. However, recognition of a compatible endophyte usually involves selective glycoproteins that specifically bind to a bacterial cell wall ligand [15]. Fluorescein-labelled sugarcane glycoproteins are able to bind to *Gluconacetobacter diazotrophicus* (an atmospheric nitrogen-fixing bacterium that behaves as a natural endophyte of sugarcane). Legaz et al. [12] report conclusive evidence about the ability of some sugarcane glycoproteins to bind to the cell wall of *G. diazotrophicus*, strain AP5, as the first step of the biological discrimination of a compatible, symbiotic endophyte, therefore resembling a mechanism of specific tolerance such as those found in the immune system of higher eukaryotics. Moreover, *G. diazotrophicus* is able to produce, in axenic culture in liquid medium, a lysozyme-like bacteriocin which inhibited the growth of *Xanthomonas albilineans* [16] (a sugarcane pathogen which causes leaf scald disease).

In this paper, the occurrence of specific receptors for sugarcane glycoproteins in the cell wall of *G. diazotrophicus* has been studied by polyacrylamide gel electrophoresis, capillary electrophoresis (CE) and affinity chromatography.

2. Experimental

2.1. Bacterial strains and growth conditions

G. diazotrophicus, strain 166 (CM-INICA), isolated from sugar cane plants, was maintained in a N-poor solid medium [17], which contained 10% sucrose, pH 5.5. Cultures were maintained for 5 days at 30 °C and the formation of acid in parallel to the culture growth was tested by adding bromothymol blue to the medium [18,19]. This indicator is green at pH 5.5 and changes to yellow at pH 5.0. The size of the inoculum and the growth rate were measured by nephelometry following the absorbance changes at 710 nm.

2.2. Plant material and preparation of glycoproteins from sugarcane juices

Stalks from 22-month-old plants of *Saccharum officinarum*, var. Jaronu 60-5, field-grown, were mechanically crushed immediately after having been cut and the crude juice was centrifuged at 5000 × *g* for 15 min at 4 °C. The pellet was discarded and the supernatant was filtered through filter paper. This centrifuged juice was then filtered through a Sephadex G-10 column (15 cm × 2.5 cm) embedded in 10 mM sodium phosphate buffer at pH 6.8. Elution was also carried out with the same buffer. Fractions (1.0 mL) 1–17 were discarded. Fractions 18–35 were collected and considered as a mixture of soluble polysaccharides and glycoproteins, MMMGS and HMMGSS. Fractions from 60, mainly composed by sucrose, were also discarded. After this, a mixture of fractions 18–35 was filtered through a Sephadex G-50 column (30 cm × 2.5 cm) pre-equilibrated as described above. Fractions 40–60 contained HMMGSS whereas MMMGS eluted in fractions 66–100 [6]. Eluted fractions were monitored for carbohydrates according to Dubois et al. [20] and for protein according to Lowry et al. [21].

2.3. Fluorescence labelling of glycoproteins

Aliquots of 3.0 mL of both HMMGSS and MMMGS were mixed with 3.0 mL 0.3 mM fluorescein isothiocyanate for 24 h at room temperature with vigorous shaking. After this, mixtures were dialyzed against 5 L of distilled water at 4 °C in the dark until free fluorophore was completely removed Fl-HMMGSS and Fl-MMMGS were collected and used for the adsorption assay of glycoproteins to *G. diazotrophicus* cells.

2.4. Glycoproteins adsorption to G. diazotrophicus cells and desorption assay

G. diazotrophicus (40 mg dry weight of inoculum) was cultured on 500 mL of sterile Potatoe-P liquid medium. The culture was maintained at 30 °C for 11 days and then centrifuged at 12,000 × *g* for 15 min at 4 °C. Pellet was resuspended in 10 mL of 10 mM phosphate buffer, pH 6.14. Aliquots of 5.0 mL of this cell suspension were mixed with 1 mL of Fl-MMMGS or Fl-HMMGSS. Mixtures were maintained at 30 °C for 1 h with vigorous shaking and after this, bacteria were collected by centrifugation at 12,000 × *g* for 15 min at 4 °C. The intensity of the fluorescence emission from both supernatants was measured at 512 nm wavelength, using an excitation light of 468 nm wavelength.

To investigate the nature of the carbohydrate moiety related to the binding of HMMGSS and MMMGS to *G. diazotrophicus*, a competitive desorption assay was performed with 100 mM sucrose from Sigma Chemical Company (Saint Louis, MO, USA). After 2 h of shaking, bacteria were removed again by centrifugation and fluorescence of the supernatant was measured in the conditions specified above.

2.5. Extraction of cell wall proteins

G. diazotrophicus (280 mg dry weight) was sown in 500 mL of sterile Potatoe-P liquid medium. The culture was maintained at 30 °C until exponential phase of bacterial growth (7 days). Then, the culture was centrifuged at 12000 × *g* for 15 min at 2 °C. The pellet was resuspended in 20 mL distilled water. After this, mixture was newly centrifuged at 14,000 × *g* for 15 min at 2 °C. Pellet was resuspended in 40 mL of acetone and was newly centrifuged in the same conditions. The pellet was dried in a vacuum and the dry residue was resuspended in 10 mL of 0.05% (w/v)

Triton X-100. This mixture was maintained at 4 °C for 2 h. After this, the mixture was dialyzed against 5 L of distilled water at 4 °C in the dark until Triton X-100 was completely removed. Later, the dialysate was centrifuged at 14,000 × *g* for 15 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 10 mL of 2% (w/v) sodium chloride to extract cell wall proteins. To impede enzymatic degradation of proteins, 2.0 mL of protease inhibitor cocktail from Sigma Chemical Company (Saint Louis, MO, USA) were added to the mixture. This mixture was maintained at 4 °C for 24 h and later, was centrifuged at 12,000 × *g* for 15 min at 2 °C. Supernatant was dialyzed for 3 days in the conditions specified above. Dialysate was collected and 3.0 mL of protease inhibitor cocktail was added. This mixture was used to purify cell wall receptors for sugarcane glycoproteins.

2.6. Purification of cell wall receptors of G. diazotrophicus

To isolate cell wall receptors of MMMGS and HMMGS affinity chromatography in 8% cyanogen-bromide agarose (Sigma Chemical Co.) was used. Two beads of 8% cyanogen-bromide agarose (5.0 cm × 1.0 cm I.D.) were prepared and 3.0 mL of MMMGS were added to one bead, while 3.0 mL of HMMGS was loaded onto the second bead. Columns were closed for 2 h at room temperature. During this time, glycoproteins were joined to activated agarose. After this, beads were washed with 10 mL of distilled water [22]. Then, 3.0 mL of cell wall proteins of *G. diazotrophicus* were added and retained in contact with the matrix for 2 h at room temperature. After this, beads were washed with 100 mL of distilled water to remove non-retained bacterial glycoproteins. Fractions of 3.0 mL were assayed for their protein content by the Warburg and Christian method [23]. Later, 100 mL of 100 mM sucrose was added to each bead to desorbe retained cell wall receptors [12]. Eluates of 3 mL were assayed for their protein content by the Warburg and Christian method [23].

2.7. SDS–PAGE

Samples were prepared and resolved by SDS–PAGE according to the standard protocols. Non-adhered glycoproteins from cell walls of *G. diazotrophicus* to HMMGS and MMMGS and total cell walls proteins from this bacterium were concentrated with a Spectra/Gel Absorbent (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and resuspended in Laemmli buffer containing 5 μM 2-mercaptoethanol [24]. Then samples were clarified of debris by centrifugation in a labtop centrifuge at 20,000 × *g* for 15 min at 4 °C, boiled for 5 min at 100 °C, loaded on a 15% polyacrilamide gel and subjected to SDS–PAGE employing 50 mM Tris/0.1 M glycine/0.1% SDS as running buffer. When samples reached the end of the gel slab, electrophoresis was stopped, and the gel was stained for 30 min with Coomassie Brilliant Blue reagent (Sigma Chemical Co., St. Louis, MO, USA). Destaining was performed by immersion of the gel in 20% methanol/10% acetic acid overnight. The gel was then vacuum-dried and scanned. Prestained broad molecular weight markers were purchased from BioRad Laboratories (Hercules, CA, USA). Densitometry of gels was achieved by using the program L Process V1 from Fuji Photo Film, Inc. (Tokyo, Japan).

2.8. Capillary electrophoresis (CE)

Desorbed glycoproteins with 100 mM sucrose from affinity beads were used for CE analysis. Zone electrohoresis was performed using a Spectraphoresis 500 system from Spectra-Physics (Fremont, CA, USA). Microbore fused-silica tubing coated with polyimide (Scientific Glass Engineering. Milton Keynes. UK) of 75 μm I.D. and 190 μm outer diameter (O.D.) with a total length of 70 cm and a separation length of 63 cm were used. The capillary was enclosed in a cassette for easy handling. On-line detection was performed with a variable-wavelength UV–vis detector of 6 nm band width (Spectra-Physics, Fremont, USA). Detection of glycoproteins was monitored at 280 nm and electrophoregrams were recorded using a SP 4290 integrator (Spectra-Physics, Fremont, USA). Benzene was used as neutral marker.

New capillaries were conditioned with 1 M NaOH for 10 min at 60 °C, 0.1 M NaOH for 10 min at 60 °C and Milli-Q grade water for 10 min at 60 °C. Equilibration of the capillary was then performed by washing with 25 mM sodium borate buffer, pH 9.2 for 30 min at 25 °C and finally with the same buffer for 30 min at 25 °C under applied voltage of 17 kV. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence: 0.1 M NaOH for 10 min. Milli-Q grade water for 10 min and 25 mM sodium borate buffer, pH 9.2 for 15 min. The buffer used as electrolyte was 25 mM sodium borate buffer, pH 9.2 [25].

To know the isoelectric point of these glycoproteins, a straight line of log of isoelectric point versus electrophoretic mobility was constructed by using standard proteins. Isoelectric point markers were invertase, ferritin, bovine serum albumin, alcohol dehydrogenase, catalase, carbonic anhydrase, myoglobin and cytochrome c (all from Serva Feinbiochemica GmbH & Co., Heidelberg, Germany). Standard proteins as well as bacterial proteins were prepared in 10 mM sodium borate buffer, pH 9.2, containing 5.0 μL 100% benzene. Voltage was applied in such manner that ions migrated from the anode to the cathode.

3. Results and discussion

3.1. Labelling MMMGS and HMMGS

To study the capability of sugarcane glycoproteins to bind to cell walls of *G. diazotrophicus* sugarcane juice was filtered through Sephadex G-10 column. The elution profile of sugarcane polysaccharides is shown in Fig. 1A.

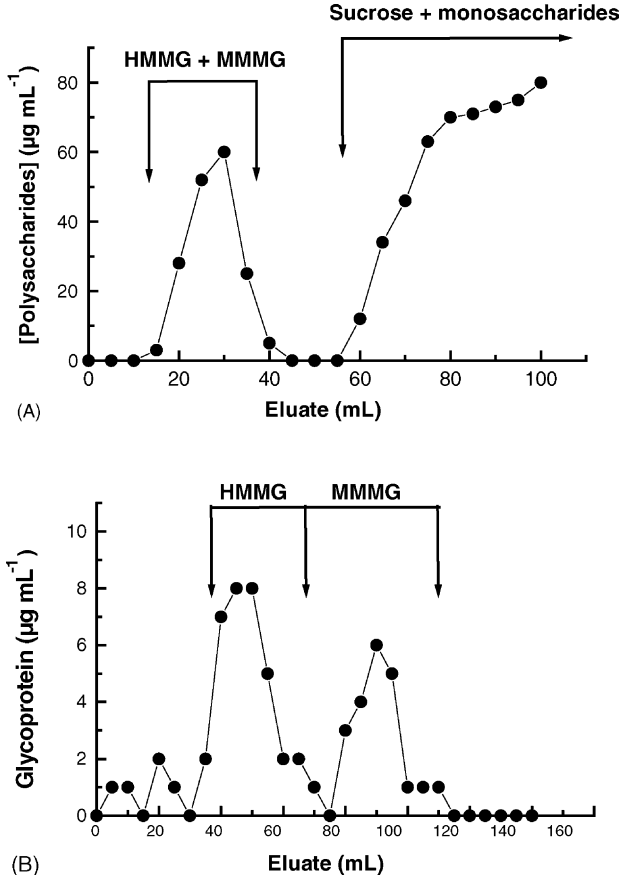

Fig. 1. (A) Elution profile of a mixture of high and mid-molecular mass glycoproteins HMMGS and MMMGS, respectively from a Sephadex G-10 column. (B) Separation of HMMGS from MMMGS by filtration of the mixture through a Sephadex G-50 column.

Two main peaks could be observed, the first peak being a mixture of both HMMGS and MMMGS (8 mL, 0.24 mg of carbohydrates), which were recovered in fractions 18–35, whereas the second one was sucrose, the main component of sugarcane juice. Fractions 18–35 were mixed and filtered through a Sephadex G-50 column. Elution profile of eluted glycoproteins (Fig. 1B) showed two well-defined peaks, the first containing a total of 12 mg of HMMGS (fractions 40–60) and a second containing 0.108 mg of MMMGS (fractions 66–100). Separation of both MMMGS and HMMGS in these experimental conditions was very similar to other production patterns previously described [26].

Aliquots of 3.0 mL of HMMGS and MMMGS were mixed with 3.0 mL of 10 mM fluorescein isothiocianate for 18 h at room temperature with shaking. After this, fluorescence emission at 512 nm wavelength was measured in this mixture, using a excitation light of 468 nm wavelength (Fig. 2).

3.2. Adsorption of labelled glycoproteins to G. diazotrophicus cells and desorption with sucrose

To ascertain the ability of juice glycoproteins to bind to *G. diazotrophicus*, binding assays were performed in which

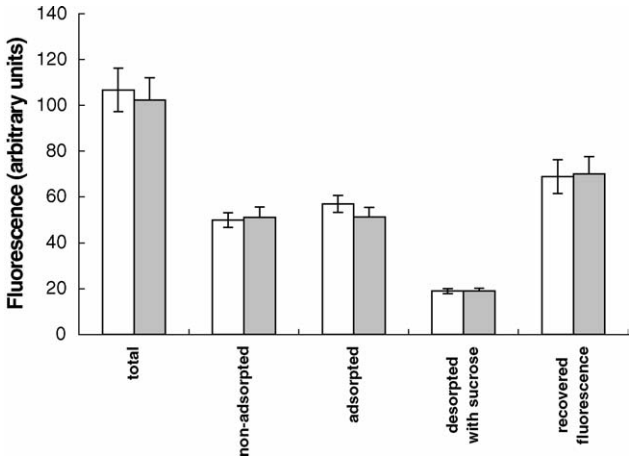

Fig. 2. Binding and desorption with 100 mM sucrose of fluorescein isothiocyanate-labelled HMMGS (grey rectangles) and MMMGS (white rectangles) to cells of *Gluconacetobacter diazotrophicus*. Total protein is referred to the amount of labelled HMMGS and MMMGS supplied to the identical amount of bacterial cells. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

cells were incubated with fluorescein-coupled HMMGS (Fl-HMMGS) or MMMGS (Fl-MMMGS) for 1 h at room temperature. Then, cells were removed by centrifugation and fluorescence emission of the supernatant was measured. The emission fluorescence values obtained indicated that about 49.9% of HMMGS and 46.7% of MMMGS were not retained by bacterial cells (Fig. 2). Then, 50.1% of total labelled-HMMM and 53.3% of MMMGS bind to bacterial cell walls.

Bound sugarcane glycoproteins were desorbed from bacterial cell walls by addition of 100 mM sucrose. Emission fluorescence was measured in the supernatans after removing bacteria. Sucrose desorbed about 34% of retained Fl-MMMGS and 36% of retained Fl-HMMGS by cell walls.

3.3. Cell wall receptors of G. diazotrophicus for sugarcane glycoproteins

To isolate receptors of *G. diazotrophicus* for sugarcane glycoproteins, affinity chromatography on bromide-activated agarose was carried out. Elution profiles of bacterial cell wall proteins that were not retained proteins by MMMGS- and HMMGS-agarose beads, eluted with distilled water, are shown in Fig. 3. Fractions 1 (3.0 mL) eluted from both beads were concentrated and used for SDS–PAGE analysis. After this, retained proteins from bacterial cell walls were eluted from both MMMGS- and HMMGS-agarose beads with 100 mM sucrose. Elution profiles MMMGS and HMMGS receptors are shown in Fig. 4. Three main peaks of receptors of HMMGS were observed, eluting at 3.0, 18–21 and 36–39 mL, respectively whereas five main peaks of MMMGS receptors were observed at 6.0, 12, 18, 30 and 42 mL of eluted volume from the agarose bead.

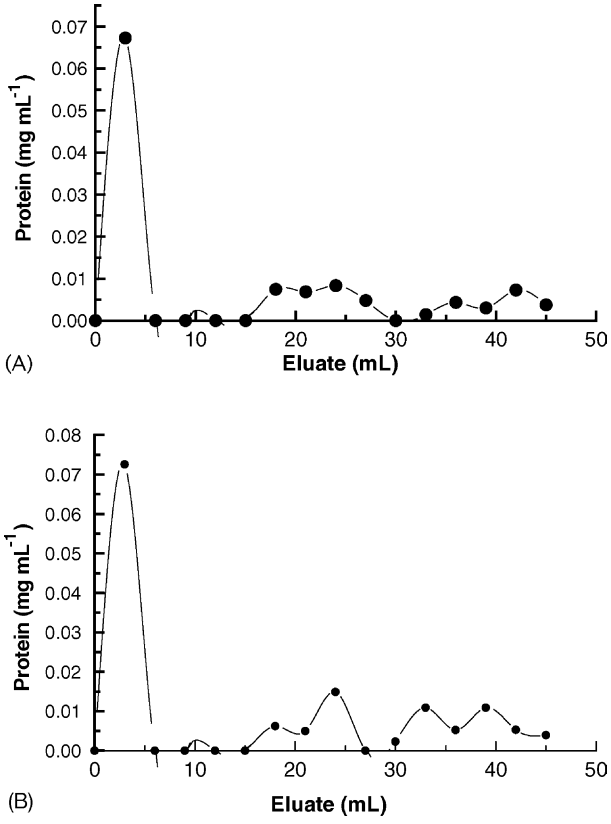

Fig. 3. Elution profiles of bacterial cell wall proteins loaded onto beads of: MMMGS (A) and HMMGS (B) and eluted with distilled water.

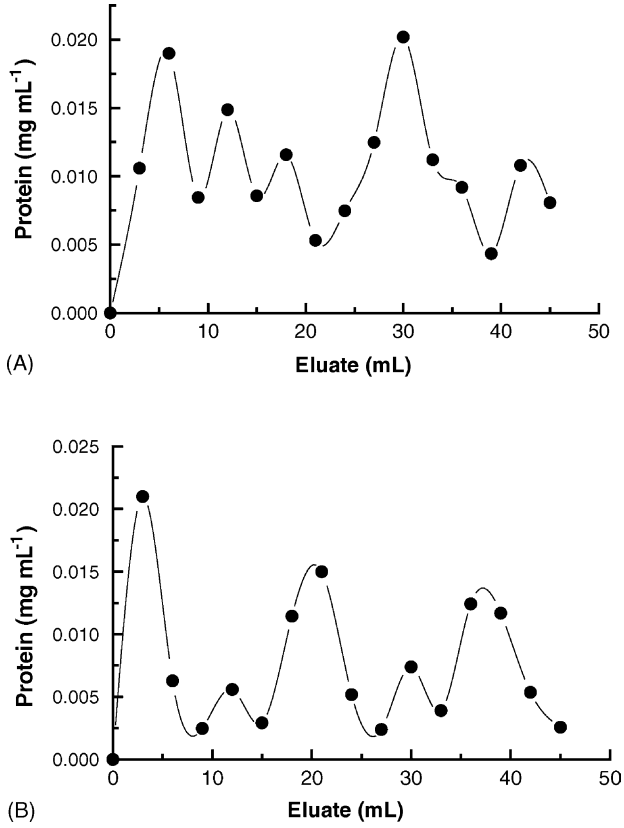

Fig. 4. Elution profiles of bacterial cell wall proteins retained in beads of: MMMGS (A) and HMMGS (B) and eluted with 100 mM sucrose.

3.4. Identification of bacterial cell wall receptors by SDS–PAGE

Separation by SDS–PAGE of proteins extracted from bacterial cell walls is shown in Fig. 5 (lane cwp). Lanes corresponding to eluates from agarose beads containing HMMGS and MMMGS (lanes HMMGs and MMMGs, respectively, in Fig. 5) found that the affinity of MMMGS for some proteins extracted from bacterial cell walls was higher than that found for HMMGS but, definitively, only two main bands completely disappears after filtration of bacterial proteins through both affinity beads, those identified as band 6 and band 12, with molecular masses of 35 and 25 kDa, respectively. This indicated that both protein were completely retained by MMMGS and partially by HMMGS, and this was confirmed by the corresponding densitometric traces (Fig. 5).

3.5. Analysis of bacterial cell wall receptors by capillary electrophoresis

Analysis by capillary electrophoresis of sugarcane glycoprotein receptors was carried out by using main peaks obtained from elution profiles of bacterial cell wall glycoproteins retained on MMMGS-agarose and HMMGS-agarose and eluted with sucrose. Highly repeated electrophoregrams corresponding to the first and second peaks of bacteria receptors eluted from MMMGS as well as the first and third peaks of the glycoproteins eluted from HMMGS were shown in Figs. 6 and 7, respectively. By assuming that each peak desorpted with sucrose from the affinity column could correspond to an unique glycoprotein, multiple peaks in the corresponding electropherogram indicated microheterogeneity within the same glycoprotein. Variation in the charge-mass ratio could explain this microheterogeneity [27]. The charge would be only related to the protein but the mass could vary when a polysaccharide moiety of variable length binds to the invariable polypeptide chain.

Bacterial receptors for both MMMGS and HMMGS seemed to be anionic proteins (Figs. 6 and 7), because absolute retention time values for each peak were always higher than that of the benzene used as neutral marker. Therefore, electrophoretic peaks would be interpreted as anionic proteins containing sugar chains of variable length.

Four electrophoretic main peaks with an area counts value higher than 9% of the total area (those with electrophoretic mobility values of 16.71, 18.46, 19.94 and 21.83 min (Fig. 6A) were observed in the electropherogram obtained from the first peak of glycoproteins eluted from MMMGS-agarose, whereas only two main peaks, those with electrophoretic mobilities of 20.64 and 22.36 min were obtained from the second peak (Fig. 6B). The same behaviour was observed for electrophoregrams obtained for other peaks

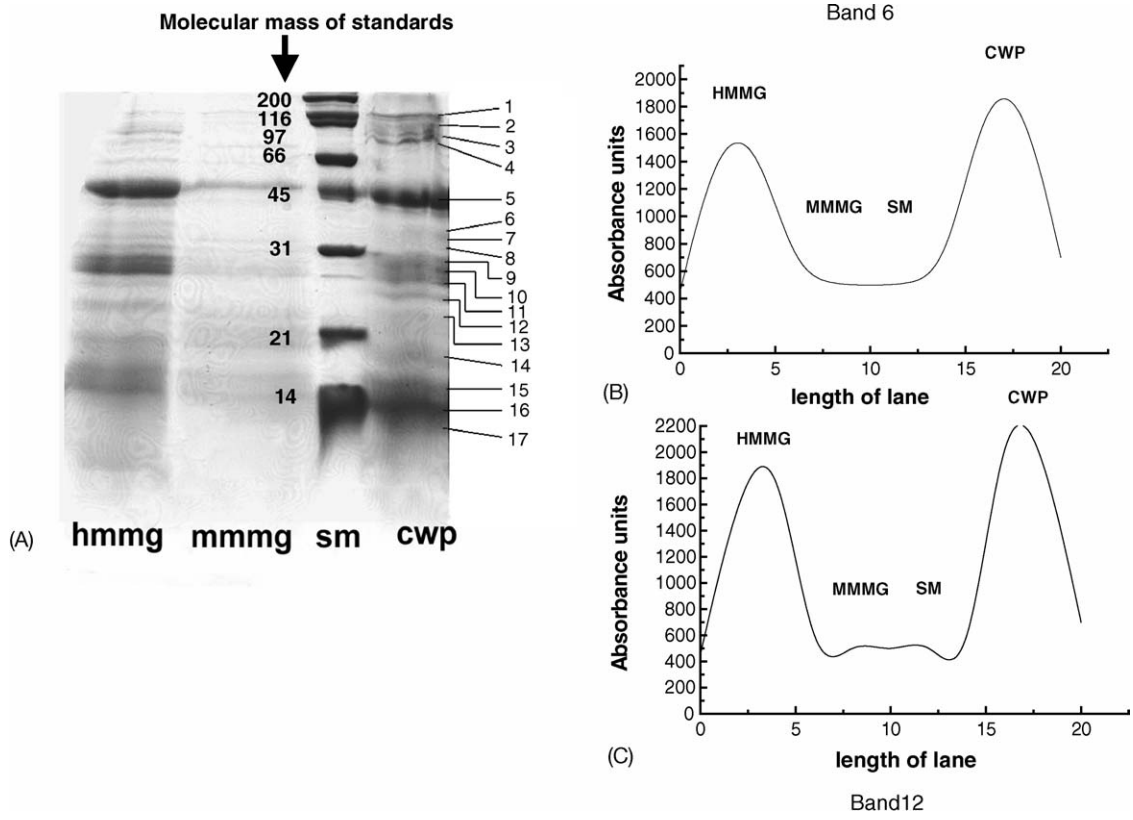

Fig. 5. (A) Separation by SDS–PAGE of bacterial proteins extracted from the cell wall before (lane cwp) and after affinity chromatography on beads of HMMGS-agarose (HMMGs) and MMMGS-agarose (MMMGs). Numbers at left of standard markers (sm) lane indicated their molecular mass in kDa. (B) Densitometric trace of the band 6 in the lane cwp. (C) Densitometric trace of the band 12 in the lane cwp.

eluted from MMMGS-agarose (data not shown). Calculated values of pI of the main peaks of MMMGS receptors are shown in Table 1.

Since five electrophoretic main peaks with an area value higher than 9%, those that migrated at 16.10, 17.99, 19.29, 19.73 and 27.00 min, were observed in the electrophoregram obtained from the first peak eluted with sucrose from HMMGS-agarose bead (Fig. 7A), the degree of micro-heterogeneity of this glycoprotein was higher than that shown in the electrophoregram obtained from the third peak

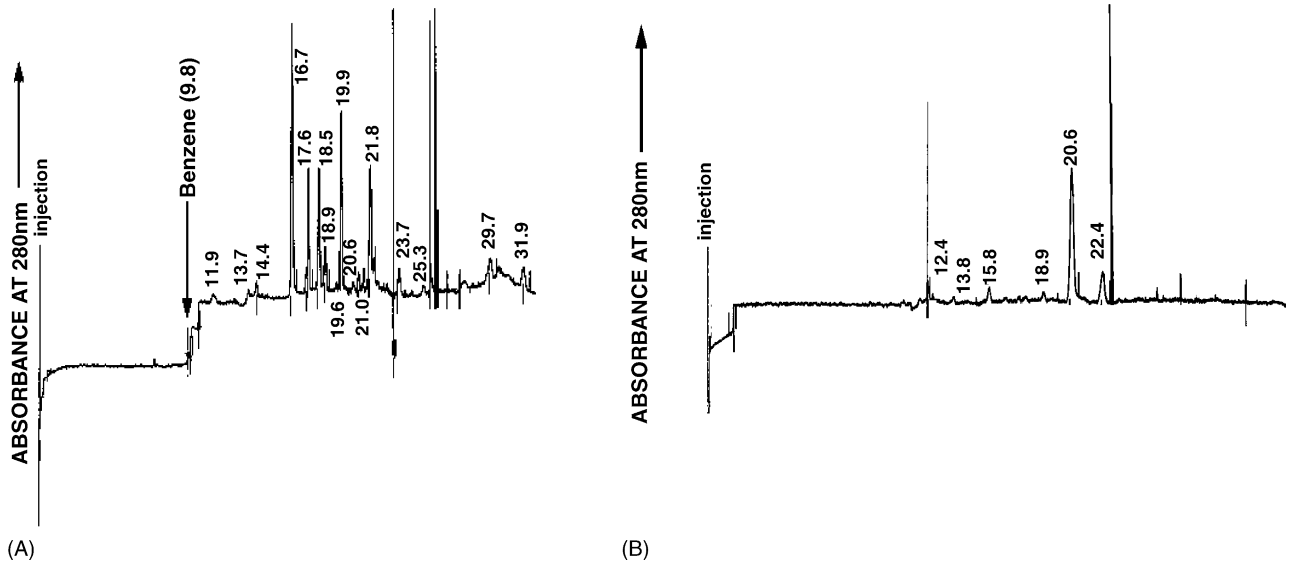

Fig. 6. Electrophoregrams corresponding to the first (A) and second (B) peaks of bacterial receptors eluted with 100 mM sucrose from MMMGS-agarose bead. Numbers near the peaks indicate absolute value of electrophoretic mobility in min. Arrows indicate the position of the neutral marker (benzene).

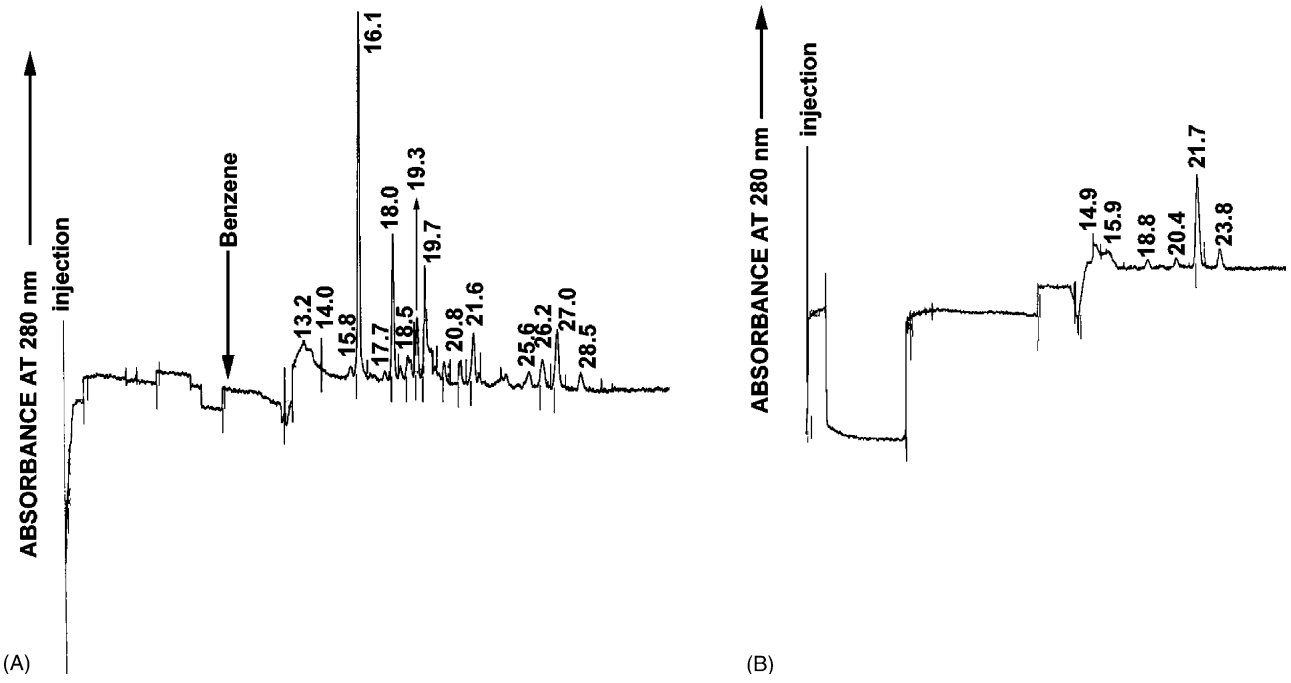

Fig. 7. Electrophoregrams corresponding to the first (A) and third (B) peaks of bacterial receptors eluted with 100 mM sucrose from HMMGS-agarose bead. Numbers near the peaks indicate absolute value of electrophoretic mobility in min. Arrows indicate the position of the neutral marker (benzene).

Table 1
Determination by capillary electrophoresis of the pI value of different receptors isolated from *G. diazotrophicus* cell walls by affinity chromatography

First peak in the elution profile with sucrose from MMMGS-agarose		Second peak in the elution profile with sucrose from MMMGS-agarose		First peak in the elution profile with sucrose from HMMGS-agarose		Third peak in the elution profile with sucrose from HMMGS-agarose	
Relative electrophoretic mobility	pI	Relative electrophoretic mobility	pI	Relative electrophoretic mobility	pI	Relative electrophoretic mobility	pI
1.71	6.43	1.66	6.17	1.51	5.37	1.69	6.33
1.88	7.33	1.80	6.90	1.69	6.33		
2.04	8.18			1.81	6.96		
2.23	9.19			1.85	7.17		
				2.54	10.84		

of glycoproteins eluted with sucrose from HMMGS-agarose bead (Fig. 7B), where only one main electrophoretic peak with area counts higher than of 9% of the total area, at 21.69 min, was observed. Calculated values of pI of the main peaks of HMMGS receptors are shown in Table 1.

Since sugarcane glycoproteins (HMMGS and MMMGS) were linked to the cyanogen bromide-activated agarose and desorption was carried out with sucrose, the polypeptide domain of the cell wall bacterium glycoproteins might interact with the polysaccharidic moiety of the corresponding sugarcane glycoprotein, which contains large segments of β-(1 → 2) fructofuranoside. On the basis of these results, and assuming that *G. diazotrophicus* as an endosymbiont of sugarcane plants is located in the intercellular apoplastic spaces [19], its cell wall glycoproteins identified as ligands of sugarcane glycoproteins would interact with the saccharide domain of both HMMGS and MMMGS, located in the cell walls of the parenchymatous cells from sugarcane stalks [10].

Acknowledgments

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