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A glycoproteomic approach reveals that the S-layer glycoprotein of *Lactobacillus kefiri* CIDCA 83111 is *O*- and *N*-glycosylated

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ABSTRACT

In Gram-positive bacteria, such as lactic acid bacteria, general glycosylation systems have not been documented so far. The aim of this work was to characterize in detail the glycosylation of the S-layer protein of *Lactobacillus kefiri* CIDCA 83111. A reductive β-elimination treatment followed by anion exchange high performance liquid chromatography analysis was useful to characterize the *O*-glycosidic structures. MALDI-TOF mass spectrometry analysis confirmed the presence of oligosaccharides bearing from 5 to 8 glucose units carrying galacturonic acid. Further nanoHPLC-ESI analysis of the glycopeptides showed two *O*-glycosylated peptides: the peptide sequence SSASSASSA already identified as a signature glycosylation motif in *L. buchneri*, substituted on average with eight glucose residues and decorated with galacturonic acid and another *O*-glycosylated site on peptide 471–476, with a $Glc_{5-8}GalA_2$ structure. As ten characteristic sequons (Asn-X-Ser/Thr) are present in the S-layer amino acid sequence, we performed a PNGase F digestion to release N-linked oligosaccharides. Anion exchange chromatography analysis showed mainly short N-linked chains. NanoHPLC-ESI in the positive and negative ion modes were useful to determine two different peptides substituted with short *N*-glycan structures. To our knowledge, this is the first description of the structure of *N*-glycans in S-layer glycoproteins from *Lactobacillus* species.
Significance: A detailed characterization of protein glycosylation is essential to establish the basis for understanding and investigating its biological role. It is known that S-layer proteins from kefir-isolated *L. kefiri* strains are involved in the interaction of bacterial cells with yeasts present in kefir grains and are also capable to antagonize the adverse effects of different enteric pathogens. Therefore, characterization of type and site of glycosidic chains in this protein may help to understand these important properties. Furthermore, this is the first description of *N*-glycosidic chains in S-layer glycoprotein from *Lactobacillus* spp.

1. Introduction

Surface-layer (S-layer) proteins are ubiquitous in both Bacteria and Archaea. S-layers are arrays of a single protein that constitutes the outermost cell envelope and have been considered to function as protective coats, maintenance of cell shape, and adhesion to specific hosts [1–3]. These proteins are present in some *Lactobacillus* species that are normal inhabitants of the oral and genital cavities and the gastrointestinal tract of humans and animals and also have been isolated from plants and food products.

The functions of bacterial S-layer proteins have been explained only in few cases, and many of them are still hypothetical. These lattices could function as protective agents against a hostile environment, as molecular sieves and ion traps, as structures involved in cell adhesion, surface recognition and inhibition of pathogens [1–3].

In Gram-positive bacteria, such as lactic acid bacteria, general glycosylation systems have not been documented so far. Although, most of the lactobacilli S-layer proteins were determined to be non-glycosylated [1], the glycoprotein nature of the S-layer proteins has been reported for *Lactobacillus buchneri* 41021/251, CD034 and NRRL B-30929 [4,5], *L. helveticus* ATCC12046 [6], *L. acidophilus* NCFM [7], *L. plantarum* 41021/252 [4] and several *Lactobacillus kefiri* strains [8]. However, only for *L. buchneri* 41021/251 and CD034, the S-layer glycan structures were studied, finding glucose oligomers attached to serine residues [5]. Further studies have revealed the importance of lactobacillar cell surface glycosylation for adhesion and biofilm formation [9] as well as gastrointestinal persistence [10] and adaptation [11].

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The presence of S-layer *L. kefiri* strains isolated from kefir, a probiotic fermented milk, was described some years ago [12]. It has been demonstrated that these S-layer proteins are involved in the interaction of bacterial cells with yeasts present in kefir grains [13]. They are also able to inhibit the invasion of *Salmonella enterica serovar* Enteritidis to Caco-2 cells [14], to antagonize the effect of *Clostridium difficile* toxins [15] and to protect bacterial cells against the deleterious effect of lead ions [16]. These glycoproteins show apparent molecular masses ranging from 66 to 71 kDa, and a high heterogeneity among aggregating and non-aggregating strains of *L. kefiri* have been demonstrated [8]. However, recent studies have revealed some similarities with the S-layer proteins of other lactobacilli in terms of amino acid composition, such as a high content of hydrophobic amino acids (34.9–38.4%) and hydroxylated amino acids (24.6–29.2%), and the absence of cysteine residues [17].

Since the amino acid sequence of the S-layer glycoprotein from *L. kefiri* CIDCA 83111 was recently reported, the aim of this work was to characterize in detail the glycosylation of this protein. To achieve this purpose, HPAEC-PAD and MALDI mass spectrometry were used to study the sugar moieties and nanoHPLC-ESI m.s. for glycopeptides. Interestingly, two *O*-glycosidic and two *N*-glycosidic chains were characterized.

2. Experimental

2.1. Bacterial strain and culture conditions

Lactobacillus kefiri CIDCA 83111 was used [18]. Bacteria were cultured in deMan-Rogosa-Sharpe (MRS)-broth (DIFCO, Detroit, USA) at 37 °C for 48 h, under aerobic conditions.

2.2. S-layer protein extraction

Extraction was performed using 5 M LiCl as described previously [15]. The homogeneity of the protein extract was tested by SDS-PAGE, stained with Colloidal Comassie Blue staining.

2.3. In gel-reductive β-elimination

The gel band was excised and then treated with 0.05 M NaOH/1 M $NaBH_4$ (0.5 mL) at 50 °C during 16 h. The solution was separated, acetic acid was added until pH 7 followed by repeated evaporation with methanol. The sample was dissolved in water, desalted in a Dowex 50 W (H^+) (Fluka) column and dried in SpeedVac.

2.4. Acid hydrolysis

The β-eliminated sample was further hydrolyzed in 2 N TFA for 4 h at 100 °C. The acid was eliminated by evaporation and the hydrolysate was re-suspended in water for HPAEC-PAD analysis.

2.5. Analysis of the sugar composition of the S-layer glycoprotein by HPAEC (high performance anion exchange chromatography)

Analysis was performed in a DX-500 Dionex BioLC system (Dionex Corp.) with a pulse amperometric detector. The following columns and conditions were employed: (*a*) neutral and aminosugar analysis was performed in a Carbopack PA-1 column using a 18 mM NaOH isocratic program, flow rate was 0.4 mL/min; (*b*) acidic monosaccharide analysis was performed in a Carbopack PA-1 column with a 48 mM NaOH/140 mM sodium acetate isocratic program was used, flow rate was 0.4 mL/min; c) for alditols, a CarboPac MA 1 column was used with a 0.4 M NaOH isocratic program and a flow rate of 0.4 mL/min. d) for oligosaccharides a Carbopack P-100 microbore column equipped with a P-100 pre-column was used and a gradient elution with 100 mM NaOH, 70–400 mM sodium acetate for 45 min, with a flow rate of 0.25 mL/min.

2.6. Release of N-glycosidic chains by PNGase F treatment

The protein band corresponding to the S-layer glycoprotein was cut out from the gel, frozen for 3 h, and washed (mixing for 30 min) with (*a*) acetonitrile, (*b*) 20 mM $NaHCO_3$, pH 7, and (*c*) acetonitrile. The gel pieces were dried, and the *N*-glycans were released by incubation with PNGase F (20 milliunits) (New England Biolabs Inc., Beverly, MA) overnight at 37 °C in 20 mM $NaHCO_3$, pH 7 (30 μL). The gel pieces were thoroughly washed, and the supernatants were removed and dried. Glycans were filtered through an Ultrafree McFilter (*M*r 5000), dried, resuspended in 0.1% (v/v) formic acid (20 μL), and left at room temperature for 40 min. Finally, the sample was dried and suspended in water.

2.7. Glycoprotein digestion

The protein band corresponding to the S-layer glycoprotein was cut out from the gel and washed with acetonitrile. The gel pieces were reduced with 10 mM DTT in 50 mM NH_4HCO_3 at 55 °C for 30 min. They were further washed with acetonitrile and alkylated with 55 mM IAA in 50 mM NH_4HCO_3 for 20 min at room temperature in darkness. After washing with 50 mM NH_4HCO_3 for 10 min and with acetonitrile for 5 min, they were dried in a SpeedVac. The gel slices were rehydrated with 20 ng/μL trypsin (Sigma) in 40 mM NH_4HCO_3, 9% acetonitrile and incubated at 37 °C overnight. Further digestion was carried upon the addition of 20 ng/μL Glu-C (V8) protease (Promega) in 50 mM NH_4HCO_3, pH 8, at 37 °C overnight. After incubation, the supernatant was separated and taken to dryness.

2.8. Mass spectrometry analysis

Matrices and calibrating chemicals were purchased from Sigma-Aldrich. Oligosaccharide analysis was performed in an Ultraflex II TOF/TOF mass spectrometer equipped with a high performance solid-state laser (λ 355 nm) and a reflector. The system is operated by the Flexcontrol version 3.3 software package (Bruker Daltonics GmbH, Bremen, Germany). Samples were irradiated with a laser power of 25–50% and measured in the linear and the reflectron modes, in positive and negative ion polarities.

2.9. Laser-induced dissociation tandem mass spectrometry (LID-MS/MS) analysis in the MALDI-TOF/TOF-MS/MS instrument

The Ultraflex II MALDI-TOF/TOF mass spectrometer was used. For all experiments using the tandem time-of-flight LIFT mode, the ion source voltage was set at 8.0 kV with a precursor ion mass window of 3 Da. Precursor ions generated by LID were accelerated at 19.0 kV in the LIFT cell. The reflector voltage was set at 29.5 kV. 2,5-Dihydroxybenzoic acid was used as matrix. The samples were loaded onto a MTP 384 ground steel target (Bruker Daltonics GmbH) using the sandwich method. Mass spectra were the sum of 100–300 single laser shots, depending on the sample conditions.

2.10. Spectrum calibration

External calibration reagents were used (commercial proteins bradykinin 1–7, *M*r 757.399; angiotensin I, *M*r 1296.685; renin substrate, *M*r 1758.933; insulin β-chain, *M*r 3494.6506) with β-cyclodextrin (cyclohepta amylose, *M*r 1135.0) and γ-cyclodextrin (cyclooctaamylose, *M*r 1297.1) with 2,5-dihydroxybenzoic acid as matrix.

2.11. Glycopeptide analysis

The glycopeptide mixtures obtained after protease digestions were purified by cotton HILIC SPE micro tips [19]. The enriched

glycopeptide mixtures were re-suspended in 50% ACN -1% formic acid in water, 1:1.

The digests were analyzed in a nanoLC 1000 coupled to an EASY-SPRAY Q Exactive Mass Spectrometer (Thermo Scientific) with a HCD (High Collision Dissociation) and an Orbitrap analyzer. An Easy Spray PepMap RSLC C18 column (50 μm × 150 mm, particle size 2.0 μm, pore size: 100 Å) at 40 °C was used for separation. Separation was achieved with a linear gradient from 5% to 35% solvent B developed in 75 min, at a flow of 300 nL/min(mobile phase A: water-0.1% formic acid; mobile phase B: ACN-0.1% formic acid). Injection volume 2 μL. Spray voltage (+): 3.5 kV; (−): 3.0 kV. A full-scan survey MS experiment (*m/z* range from 400 to 2000; automatic gain control target $3\ 10^6$; maximum IT: 200 ms, resolution at 400 *m/z*: 70000.

Data Dependent MS^2 method was set to the centroid mode, resolution 17500; maximum IT 50 ms; automatic gain control target 10^5; fragment the top 15 peaks in each cycle; NCE: 27.

2.12. Data interpretation

Data from de nanoHPLC-ESI-Orbitrap experiments were manually evaluated. Automatic search of peptides and glycopeptides was assisted by SequestHT on Proteome Discoverer 1.4 (Thermo Fisher Sc.)

Trypsin and Glu-C were selected as the enzymes and one missed cleavage was permitted. The mass accuracy tolerance was set to 10 ppm for precursor ions. The static modification was carbamidomethylation in the Cys residues. $HexNAc_2$; $HexNAc_2dHex$, $HexNAc_2Hex$; $HexNAc_2Hex_3$ and $HexNAc_2Hex_3dhex$ were selected as dynamic carbohydrate modifications on Asn. A maximum of two modifications per peptide was permitted. Deconvolution was assisted by Xtract on Thermo Xcalibur 3.0.63.

3. Results

3.1. Sugar components analysis

The glycosylated nature of S-layer protein extracted from *L. kefiri* strain CIDCA 83111 was previously reported by PAS staining [8]. In order to perform a sugar analysis of this S-layer protein, the gel band was excised and subjected to reductive β-elimination. When the β-eliminated sample was further subjected to a total acid hydrolysis and analyzed by HPAEC-PAD (Fig. 1A) under conditions where neutral and aminosugars are separated (condition a), the presence of a main peak corresponding to glucose was observed. Minor peaks coincident with glucosamine, galactose and mannose were also evident. The same sample was further analyzed under conditions where acidic sugars are separated (condition b) (Fig. 1B). Interestingly, a peak coincident with an authentic standard of galacturonic acid was detected. In addition, when an alditol analysis was performed (condition c) (Fig. 1C), a peak coincident with sorbitol was detected indicating that glucose is the sugar that links the O-linked chain to the peptide backbone. To complete the chromatographic analysis, the β-eliminated sample was analyzed under conditions where oligosaccharides are separated (condition d) (Fig. 1D). Main peaks corresponding to reduced maltooligosaccharides among 5 and 8 glucose units were determined. Up to this point the presence of *O*-glycosidic chains bearing glucose units was assured.

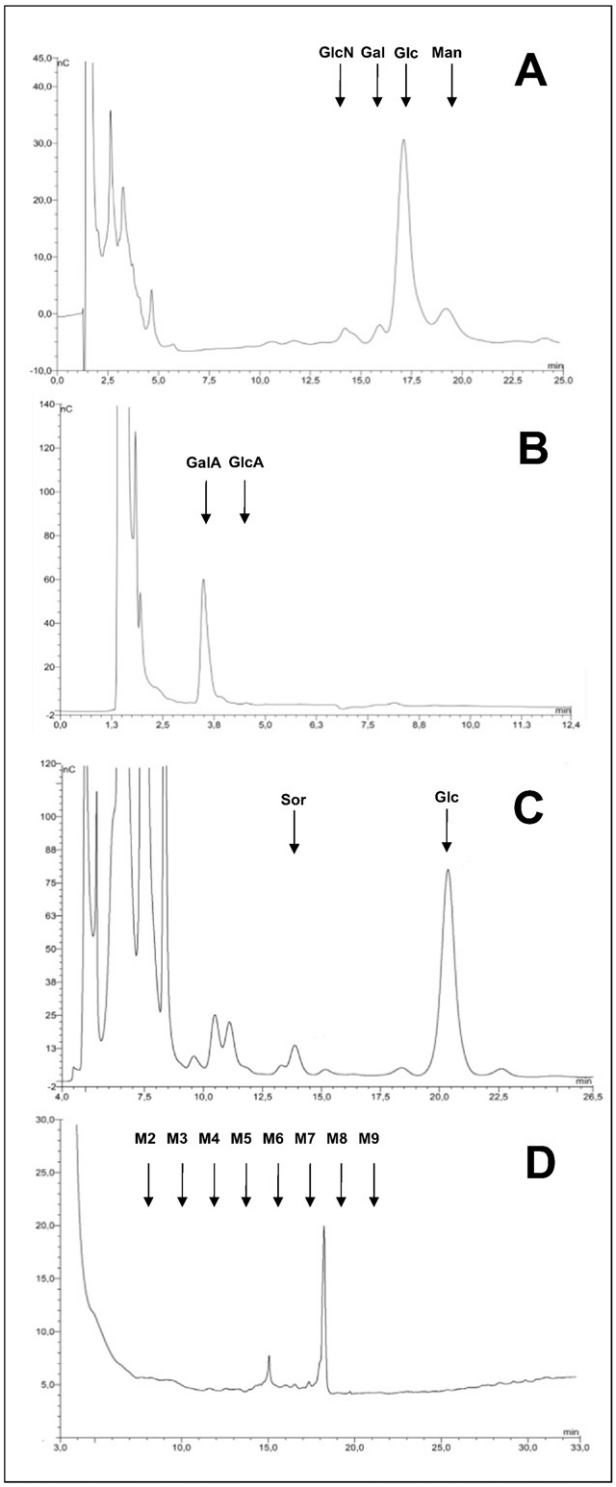

Fig. 1. HPAEC-PAD analysis of the S-layer glycoprotein subjected to a reductive β-elimination treatment. A) The sample was further hydrolyzed and neutral and aminosugar components were analyzed; B) Idem A, analyzed for acidic components; C) Idem A, analyzed for reduced monosaccharides; D) Analysis of the reduced oligosaccharides. Glc: glucose; Sor: sorbitol; Gal: galactose; Man: mannose; GlcN: glucosamine; GalA galacturonic acid; GlcA: glucuronic acid. M3-M9: reduced maltooligosaccharides containing from 3 to 9 glucose units.

3.2. Analysis of O-linked glycans by MALDI-TOF m.s.

In order to get deeper into the *O*-linked sugar structure, the reduced fraction obtained by β-elimination was subjected to MALDI-TOF m.s. analysis in the negative ion mode using 2,5-dihydroxibenzoic acid as matrix (Fig. 2). Major ion at *m/z* 829.0 (calc. *m/z* 829.2903 $C_{30}H_{53}O_{26}^-$) corresponds to the reduced oligosaccharide bearing five hexose units. A minor signal at *m/z* 667.0 (calc. *m/z* 667.2297, $C_{24}H_{43}O_{21}^-$) corresponds to the reduced oligosaccharide constituted by four hexose units. On the other hand, ion at *m/z* 991.5 (calc. *m/z* 992.3432; $C_{36}H_{63}O_{31}^-$) corresponds to six hexose units and ion at *m/z* 1005.0 (calc. *m/z* 1005.3151; $C_{36}H_{61}O_{32}^-$), to a Hex_5HexA reduced structure. Two additional signals at *m/z* 1167.0 (calc. *m/z* 1167.3680; $C_{42}H_{71}O_{37}^-$) and *m/z* 1328.4 (calc. *m/z* 1329.4208; $C_{48}H_{81}O_{42}^-$) corresponding to reduced Hex_6HexA and Hex_7HexA respectively, were also detected.

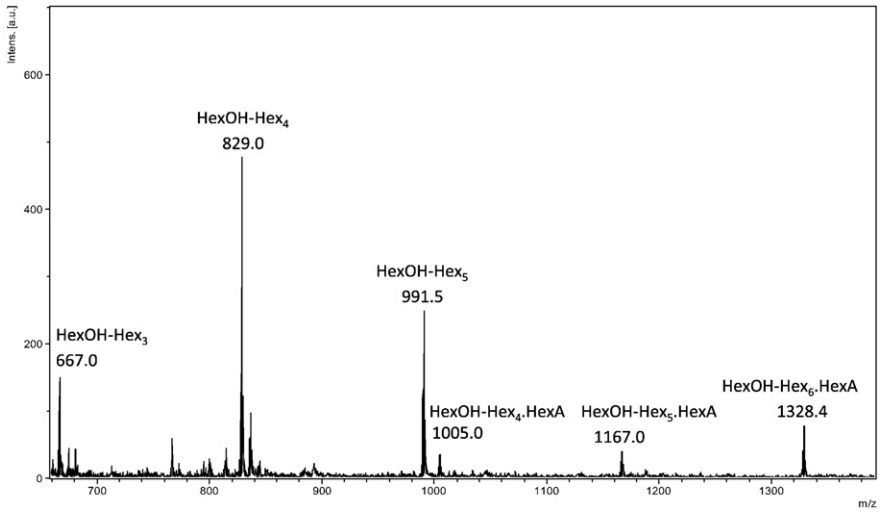

Fig. 2. MALDI-TOF mass spectrum in the negative ion mode of the oligosaccharides obtained from the S-layer glycoprotein of *L. kefiri* CIDCA 83111 after reduced β-elimination treatment of the corresponding SDS-PAGE gel band.

3.3. Analysis of O-glycosylated peptides by HPLC-ESI

Next step consisted in determining the site of linkage of this oligosaccharide to the protein. Therefore, a sequential enzymatic digestion with trypsin and Glu-C was performed (S1) on the excised gel band corresponding to the S-layer glycoprotein of *L. kefiri* and an enriched glycopeptide fraction was obtained after cotton-hilic chromatography. The glycopeptide enriched fraction was further analyzed by HPLC-ESI. Fig. 3 shows the total ion chromatogram (TIC) obtained by LC/MS/MS in the positive ion mode with MS range *m/z* 400–2000 (panel A). In order to locate the glycopeptide peaks and determine *m/z* and charge state, the intensity of the oxonium Hex^+ (*m/z* 163.06) (panel B) that arose by data dependent MS/MS was depicted as an extracted chromatogram. Interestingly, the oxonium Hex^+ ion location in the extracted chromatogram suggested the presence of *O*-linked structures in the initial minutes of the HPLC run.

Searching for glycopeptides in the nanoHPLC-ESI analysis acquired in the positive ion mode, at 5.64 min, a family of glycopeptides with z = +3 (*m/z* 1544.8824; 1652.9221; 1706.9264; 1760.9561; 1814.9486; 1868.9785; 1922.9988; 1977.0089) were detected (Fig. 4A). Deconvolution of these ions leads to peptide ^{147}SASASSASSASSTE^{160} substituted with 21 to 29 hexose units. Accordingly, also a family of ions z = +4 were detected (*m/z* 1564.2961; 1604.5615; 1645.0615; 1685.8400; 1726.0867; 1766.5977; 1807.3851; 1847.6289; 1888.4052). In this case peptide 147–160 substituted with 31–39 hexose units was detected. (Fig. 4B). Table 1 shows the glycoform structures corresponding to the peptide, calculated masses, experimental masses and errors. In S2 some examples of MS/MS spectra of the corresponding ions

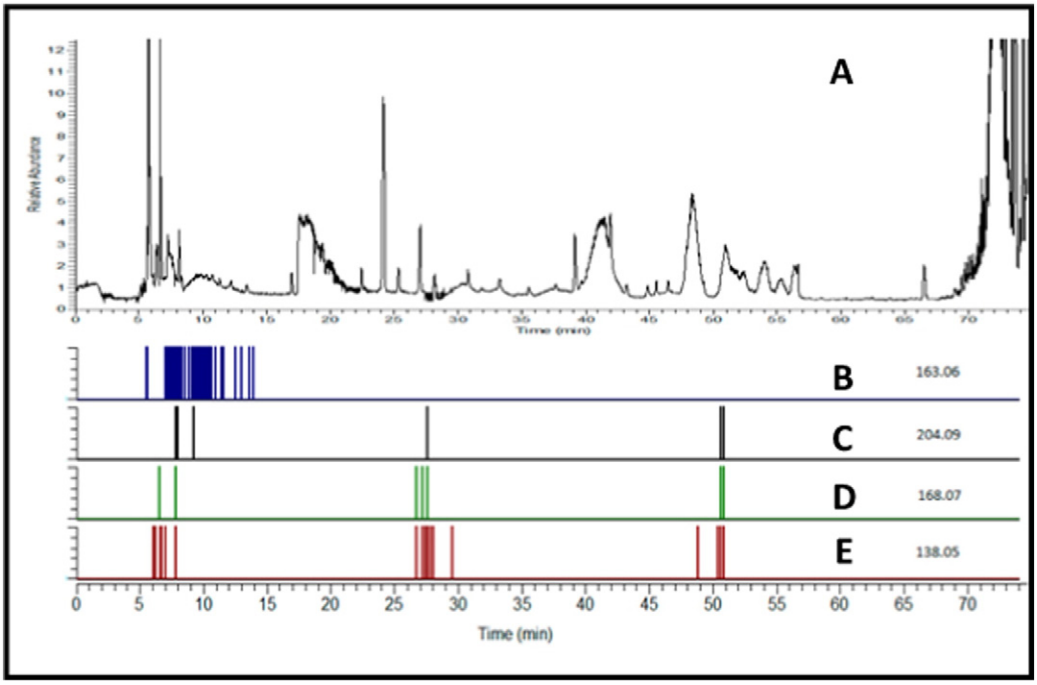

Fig. 3. Extracted ion chromatograms of *N*- and *O*-glycopeptides from S-layer glycoprotein obtained by C18-nano LC-ESI-Orbitrap-MS/MS analysis after trypsin treatment and diagnostic ions that arose by data dependent MS/MS. a) Total ion chromatogram (TIC) obtained by LC/MS/MS in the positive ion mode with MS range *m/z* 400–2000; b) Diagnostic oxonium Hex^+ (*m/z* 163.06); c) Diagnostic $HexNAc^+$ (*m/z* 204.09); d) Diagnostic $[HexNAc\text{-}2H_2O]^+$ (*m/z* 168.07); e) Diagnostic $[HexNAc - CH_6O_3]^+$ (*m/z* 138.05).

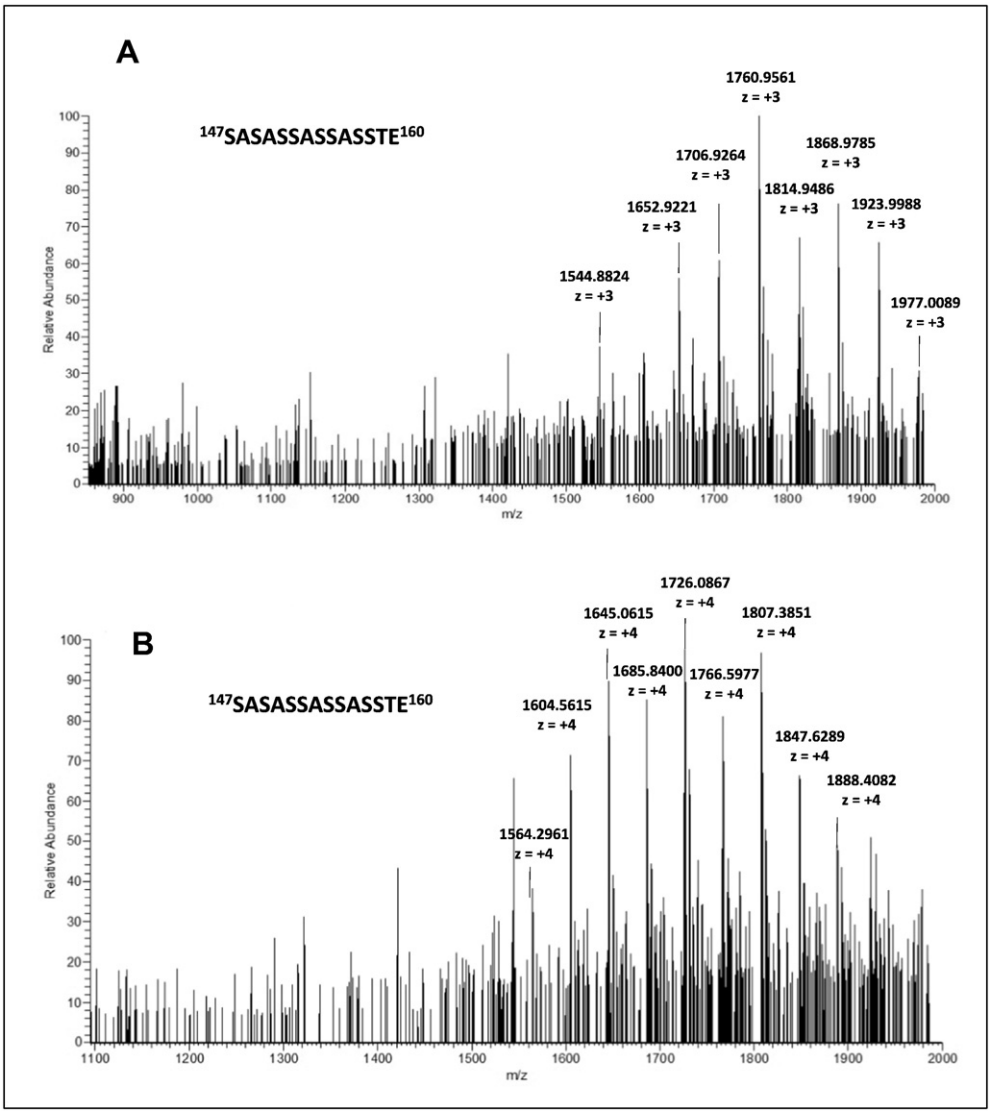

Fig. 4. HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 147–160. a) Spectrum performed in the positive ion mode acquired at Rt = 5.64 min showing cluster ions with z = +3. b) Spectrum performed in the positive ion mode acquired at Rt = 5.67 min showing cluster ions with z = +4. (See Table 1 for structures.)

containing the characteristic reporter fragments (*m/z* 145.05; 163.06 and 325.11) in the low mass region are shown.

Furthermore, at retention time 7.51 min, another cluster of +4 charged ions was shown (*m/z* 1463.8036; 1504.3110; 1544.8226; 1625.8561; 1666.3723; 1706.8783; 1747.4017; 1787.9008; 1828.4177; 1868.9435; 1909.4424; 1949.9532; 1990.4730). Deconvolution of these ions points out to the peptide ^{147}SASASSASSASSTEQTTALTDAQK^{170}, containing the previous peptide with one miscleavage, substituted with 23–35 hexose units. (Fig. 5A). Also minor signals with z = +5 were shown. Among them, the largest glycopeptide detected (*m/z* 1981.9026) bears 47 hexose units (not shown). In S2, MS/MS spectra of major ions containing diagnostic carbohydrate fragments are shown. A similar glycosylation pattern has been described as an *O*-glycosylated signature motif S-S-A-S-S-A-S-S-A in the S-layer glycoprotein of *L. buchneri* [20]. When the analogous signals were searched in the HPLC-ESI analysis performed in the negative ion mode (Fig. 5B) a similar cluster of ions with z = −4 was detected. Main peaks (*m/z* 1380.7684; 1421.2803; 1461.7980; 1502.3051; 1542.8199; 1623.8507; 1664.3583; 1704.8676; 1745.3832; 1785.895; 1826.4084; 1866.9222; 1907.4371; 1947.9553) correspond to peptide 147–170 carrying from 20 to 34 hexose units. However, in this case, it was interesting to note that among main signals, another cluster of minor ions was shown (inset). Mass differences with the major signals indicate the presence of a $Hex_{28-30}HexA_{1-3}$ structures. These results are in accordance with the MALDI-TOF m.s. analysis of the β-eliminated *O*– chains described above. Table 1 shows the structures corresponding to each peptide, calculated masses, experimental masses and errors.

In addition, between 5.14 and 5.45 min of the nanoHPLC-ESI analysis acquired in the positive ion mode another cluster of ions with z = +2 was detected (*m/z* 845.7368; 926.7651; 1007.7856; 1088.8151) (Fig. 6A). Deconvolution of these ions pointed out to peptide ^{469}DKTTTSAE^{476} glycosylated with a $HexA_2Hex_{3-8}$ structure. In this case the low abundance of these signals precluded the correct determination of the experimental monoisotopic value, leading to larger mass errors (See Table 1). S2 shows MS/MS spectra of major ions containing in the low mass region, characteristic reporter fragments. In order to verify the presence of this glycopeptide structure, another experiment in the HPLC-ESI acquired in the negative ion mode was performed. Although the glycopeptide described above could not be detected, it was possible to determine in the same region, the presence of a cluster of signals with z = −2 (*m/z* 796.2504; 877.278; 958.2607; 1039.2854; 906.2847; 987.3111) (Fig.6B). In this case, deconvolution of these ions pointed out to peptide ^{471}TTTSAE^{476}, the same peptide determined in the positive ion mode without the miscleavage, glycosylated with a $HexA_{1-2}$ Hex_{5-8} structure.

Table 1
O-glycosylated peptides detected in *Lactobacillus kefiri* CIDCA 83111 S-layer glycoprotein by HPLC-ESI.

Peptide	Modification	Charge	*m/z* calc.	*m/z* exp.	Error (ppm)
^{147}SASASSASSASSTE^{160}	Hex_{20}	3+	1490.8611	1490.8721	7.37
	Hex_{21}		1544.8787	1544.8824	2.37
	Hex_{23}		1652.9139	1652.9221	4.94
	Hex_{24}		1706.9315	1706.9264	−3.01
	Hex_{25}		1760.9491	1760.9561	3.96
	Hex_{26}		1814.9667	1814.9486	10.51
	Hex_{27}		1868.9843	1868.9785	−3.12
	Hex_{28}		1923.0019	1922.9988	−1.63
	Hex_{29}		1977.0195	1977.0089	-5.38
^{147}SASASSASSASSTE^{160}	Hex_{31}	4+	1564.042875	1564.2961	161.90
	Hex_{32}		1604.556075	1604.5615	3.38
	Hex_{33}		1645.069275	1645.0615	−4.73
	Hex_{34}		1685.582475	1685.84	152.78
	Hex_{35}		1726.095675	1726.0867	−5.20
	Hex_{36}		1766.608875	1766.5977	−6.33
	Hex_{37}		1807.122075	1807.3851	145.55
	Hex_{38}		1847.635275	1847.6289	−3.45
	Hex_{39}		1888.148475	1888.4082	137.56
^{147}SASASSASSASSTEQTTALTDAQK^{170}	Hex_{20}	4+	1382.7828	1382.7823	−0.34
	Hex_{21}		1423.2960	1423.2928	−2.23
	Hex_{22}		1463.8092	1463.8036	−3.81
	Hex_{23}		1504.3224	1504.311	−7.56
	Hex_{24}		1544.8356	1544.8226	−8.40
	Hex_{26}		1625.8600	1625.8561	−2.41
	Hex_{27}		1666.3752	1666.3723	−1.73
	Hex_{28}		1706.8884	1706.8783	−5.90
	Hex_{29}		1747.4016	1747.4017	0.07
	Hex_{30}		1787.9148	1787.9008	−7.82
	Hex_{31}		1828.4280	1828.4177	−5.62
	Hex_{32}		1868.9412	1868.9435	1.24
	Hex_{33}		1909.4544	1909.4424	−6.27
	Hex_{34}		1949.9676	1949.9532	−7.37
	Hex_{35}		1990.4808	1990.473	-3.91
^{147}SASASSASSASSTEQTTALTDAQK^{170}	Hex_{20}	4−	1380.7682	1380.7684	0.16
	Hex_{21}		1421.2814	1421.2803	−0.76
	Hex_{22}		1461.7946	1461.798	2.34
	Hex_{23}		1502.3078	1502.3051	−1.76
	Hex_{24}		1542.8210	1542.8199	−0.70
	Hex_{26}		1623.8454	1623.8507	3.25
	Hex_{27}		1664.3606	1664.3583	−1.37
	Hex_{28}		1704.8738	1704.8676	−3.62
	Hex_{29}		1745.3870	1745.3832	−2.16
	Hex_{30}		1785.9002	1785.895	−2.90
	Hex_{31}		1826.4134	1826.4084	−2.72
	Hex_{32}		1866.9266	1866.9222	−2.34
	Hex_{33}		1907.4398	1907.4371	−1.40
	Hex_{34}		1947.9530	1947.9553	1.19
^{147}SASASSASSASSTEQTTALTDAQK^{170}	$Hex_{29}HexA$	4−	1789.3950	1789.3876	−4.13
	$Hex_{28}HexA_2$		1792.8898	1792.8851	−2.63
	$Hex_{28}HexA_2 + H_2O$		1797.3925	1797.3877	−2.65
	$Hex_{30}HexA$		1829.9082	1829.8981	−5.52
	$Hex_{29}HexA_2$		1833.4030	1833.3987	−2.36
	$Hex_{29}HexA_2 + H_2O$		1837.9057	1837.9108	2.79
	$Hex_{28}HexA_3 + H_2O$		1841.4005	1841.4114	5.92
	$Hex_{31}HexA$		1870.4214	1870.4252	2.03
	$Hex_{30}HexA_2$		1878.4189	1878.4196	0.39
	$Hex_{30}HexA_2 + H_2O$		1882.9208	1882.9182	-1.36
^{469}DKTTTSAE^{476}	Hex_3HexA_2	2+	845.8219	845.7368	89.74
	Hex_4HexA_2		926.8386	926.7651	79.29
	Hex_5HexA_2		1007.865	1007.7856	78.77
	Hex_6HexA_2		1088.8914	1088.8151	70.06
^{471}TTTSAE^{476}	Hex_5HexA	2−	796.2734	796.2504	−28.88
	Hex_6HexA		877.2998	877.278	−24.85
	Hex_7HexA		958.3262	958.2607	−68.35
	Hex_8HexA		1039.3526	1039.2854	−64.66
	$Hex_5HexA_2Na_2$		906.27174	906.2847	14.30
	$Hex_6HexA_2Na_2$		987.29814	987.3111	13.13

3.4. Analysis of N-glycosidic chains by HPLC-ESI

The peptide sequence of the S-layer glycoprotein (17, S1), shows ten consensus sequences for putative *N*-glycosylation (N-X-S/T). Therefore, as this type of substitution has not been determined in Lactobacillus so far, we performed a PNGase digestion on the gel band corresponding to the S-layer glycoprotein in order to search for its presence. Analysis of the released material by HPAEC-PAD (Fig. 7A) showed a main oligosaccharide migrating very near the classical *N*-glycosidic core ($GlcNAc_2Man_3$). Minor peaks corresponding to larger structures were also detected. Next step consisted in searching in the glycopeptide HPLC-ESI analysis, for the intensity of

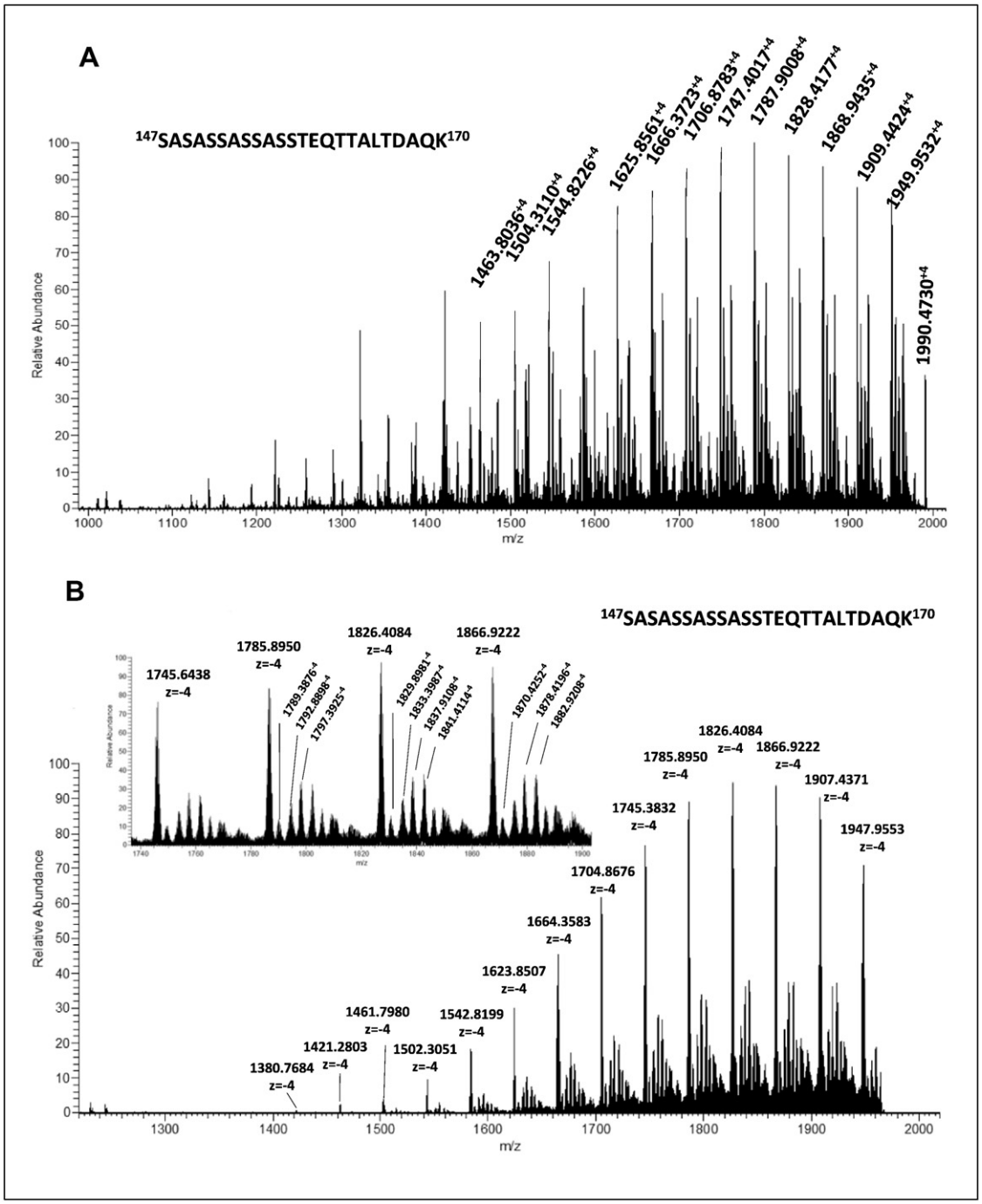

Fig. 5. HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 147–170. a) Spectrum performed in the positive ion mode acquired between Rt = 10–15 min showing cluster ions with z = +4. b) Spectrum performed in the negative ion mode showing cluster ions with z = −4. The inset is a magnification of range *m/z* 1700–1900. (See Table 1 for structures.)

the oxonium ions $HexNAc^+$ (*m/z* 204.09) (Fig. 3, panel C), $[HexNAc\text{-}2H_2O]^+$ (*m/z* 168.07) (Fig. 3, panel D) and $[HexNAc - CH_6O_3]^+$ (*m/z* 138.05) (Fig. 3, panel E) characteristic for *N*-glycosidic moieties that arose by data dependent MS/MS. Notably, three defined regions in the HPLC run bearing these reporter ions were observed: 5–8 min; 26–30 min and 48–55 min. Due to the fact that glycopeptides may acquire multiple charges and that in the positive ion mode they may be protonated and/or sodiated making sometimes complicated the mass spectrometry analysis, we decided to perform first a manual inspection of the spectra acquired in the negative ion mode. As expected, at an elution time of 7.58 min, it was easy to detect a signal of *m/z* 1130.5466 with z = −1 and the corresponding signal, of *m/z* 564.7698 with z = −2, attributed to peptide ^{233}IADT**N**^{237}ATNGQK^{243} containing an *N*-glycosylation site (Fig. 7B). Next to these signals, a mixture of glycosylated forms with z = −2 matching with substitution at ^{237}N were observed. Signal at *m/z* 921.9228 corresponds to the peptide bearing a $HexNAc_2HexdHex$ structure; ion at *m/z* 1296.0752 corresponds to the addition of two hexoses, two HexNAc units plus H_2O to the latter and *m/z* 1319.0042 matches with $HexNAc_2Hex_5dHex_2$ structure. Interestingly, the fact that the non-glycosylated peptide was also found suggests only a partial occupation of this *N*-glycosylation site. In the HPLC-ESI analysis acquired in the positive ion mode only two signals in this region could be assigned: one corresponding to the naked peptide with z = +2 (*m/z* 566.7837) and one corresponding to the same peptide substituted with two HexNAc units plus H_2O (*m/z* 778.8825) (S3). In S4, MS/MS spectra of major ions containing in the low mass

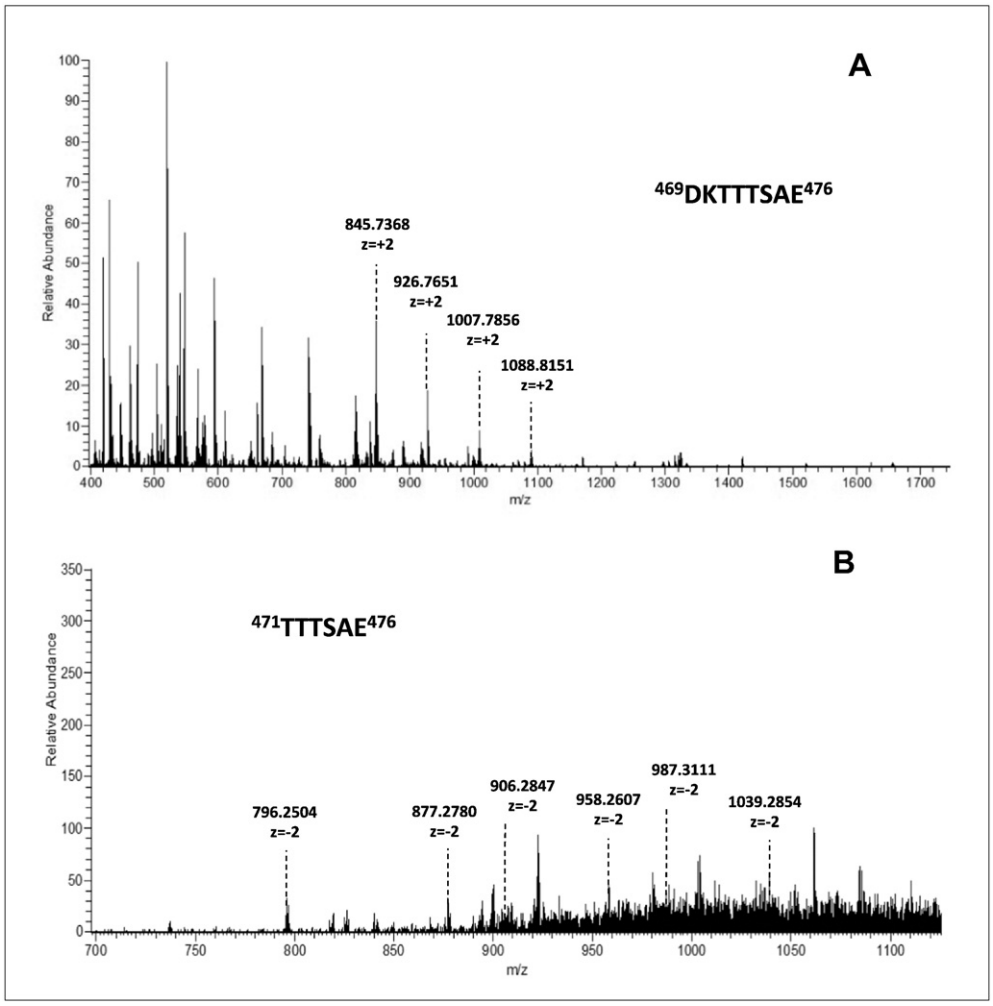

Fig. 6. HPLC-ESI analysis of *O*-linked glycopeptides corresponding to peptide 469–476 and 471–476. a) Spectrum performed in the positive ion mode acquired between Rt = 5.14–5.45 showing cluster ions with z = +2 corresponding to peptide 469–476. b) Spectrum performed in the negative ion mode showing cluster ions with z = −2 corresponding to peptide 471–476.

region diagnostic 204.09 fragment are shown. However after the use of SequestH algorithm, the peptide ^{233}IADT**N**^{237}ATNGQKINGWIK^{249} containing the same *N*-glycosidic sequon bearing $HexNAc_2dHex$ was identified with an elution time of 56.51 min (S5). Manual inspection of this region showed a signal with z = +3 at *m/z* 804.7286 matching with the named peptide carrying a $HexNAc_2Hex$ structure and *m/z* 873.4028 assigned to $HexNAc_2Hex_2$ were detected. (See S4 for MS/MS.)

Looking into the second region, at Rt = 30.02 min, a signal with z = −2 (*m/z* 770.3560) corresponding to peptide ^{203}TVTDATPYA**N**^{212}DTFK^{216} was detected. Nearby, a signal with *m/z* 1046.4855 was assigned to peptide 203–219 substituted with a $HexNAc_2dHex$ structure (Fig. 7C). In order to assure the glycopeptide structure, the analysis performed in the positive ion mode was investigated. As expected, the corresponding signals with z = +2 were detected (*m/z* 772.0980 and *m/z* 1048.4679) (S3).

Finally, in the third region, at an elution time of 52.77 min, the automatic search (S5) found peptide ^{125}SVTAFAGGIASF**N**TTTAPAAAK^{146} glycosylated with $HexNAc_2dHex$ but, the low abundance of the corresponding signals precluded the detection of diagnostic fragments in the MS/MS.

4. Discussion

This is the first detailed report of the *O*- and *N*- glycosylation of the S-layer glycoprotein of *Lactobacillus kefiri*. Classical methodology as reductive β-elimination and HPAEC-PAD analysis were useful to characterize the *O*-glycosidic structure. In addition, MALDI-TOF m.s. analysis confirmed the presence of oligosaccharides bearing from 5 to 8 glucose units carrying galacturonic acid. Further nanoHPLC-ESI analysis in the negative and positive ion modes of the glycopeptides obtained after trypsin and Glu-C digestion, showed two *O*-glycosylated peptides (Table 1). The peptide SSASSASSA has been already identified as a signature glycosylation motif in *L. buchneri*, with four serine residues within the sequence S-S_{152}-A-S_{154}-S_{155}-A-S_{157}-SA substituted on average with seven glucose residues [5]. In *L. kefiri*, the same peptide sequence was determined [17] but, unlike in *L. buchneri*, it is substituted on average with eight glucose residues decorated with galacturonic acid. Furthermore, another *O*-glycosylated site, peptide 471–476, substituted with a $Glc_{5-8}GalA_2$ structure was also determined. Since the cell envelope is the first target of physicochemical and environmental stress, several studies indicate that there is a correlation between the different structural and chemical characteristics of the S-layer proteins with the surface properties of lactobacilli [1,20]. Lactobacilli are often exposed to changes in the osmolarity of their environment which can compromise essential cell functions. Changes in solute concentrations in the environment, cause changes in cell turgor pressure which lead to changes in cell volume [21]. In this sense, it can be speculated that a high degree of glycosylation, as shown in *L. kefiri*, may be protective against the harsh environmental conditions. In addition, a negative surface charge, in this case due to the presence of GalA, is thought to stabilize proteins

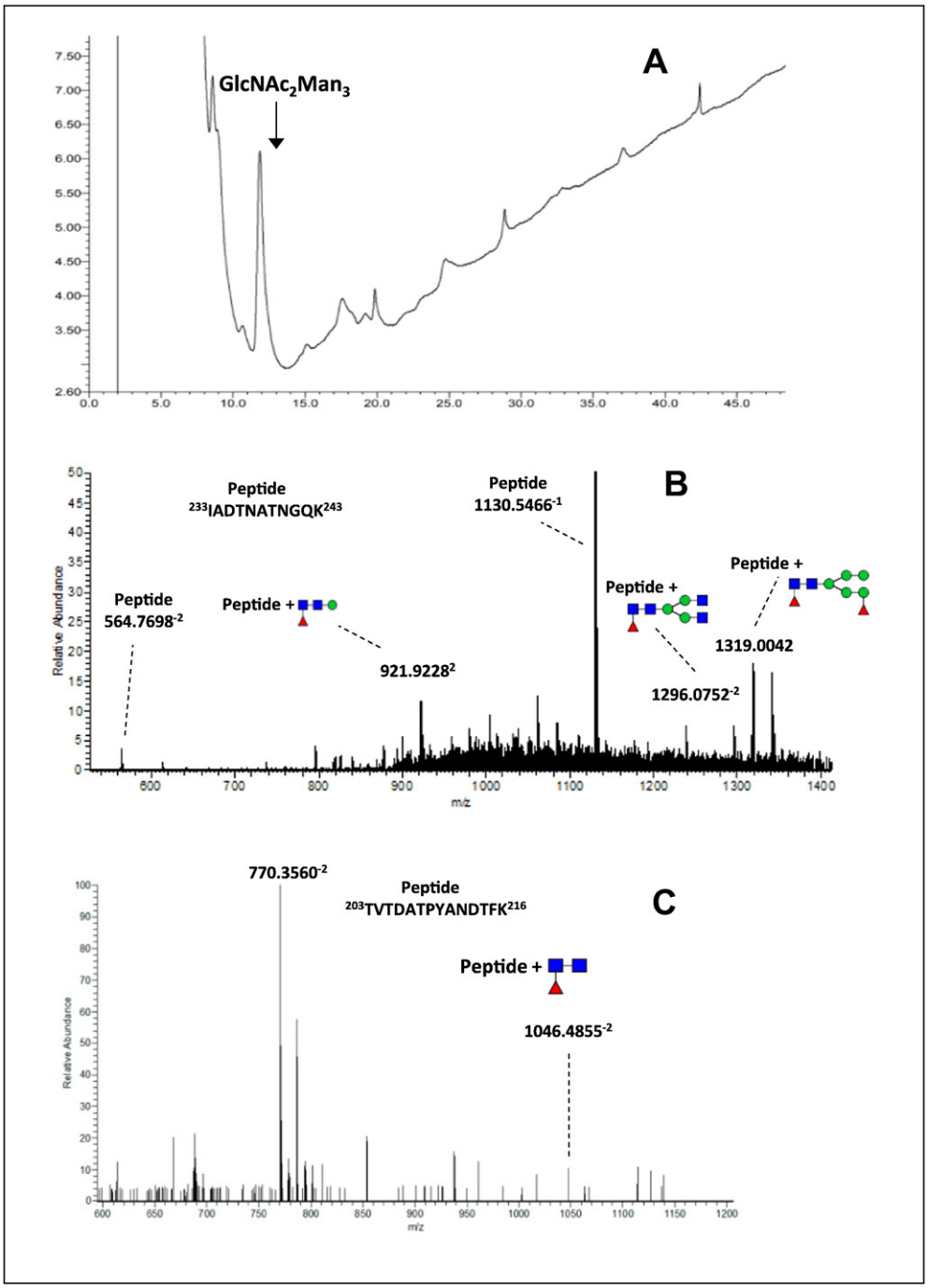

Fig. 7. Analysis of *N*-linked glycopeptides. a) HPAEC-PAD analysis of the oligosaccharides released by PNGase F from the SDS-PAGE gel band. b) HPLC-ESI analysis in the negative ion mode obtained at Rt = 7.58 min showing signals corresponding to peptide ^{233}IADT**N**ATNGQK^{243} and different glycoforms. c) HPLC-ESI analysis in the negative ion mode obtained at Rt = 30.02 min showing peptide ^{203}TVTDATPYA**N**DTFK^{216} and different glycoforms. ■ = HexNAc; ▲ = dHex; ● = Hex.

in high salt conditions, possibly via the formation of an energetically favorable protein-water-salt hydration network, as proposed for other S-layer glycoproteins [22]. In a separate procedure, the presence of *N*-glycosidic modifications was studied. Although it has been reported that tunicamycin did not affect the SDS-PAGE pattern of the *L. kefiri* S-layer glycoprotein [8] the fact that ten characteristic sequons (Asn-X-Ser/Thr) are present in the S-layer amino acid sequence led us to search for this type of modification. PNGase F treatment of the corresponding gel band followed by HPAEC-PAD analysis showed that mainly short *N*-linked oligosaccharides were released. Therefore, a glycoproteomic approach by nanoHPLC-ESI in the positive and negative ion mode was performed allowing us to determine at least two different peptides substituted with short structures. This result is in agreement with a recent report showing that *L. kefiri* JCM 5818 S-layer is effective on preventing *L. acidophilus* binding to hDC-SIGN and also it has strong activity against infection of DC-SIGN expressing cells, but deglycosylation by PNGase F remarkably reduced its activity suggesting the presence of *N*-glycosidic chains and their involvement in the adhesion process [23]. Table 2 shows a summary of the glycosidic structures described.

5. Conclusion

In this work, we provide evidence that the S-layer glycoprotein present in *L. kefiri* CIDCA 83111 is *O*- and *N*-glycosylated. In fact, as far as we

Table 2
Summary of the glycoforms found in the S-layer glycoprotein of *L. kefiri* CIDCA 8111.

	Glycopeptide		Oligosaccharide	Peptide sequence
	Ret. time (min)	Charge	Modification	
O-glycosylation	5.14–5.45	+2	$Glc_{3-6}GalA_2$	^{469}DKTTT**S**AE^{476}
	5.14–5.45	−2	$Glc_{5-8}GalA_{1-2}$	^{471}TTT**S**AE^{476}
	5.64	+3	Glc_{20-29}	^{147}SASASSASSASSTE^{160}
	5.64	+4	Glc_{31-39}	^{147}SASASSASSASSTE^{160}
	7.51	+4	Glc_{20-47}	^{147}SASASSASSASSTEQTTALTDAQK^{170}
	7.51	−4	$Glc_{20-34}GalA_{0-3}$	^{147}SASASSASSASSTEQTTALTDAQK^{170}
N-glycosylation	7.58	−2	$GlcNAc_2MandHex$; $GlcNAc_4Man_3dHex$; $GlcNAc_2Man_5dHex_2$	^{233}IADT**N**ATNGQK^{243}
	7.58	+2	$GlcNAc_2$	^{233}IADT**N**ATNGQK^{243}
	54.33	+3	$GlcNAc_2Man$; $GlcNAc_2Man_2$	^{233}IADT**N**ATNGQKINGWIK^{249}
	30.02	−2	$GlcNAc_2dHex$	^{203}TVTDATPYA**N**DTFK^{216}
	30.02	+2	$GlcNAc_2dhex$	^{203}TVTDATPYA**N**DTFK^{216}

know, this is the first description of the structure of *N*-glycosidic chains in S- layer glycoprotein from *Lactobacillus* species.

The knowledge of type and site of glycosylation of the S-layer glycoprotein of *L. kefiri* gives the exciting possibility to study in depth the structure-activity relationships. Although glycosylation may be only part of the puzzle, it is nowadays evident that in *L. kefiri*, carbohydrates play a prominent role as protective coats and as promoters for cell adhesion and surface recognition. Future comparative studies on wild-type strains with different characteristics will help to elucidate the functional significance of the carbohydrate moieties of S-layer glycoproteins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.04.007.

Conflict of interest

The authors declare that this manuscript has no conflict of interest.

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