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THE DIFFERENT FORMS OF PNS MYELIN P0 PROTEIN WITHIN AND OUTSIDE LIPID RAFTS

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ABSTRACT

It is now well established that plasma membranes, such as the myelin sheath, are made of different microdomains with different lipid and protein composition. Lipid rafts are made mainly of sphingolipids and cholesterol, whereas the non-raft regions are made mainly of phosphoglycerides. Most myelin proteins may distribute themselves in raft and non-raft microdomains but the driving force that gives rise to their different distribution is not known yet. In this paper, we have studied the distribution of P0, the most representative protein of PNS myelin, in the membrane microdomains. To this end, we have purified P0 from both non-raft (soluble P0, P0-S) and raft (P0-R) regions of PNS. Purified proteins were analyzed by bidimensional gel electrophoresis and identified and characterized by MALDI ToF mass spectrometry. A detailed structural description of the two P0 forms is given in terms of amino acid sequence, post-translational modifications and composition of associated lipids. Our findings suggest that structural differences between the two proteins, mainly related to the glycogroups, might be responsible for their different localization.

Key words: Myelin, P0, PNS, lipid rafts, mass spectrometry, glycans.

Running title: Protein P0 partitioning in PNS myelin

INTRODUCTION

Myelin is the insulating lipid-rich membranous sheath discontinuously wrapped around nerve axons to form compact multilamellar structures that allow for saltatory impulse conduction along the axon both in CNS and PNS (Kirschner and Blaurock 1992). In the PNS, the myelin sheath is an extension of the Schwann cell plasma membrane and consists of an exclusive set of proteins, which have been largely investigated for their putative role in membrane assembly and compaction as well as for their degradation in specific demyelinating diseases (Greenfield et al. 1973; Avila et al. 2005).

The most important protein of PNS myelin (more than 70% of total protein fraction in mammalian PNS) is the protein zero (P0) (Lemke and Axel, 1985). This is a 28 kDa transmembrane glycoprotein with an immunoglobulin-like extracellular domain (Kirschner et al. 2004; Filbin et al. 1990; D'Urso et al. 1990; Lemke et al. 1988). P0 tetramers are proposed to account for myelin compaction (Shapiro et al. 1996; Inouye et al. 1999; Thompson et al. 2002).

P0 has a single N-linked glycosylation site. When extracted with denaturant methods from PNS myelin, the P0 protein shows the presence of a series of N glycans HNK1 carbohydrate epitopes (Voshol et al. 1996) whose structure has been well characterized (Gallego et al. 2001). The importance of P0 glycans is connected to the adhesive role of P0 in cell culture aggregation assay, probably due in part to its sugar residues (Filbin and Tennekoon 1992, 1993; Yazaky et al. 1992).

Recent renewed input in the study of PNS myelin and P0 come from the interest in distribution and sorting of the proteins in myelin, as a new model for biological membranes has been proposed (Simons and Ikonen 1997). According to this model, myelin and other plasma membranes contain lipid platforms consisting of glycosphingolipids and cholesterol

microdomains called *Lipid Raft*s (LR) distributed in a more fluid *non-raft region* consisting prevalently of glycerophospholipids. LR can be involved in a series of biological processes such as signal transduction pathways, apoptosis, cell adhesion and protein sorting (Brown and London 1998; Simons and Toomre 2000; Harris and Siu 2002; Tsui-Pierchala et al. 2002).

Raft and non-raft microdomains are usually recognized by their different solubility in detergents. Non-raft microdomains are soluble in mild detergents as CHAPS [(3-[(3 cholami dopropyl) dimethylammonio] propanesulfonic acid] or Triton-X100, whereas LR are not. Accordingly, purified myelin proteins extracted with CHAPS have been found to be associated mainly with glycerophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE). Lipid-bound myelin basic protein (MBP) from CNS (Riccio et al., 1984, 1994) and lipid-bound P2 from PNS myelin (Riccio et al. 1998; Sedzik et al. 2003) were the first good examples of proteins belonging to the non raft microdomains. Other myelin proteins have been mainly classified as LR proteins. In particular, with regard to PNS, it has been shown that P0 is associated to the raft domains (Hasse et al. 2002). However, exclusive partitioning of myelin proteins in raft and non-raft microdomains, which are obtained by selective membrane extraction with different detergents in the cold, might not correspond to a general behavior and it is now almost accepted that most myelin proteins may distribute themselves in both microdomains. In this case, the driving force that gives rise to their different distribution in raft/non-raft domains is not known yet.

To assess whether P0 is present in different membrane microdomains, we have purified P0 from both non-raft (soluble P0, P0-S) and raft (P0-R) regions of PNS. Purified proteins were analyzed by bidimensional gel electrophoresis and identified and characterized by MALDI ToF mass spectrometry. A detailed structural description of the two P0 forms is given in terms of amino acid sequence, post-translational modifications and composition of

associated lipids. Our findings suggest that structural differences between the two proteins might be responsible for their different localization.

EXPERIMENTAL PROCEDURE

Trypsin, dithiothreitol, iodoacetamide, glycerol and thioglycerol were purchased from Sigma. CHAPS and EDTA were either from Boehringher or from Amersham. Peptide N-glycosidase A (PNGase A) was from Roche. Pre-packed PD-10 gel filtration cartridges were from Pharmacia. Pre-packed Sep pak C18 cartridges were purchased from Waters. All other reagents and solvents were of the highest purity available from Carlo Erba, Italy.

Myelin preparation

Bovine spinal roots were obtained from 18 months old cows of the strains Brown Swiss or Holstein Friesian in a local slaughterhouse (Mattatoio Saponaro/Ciavarella, Noicattaro, Bari), transported on ice in the lab, cut into pieces and stored at –70 °C until use.

Peripheral nerve myelin was prepared as described previously (Riccio et al. 1998; Sedzik et al. 2003). Briefly, bovine spinal roots were homogenized at 0.3 g tissue/ml in 0.32 M sucrose solution containing proteases inhibitors i.e. 1m M EDTA/ 1 mM β-mercapto-ethanol (β-SH)/ 0.25 mM PMSF. The suspension was layered on 0.85 M sucrose, containing the same inhibitor mixture, and centrifuged at 140,000 x g for 40' at 4 °C. Myelin was recovered at the interface of the two different sucrose concentrations, washed twice with water and subjected to a second sucrose gradient stratification. Myelin recovered after the second sucrose gradient and two washes with water in the presence of proteases inhibitors, was treated with 500 mM NaCl in 20 mM Tris pH 8.5 to remove saline-soluble proteins, in particular some loosely-associated proteases. The NaCl-treated myelin (NaCl-TM) was stored at -70° until extraction

with detergent. In all steps protein content was determined according to Bradford procedure using myelin basic protein (MBP), purified with the Deibler et al. (1972) procedure, as standard (Riccio et al. 1994, 1998; Sedzik et al. 2003).

Lipid analysis by High-Performance Thin-Layer Chromatography (HPTLC)

Lipids were extracted from samples of 60 μg protein according to procedure of Folch et al. (1951) by adding four volumes of chloroform : methanol (2 : 1 v/v). The mixture was centrifuged for 2 min at 13.000 rpm. Next, 4/3 vol H_2O and 4/3 vol chloroform were added. After centrifugation for 5 min at 13.000 rpm, the upper (methanol) phase and the protein phase (at the interface between the two phases) were discarded. HPTLC was carried out on Whatman LHPK 60 Å plates as described in Ganser et al. (1988). The lipids were detected by 10% $CuSO_4$ in 8% H_3PO_4 . The eluent was methylacetate : 1-propanol : chloroform : methanol : 0.25 % NaCl : acetic acid (25 /25 /25 /10 /9 / 0.3 v/v).

For neutral lipids the plate was first developed with chloroform : methanol : acetic acid (93.1 / 1.9 / 0.1) to 1.5 - 2.0 cm from the top of the plate, and then redeveloped with a mixture of hexane - ethyl ether - acetic acid (89.3 : 5.7 : 0.1) in the same direction and to the top of the plate. Lipids were detected by 10% $CuSO_4$ in 8% H_3PO_4.

Computerized densitometric analysis of HPTLC plates was carried out in triplicate with the Image Master 1D, Pharmacia Biotech, Uppsala, Sweden. Results are expressed as relative percentage of each lipid referred to the total OD x mm^2 in the same lane or by representing the OD x mm^2 directly.

Extraction with CHAPS of the myelin residue after treatment with salts: the P0-S protein

Salt-treated myelin was treated with 2.5 % CHAPS / 500 mM NaCl / 20 mM Tris-HCl, pH 8.0 in the presence of protease inhibitors (1 ml/ 2.5 mg prot.) and homogenized with a Potter-Elvehjem apparatus. The homogenate was left at 4 °C for 30' and centrifuged at 161,000 x g for 40' at 4 °C. The supernatant obtained after the centrifugation was applied on a hydroxyapatite column (HA) equilibrated with 1 % CHAPS/ 20 mM TRIS/ 0.5 mM EDTA/ 0.5 mM EGTA/ 1 mM β-SH/ 1 mM $ZnCl_2$, pH 8.0, using the FPLC system of Pharmacia Biotech, at a ratio of 3 mg prot./ ml HA.

Extraction with SDS of the CHAPS- treated myelin residue: the P0-R protein

The material not extracted with CHAPS (CHAPS residue) was washed twice with 10 volume of TE buffer (25 mM TRIS/ 5 mM EDTA, pH 7.4) in order to remove the residual CHAPS detergent. The washed pellet was treated with 2 % SDS in TNE buffer (25 mM TRIS /150 mM NaCl/ 5 mM EDTA, pH 7.4) in a ratio of 3 mg prot./ml buffer. The SDS homogenate was incubated for 18 h at room temperature and then centrifuged at 161,000 x g for 40' at 20 °C. The SDS extracted proteins (raft proteins) were diluted 20 times with TNE buffer to a final concentration of 0.1% SDS and charged onto a HA column equilibrated with 0.1 % SDS/ 20 mM TRIS/ 0.5 mM EDTA/ 0.5 mM EGTA/ 1 mM β-SH/ 1 mM $ZnCl_2$, pH 8.0.

Gel Filtration

Both samples containing P0-S and P0-R proteins were applied to gel filtration on a Hi load 16/60 Superdex 200 prep grade using the FPLC system of Pharmacia Biotech. Flow rate was 0.5 ml /min and equilibration and elution buffer for P0-S was CHAPS/ 20 mM TRIS/ 150 mM NaCl/ 0.5 mM EDTA/ 0.5 mM EGTA/ 1 mM $ZnCl_2$ and 1 mM β–mercaptoethanol. In the elution buffer for P0-R 0.5 % CHAPS was substituted by 0.1% SDS. Standards proteins

were the high molecular and low molecular kits for gel filtration from GE Healthcare.

Sample preparation for 2D

Aliquots of purified P0-S and P0-R (200 µg of proteins) were treated according to Wessel and Flügge (1984) in order to remove both detergent and salt, and the obtained pellets were resuspended with 450 µl of the re-hydration solution containing 7 M urea, 2 M thiourea, 2 % CHAPS, 20 mM DTE, 0.5 % 4-7 IPG, 1 % IPG 3-11 NL, plus a trace of bromophenol blue. Before isoelectrofocusing (IEF), samples were sonicated on ice for 5'' at maximum power on a sonicator bath. The samples were loaded on 24 cm IPGphor strip holder and IEF was performed on 24 cm non-linear pH gradient 3-11 IPG Dry-Strips (Amersham Biosciences). IPG Dry-Strips were re-hydrated with the sample-containing re-hydration solution (Sanchez et al., 1997; Rabilloud et al., 1994). IEF was run on an IPGphor unit (Amersham Biosciences) for a total of 59750 Vhs.

After IEF, IPG-strips were equilibrated in the following buffers: a) 6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris-HCl pH 8.8, 1 % DTE (15 min), and b): 6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris-HCl pH 8.8, 4 % iodoacetamide plus a trace of bromophenol blue (15 min) (Görg et al., 2000).

For the second dimension, the IPG strips were positioned onto a home-made homogeneous running gel (15% acrylamide) and sealed with 2 ml hot 0.5 % agarose solution in electrode buffer (25 mM Tris, 192 mM glycine, 0.1 % w/v SDS, pH 8.3 plus a trace of bromophenol blue). SDS-PAGE was carried out on the Ettan DALT II system of Amersham Biosciences. The run was performed at 10 °C, 2 Watt / gel, until the bromophenol blue reached the bottom of the gels (about 14 hrs). Gels were stained for 1 h in 0.2 % Coomassie blue R-250 and 0.05% Coomassie blue G-250 in methanol:acetic acid:water (4:1:4, v/v/v), and the excess dye was removed with acetic acid:methanol:water (1:4:5, v/v/v). The gels were

then scanned with the ImageMaster DTS scanner of Pharmacia Biotech using the ImageMaster 2D Elite software (Amersham Biosciences).

In-gel digestion

Spots or bands of interest were excised from Coomassie-blue-stained gels. The gel pieces were put in 1 ml Eppendorf cups and destained overnight with a solution containing 25 mM ammonium bicarbonate/ 50 % acetonitrile. The supernatant was discarded and the gel pieces were dehydrated by acetonitrile, swelled in 25 mM ammonium bicarbonate and shrunk again in acetonitrile (Shevchenko et al., 1996). The liquid phase was removed and the gel pieces were dried in speed-vac. Proteins were then digested in-gel with trypsin (Promega, Madison, WI, USA) overnight at 37°C. The generated peptide mixtures were analyzed by MALDI-ToF mass spectrometry.

Chemical and enzymatic hydrolysis.

Aliquots of purified P0-S and P0-R (150 µg proteins) were reduced and alkylated under nitrogen in 1 M Tris HCl, pH 8.0, containing 6 M guanidine HCl and dithiothreitol (10-fold molar excess over the thiol groups of the protein) for 2 h at 37 °C. Iodoacetamide (Sigma, 1:5-fold molar excess over the total thiols) was then added and the mixture was incubated in the dark at room temperature for 30 min before the reaction was stopped by addition of acetic acid. Reduced and carboxyamidomethylated P0-S and P0-R were digested with trypsin (Sigma) in 50 mM ammonium bicarbonate, pH 8.5, using an enzyme/substrate ratio of 1/25 (w/w) for 6 h at 37 °C. Deglycosylation of the peptide mixtures was performed by treatment with PNGase F in 50 mM ammonium bicarbonate, pH 8.5, overnight at 37 °C using 0.1 enzyme unit per 300 µg of protein. N-linked oligosaccharide chains released by PNGase F

were separated from peptides by reverse phase chromatography on prepacked Sep-pak cartridges.

Sample preparation for MALDI-ToF mass spectrometry analysis

Identification of P0 samples fractionated by mono- and/or bidimensional gel electrophoresis was carried out by the peptide mass fingerprinting procedure using MALDI-ToF mass spectrometry analysis. Aliquots of 2 µl of each peptide mixture was mixed with 2 µl of α-cyano-4-hydroxy-cinnamic acid solution in 50 % v/v acetonitrile/0.5 % v/v TFA. Subsequently 0.3 µl of this matrix-peptides mixture was applied in triplicate to the MALDI target and allowed to air dry. Mass spectra were acquired in reflectron mode using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Calibration of the time-to-mass scale was performed using two internal matrix mixture standard peptides (ile-7AngIII, M+H 897.531, monoisotopic, and hACTH 18-39, M+H 2465.191, monoisotopic). Mass finger-printing database searching was carried out using an on-line available version of the Mascot software (http://www.matrixscience.com).

Structural characterization of the P0 glycoprotein forms and its glycosidic moieties were performed by MALDI MS analyses. Reflectron MALDI spectra and MALDI-PSD spectra were recorded on a Voyager DE STR PRO instrument (Applied Biosystems, Framingham, MA). MALDI matrices were prepared by dissolving 20 mg of DHB or 10 mg α-cyano-4-hydroxy-cinnamic acid in 1 ml acetonitrile/0.1 % trifluoroacetic acid (70:30 v/v). Typically, 1 µl of matrix was applied to the metallic sample plate and 1 µl of analyte was then added. Acceleration and reflector voltages were set up as follow: target voltage at 20 kV, first grid at 95% of target voltage, delayed extraction at 600 ns. PSD fragment spectra were acquired after pseudomolecular ion MH+ selection using the time ion selector. Fragment ions were

refocused by stepwise reducing the reflector voltage by 20%. The individual segments were then stitched together using the Applied Biosystems software.

RESULTS

Extraction of P0-S and P0-R

After treatment (negative extraction) of PNS myelin with sodium chloride, the non-extracted material was treated with CHAPS. The CHAPS extract, defined as the non-raft domain of myelin, was the starting material for the purification of P0-S, the P0 protein soluble in CHAPS.

The myelin fraction that was not extracted with CHAPS was defined as the raft-domain of myelin. The extract obtained by treatment with SDS of the CHAPS residue was the starting material for the purification of P0-R.

The flow-sheet corresponding to the different myelin extraction steps is described in Fig 1.

Aliquots (about 10 µg) of the CHAPS supernatant (soluble non-raft fraction) and the CHAPS residue (raft fraction) were analysed by SDS-PAGE to evaluate the distribution of P0 between the two regions. As shown in Figure 2A, a 30 kDa band, corresponding to the P0 protein, was present in both CHAPS supernatant and CHAPS residue (lanes 2 and 3), whereas P2 and MBP, the other abundant myelin proteins, were found in the soluble portion.

The 30 kDa bands were excised from gel, digested with trypsin and the resulting peptide mixtures were analyzed by MALDI-ToF mass spectrometry. The mass values

recorded in the spectra were used to search for a non-redundant protein databank. As a result, both bands were confirmed to be bovine P0 protein (Swiss Prot AC P10522). Figure 2B shows the quantitative evaluation of the two P0 forms obtained by densitometric analysis of the gel using ImageMaster 1D (Pharmacia Biotech). The image analysis revealed that P0-S accounted for 27% and P0-R for 73% of the total P0 protein.

Lipid composition of CHAPS extract and CHAPS residue was evaluated by HPTLC analysis of polar lipids, according to Ganser et al. (1988) (Fig 2C). The resulting lipid bands were subjected to densitometric analysis and their relative percentages are reported in Fig 2D. Lipid patterns of the two P0 forms were, as expected, different.

Purification of P0-S

CHAPS-soluble P0-S was purified on a HA column equilibrated with 20 mM TRIS buffer, 0.5 mM EDTA, pH 8.0, containing 1 % CHAPS. After elution with the equilibrating buffer of the non-adsorbed (pass-through) myelin proteins, a linear gradient of 50-250 mM NaPi was applied to elute adsorbed proteins. P0-S emerged from the column between 175 and 250 mM NaPi. All the chromatographic fractions were analyzed by SDS-PAGE and the more homogeneous fractions were pooled and stored for further analysis. The recovered P0-S accounted for about 5% of the total CHAPS extracted proteins (average of three experiments).

The HA-P0-S pool fraction was applied to gel filtration on Superdex 200. The apparent molecular mass of P0-S determined by this method was found to be 187 kDa (not shown), accounting for a hexameric assembly of P0-S, in agreement with the tendency of P0 to form dimeric and tetrameric structures (Shapiro et al. 1996; Xie et al. 2007).

Unambiguous identification of the purified protein as P0 was obtained by the peptide mass fingerprinting procedure. Gel bands corresponding to the purified P0 protein were excised, digested with trypsin and the resulting peptide mixtures directly analyzed by MALDI

ToF mass spectrometry. Protein identification was performed as reported above using the MASCOT software (www.matrixscience.com). As expected, the Mascot output assigned the highest similarity to bovine P0 myelin protein (Table1). A total of 6 peptides having positive match with peptides obtained from the theoretical digestion of P0 were identified, all of which located in either the extracellular or the cytoplasmic domain of the protein. No peptides from the transmembrane region of the protein could be detected.

P0-R purification

CHAPS insoluble proteins were extracted in SDS and purified on the HA column as previously described for P0-S. Non adsorbed proteins were eluted in the equilibrating buffer while bound P0-R was eluted with a NaPi linear gradient from 60 mM to 225 mM. The apparent molecular mass of P0-R, as determined by gel filtration on Superdex 200, was 32 kDa.

Purity of the collected fractions was assessed by SDS PAGE. Two bands with an apparent molecular mass of 33 kDa and 30 kDa respectively were detected and identified by mass spectrometry as described in the previous paragraph. Identical peptides were observed in both bands. As reported in Table 1, both matched with bovine P0 myelin protein. As in P0-S, no peptides from the transmembrane region were detected. The recovery of P0-R was estimated as 24% of the total SDS extracted protein (mean of three experiments).

Lipid analysis

In order to assess differences in the lipid composition associated with P0-S and P0-R, the lipid composition of the two protein samples was determined by two different HPTLC analyses for polar and neutral lipids, according to Ganser et al. (1988). Protein samples were

subjected to gel filtration in order to further remove non-bound lipids, before HPTLC analyses. The relative percentage of each lipid was calculated as reported above.

Figures 3A and 3B show the HPTLC analysis of the two P0 samples and the corresponding calculated relative amounts of associated lipids. Some differences in lipids association between the two protein forms could be easily detected. Cerebrosides and sulphatides were more present in association with P0-R rather than with P0-S, that alternatively showed a higher association with PE and PS as previously reported for other lipid-bound non-raft myelin proteins soluble in CHAPS, such as MBP in the CNS (Riccio et al. 1994; Haas et al. 2004) and P2 in the PNS (Sedzik et al. 2003).

Bidimensional gel electrophoresis of P0-S and P0-R

The different isoforms of both P0-S and P0-R proteins were fractionated by two-dimensional gel electrophoresis (2-DE). Before analysis, both proteins were subjected to gel filtration chromatography in order to remove traces of contaminating proteins and free lipids.

Figures 4A shows the 2-DE of P0-S. Four different spots were detected. Individual spots were submitted to mass spectrometric identification following the procedure described above. Spot 1, with an apparent molecular mass (Mr) of 43 kDa and pI of 6.7, was identified as a splicing variant of Annexin 11 (Swiss Prot entry P27214-2 VSP000289). The remaining three spots sharing an apparent molecular mass of 31 kDa and with isoelectric points ranging between 10.2-10.6 were all identified as P0 bovine myelin protein (Swiss Prot entry P10522).

The 2-DE of P0-R is shown in Fig. 4B. Only two protein spots with an apparent molecular mass of 33 kDa but different pI could be detected. Mass spectral analyses identified both species as bovine P0. Table 2 summarizes the tryptic peptides detected in both P0-S and P0-R mass spectral analyses and the protein identification scores. As shown in table 2, the

matching peptides obtained from in-gel digestion of P0-S and P0-R covered 25% and 28 % of P0 protein sequence respectively.

Structural characterization of P0-S and P0-R

P0-S and P0-R proteins were submitted to a detailed structural characterization to verify both amino acid sequence and post-translational modifications. The aim was to detect possible structural differences characteristic of the various P0 forms observed in the 2-DE experiments.

Aliquots of intact P0-S and P0-R (100 µg) were reduced and carboxyamidomethylated under denaturing conditions, digested with trypsin and deglycosylated by incubation with PNGase F. The oligosaccharides were separated from the peptides by a Sep-pak reverse phase chromatographic step and the glycan portion was stored for further analysis (see below).

The resulting peptide mixture was analyzed by MALDI MS. Mass signals were assigned to the corresponding peptides within the protein sequence on the basis of their molecular mass and the specificity of the enzyme. Results are summarized in Table 2. The mass spectral analysis led to the verification of the entire primary structure of both proteins and to the assessment of a single N-glycosylation site. As reported in the table, the mass signals at m/z 3260.5 and 2441.1 occurred 1 Da higher than the expected mass value for the peptides 80-109 and 80-101 respectively as Asn93 was converted into Asp following PNGase F hydrolysis.

As expected, the peptide mass fingerprinting of both P0-S and P0-R were essentially identical although few differences related to post-translational modifications could be detected. Figures 5A and B show the partial MALDI mass spectra of the peptide mixtures generated from P0-S and P0-R digestion in the region where differences could be observed. Two mass signals at m/z 1363.7 and 1257.6 were detected in the P0-S spectrum and attributed to the phosphorylated peptides 171-182 and 199-209 respectively. These signals were absent

in the corresponding P0-R spectrum where a new signal could be detected at m/z 1433.7 and assigned to the phosphorylated form of the peptide 56-67 modified at Thr65. All these signals disappeared following incubation of the peptide mixtures with alkaline phosphatase (data not shown)

Characterization of the oligosaccharide moieties of P0-S and P0-R.

The structure of the glycosidic portion of P0-S and P0-R was investigated by direct MALDI MS analysis of the oligosaccharide mixtures both in positive and negative ion mode. Figures 6A and B show the glycosylation profile of the two P0 forms obtained in positive ion mode demonstrating that the oligosaccharide structures occurring in P0-S and P0-R belong to both the complex and hybrid types. Both complex and hybrid glycans essentially consist of sulphated biantennary structures carrying a different number of modifying groups that include fucose and N-acetyl neuraminic acid residues.

Most of these structures were confirmed by a detailed investigation performed by tandem MS/MS experiments using MALDI PSD analyses. The PSD spectra are dominated by Y and B ions containing the non-reducing and the reducing end of the glycan moieties that define the sequence, and the modifying groups of the antennae. A number of internal fragment ions formed by multiple glycosidic cleavages could also be observed from which the branching of the antennae was inferred. As an example, Fig. 7 shows the MALDI PSD spectrum obtained by selecting the precursor ion at m/z 1808.9 in the positive spectrum. The B5 ion at m/z 1442.39 resulting from the cleavage of the glycosidic bond between the two GlcNAc core residues led to the localization of the Fuc moiety on the first GlcNAc. The B2α and B3α ions at m/z 551.6 and 388.5 respectively defined the symmetric sequence of the two antennae, whereas the poorly resolved Y1α at m/z 1662.3 indicated the loss of the Fuc residue. Finally, a number of internal fragment ions related to double cleavages could also be

detected indicating the branching of the antennae. As a whole, these PSD data identified the oligosaccharide species at m/z 1808.9 as a biantennary structure with a Fuc unit on the first GlcNAc core residue.

Table 3 summarizes the mass signals recorded in the MALDI spectra of the oligosaccharide mixtures from P0-S and P0-R and the corresponding glycan structures as inferred by MS data. As shown in the Table, P0-S and P0-R essentially contain similar N-linked glycan structures belonging to both complex and hybrid types. However, significant differences could be observed in the relative abundances of the oligosaccharide moieties between the two protein forms, with P0-S showing a larger proportion of biantennary complex sugars while hybrid structures constitute the major glycosidic moieties N-linked to P0-R.

DISCUSSION

The myelin membrane is very rich in lipids. Most of them are glycosphingolipids and cholesterol (about 56 %). It is now acknowledged that glycosphingolipids and cholesterol are required for the formation of lipid rafts and that lipid rafts are present in myelin (DeBruin and Harauz 2007; Arvanitis et al. 2005; Taylor et al. 2002). Myelin rafts originate from the oligodendrocyte but then they assume their own pattern (Gielen et al. 2006). Glycolipid storage disorders lead to mistargeting of raft-associated myelin proteins and demyelination (Saravanan et al. 2004). Myelin proteins are distributed in the raft and non-raft domains of the membrane depending on their nature and their function. The repertoire of myelin raft proteins included in the CNS: CNPase, MOG (Kim and Pfeiffer 1999), MAL (Schaeren-Wiemers et al. 2004) and PLP (Simons M. et al. 2000), but also part of myelin basic protein (MBP) (DeBruin et al. 2005), a protein which has been always considered as an extrinsic, water-

soluble, and unstructured protein but with a high tendency to bind lipids (Smith 1992; Riccio and Quagliariello 1993). In the PNS the raft-associated proteins are P0, PMP22 and plasmolipin (Hasse et al. 2002).

The findings above show that all myelin proteins, which are probably involved in cellular signaling and trafficking, myelin sheath compaction, or interaction with the axon, are part of the raft microdomains. The raft proteins are also those that are apparently involved in specific myelin diseases: MBP, PLP and MOG in multiple sclerosis (DeBruin et al. 2006, McFarland and Martin, 2007; Steinman, 2007); P0 and PMP22 in demyelinating neuropathies such as Charcot-Marie-Tooth (CMT) type 1B, Déjèrine-Sottas neuropathy and congenital hypomyelinating neuropathy (Laurà et al. 2007; Wrabetz et al. 2006).

On the other hand, it might be expected that myelin proteins do not belong exclusively to the raft or to the non-raft domain. In this case the question arises what makes a protein suitable for the one rather than for the other domain and if membrane destination might be dictated by posttranslational modifications of the protein.

On these grounds, the purpose of this study was to investigate whether, how and to which extent, a PNS protein as P0 distributes itself in the myelin membrane, between the raft microdomains and the soluble non-raft portion of the membrane. This is actually a topic of large interest since myelin has a low protein/lipid ratio and contains only few protein types.
A good example of protein partitioning in the raft/non-raft microdomains of myelin was given in the CNS by MBP, which distributes itself depending on different post-translational modifications occurring to the protein in different developmental stages of life (DeBruin et al. 2005).

Since P0 and MBP are both involved in myelin compaction, respectively in PNS and in CNS, but are very different, we found it worthy to investigate in which aspects their

mechanisms of partitioning are different. MBP is defined as an extrinsic, water-soluble, lipid-free and intrinsically unstructured protein, whereas P0 is described as a transmembrane glycoprotein with a tendency to form dimers and tetramers (Shapiro et al. 1996; Inouye et al. 1999; Thompson et al. 2002; Gallego et al. 2001).

In this paper, we have reported on the different extraction of bovine P0 from the raft and non-raft myelin microdomains and on the isolation of the corresponding two forms of P0: P0-S extracted from the non-raft region with CHAPS, and P0-R, extracted with SDS from the raft region. The two P0 proteins were purified using a phosphate gradient on HA column and gel filtration and were characterized by 2D electrophoresis, mass spectrometry and HPTLC. Isolation of the two forms can be useful in order to understand if each of them has specific roles whose loss may be important in peripheral neuropathies.

P0-S, the non-raft protein soluble in CHAPS, was purified in the hexameric form, whereas P0-R, the raft protein extracted with SDS, was purified in the monomeric form. Circular dichroism analysis, performed on the P0-S protein only (not shown) (exp. by Eugenia Polverini, Univ. of Parma), indicated the presence of 24% alpha-helix, 33% beta-sheet, 24% beta-turn, and 19% other structures, in agreement with the predicted structure (Inouye and Kirschner 1991) but different from the P0 structure in SDS (Sedzik et al. 2002). Localization of P0-S and P0-R on 2D gel electrophoresis was different. P0-S showed a certain difficulty to focalize revealing a heterogeneous population with different isoelectric points. On the contrary P0-R showed only two well-resolved spots with different pIs but with the same apparent molecular mass (Mr). Peptides resulting from gel digestion showed phosphorylated sites and a relative abundance of N-linked glycoforms. Structural analysis of the sequences of Asn 93 bound glycogroups was performed by MALDI MS mass spectrometry.

The presence of the spot corresponding to Annexin was probably due to the very small difference in the size of the two proteins. P0-S seems to be strictly associated with Annexin splicing variant, whereas P0-R appears to be pure after gel filtration. The more difficult focalization of P0-S, when compared to P0-R, suggests that there is probably a heterogeneity in the P0-S population extracted with CHAPS. Using mass spectrometry and NMR, Gallego et al. (2001) have shown the presence of different N-glycans associated with P0. Other reports have shown that the carbohydrate attachment site is mutated in one case of CMT1B suggesting a role of carbohydrate moiety in that disease (Warner et al. 1996).

A detailed mass spectrometric analysis was then separately performed on P0-S and P0-R. Previous work performed by Xie et al. (2007) had well characterized glycoforms and post translational modifications from a P0 protein in Xenopus levis peripheral nerve by using ESI-FTMSMS technique. The major glycan structures were found to belong to high mannose and hybrid type containing sialic acid and sulphate groups thus supporting the concept of species-specific variation in N-glycosylation of P0.

Here we wanted to establish whether differences in glycan composition constituted the structural basis of the different localization of P0 in the bovine myelin membrane. For this reason we performed a glycan mass spectrometry analysis separately on P0-S and P0-R. We found that, differently from MBP (which is unglycosylated), P0 distribution in myelin microdomains depends on the types of glycoforms bound to Asn93. The mass spectral results, in fact, suggested that the presence of biantennary complex glycans in P0-S and hybrid oligosaccharides structures in P0-R might represent the labels for the correct targeting of the two P0 forms to their respective microdomains. This different distribution of P0 in the membrane might also be correlated to a more or less adhesive function in myelin. The different lipid composition of P0-S and P0-R might then be considered only a a consequence of their different partitioning in myelin microdomains.

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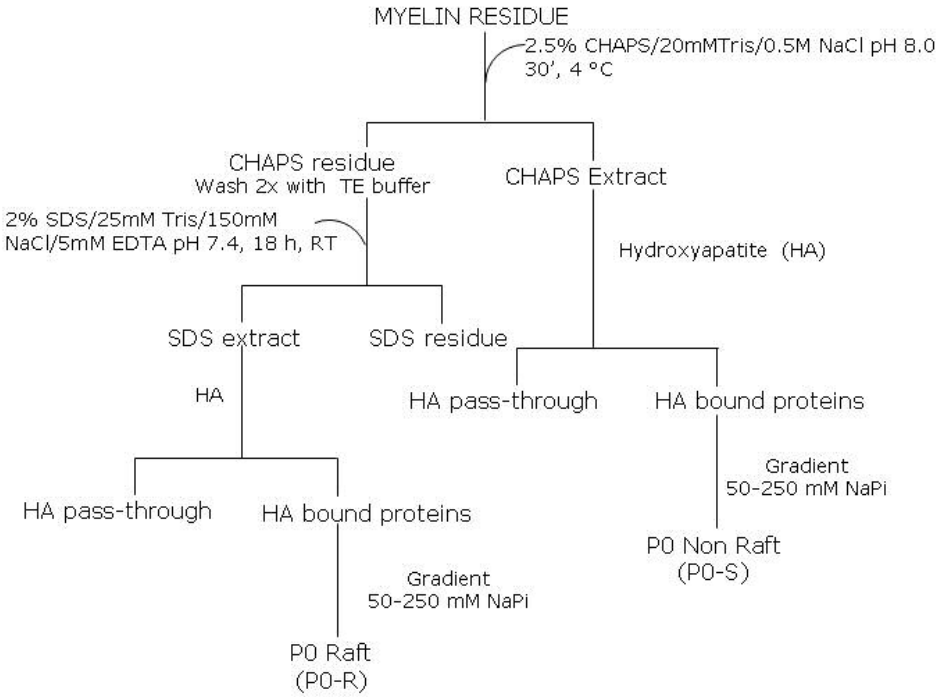

Fig. 1: Schematic representation of the different myelin extraction procedures for the purification of P0-S and P0-R.
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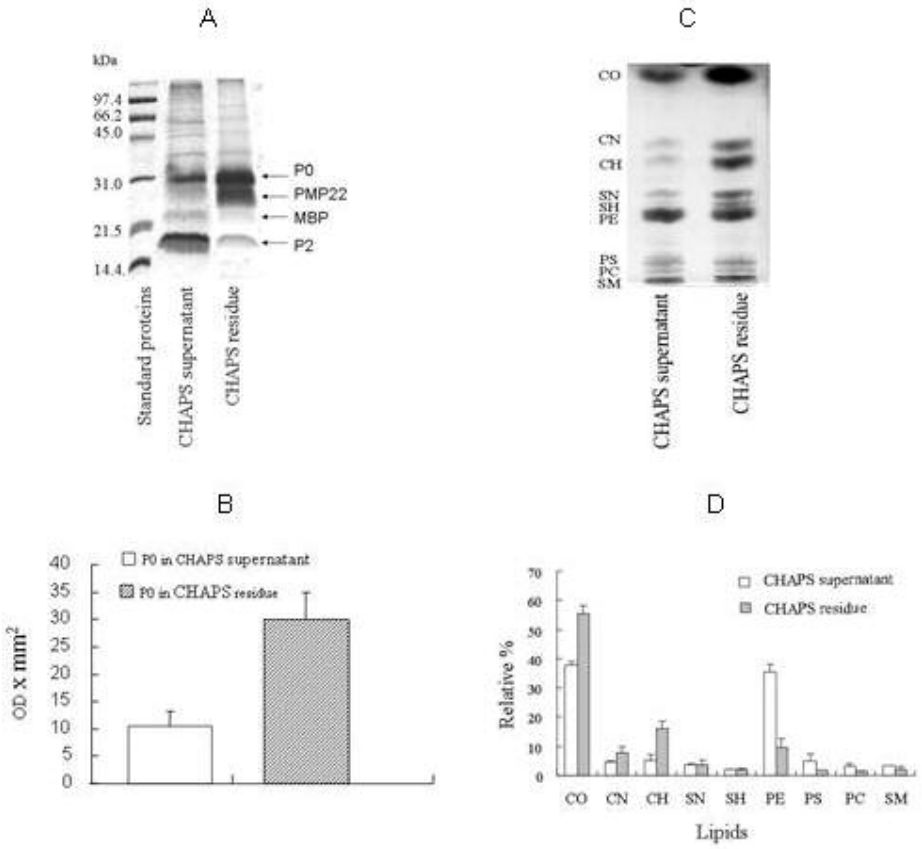

Fig. 2: SDS-PAGE of proteins (A+B) and High-performance thin-layer chromatography (HPTLC) analysis of lipids (C+D) in myelin extracts. Panel A: 15% acrylamide SDS-PAGE of PNS myelin proteins in CHAPS extract and CHAPS residue. Panel B: Densitometric analysis with ImageMaster 1D of the P0 distribution pattern in the SDS gel electrophoresis shown in panel A. Panel C: HPTLC analysis of polar lipids in CHAPS supernatant and CHAPS residue. Abbreviations: CO, cholesterol; CN and CH, nonhydroxy- and hydroxycerebrosides; SN and SH nonhydroxy- and hydroxysulphatides; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcoline; SM, sphingomyelin. Panel D: Relative lipid percentages in CHAPS extract and CHAPS residue as obtained by densitometric analysis. The values were respectively: CO, 37.9 /55.3; CN, 4.5/7.8; CH, 5.2/16.0; SN, 3.7/3.7; SH, 2.0/2.0; PE, 35.2/9.5; PS, 5.0 /1.9; PC, 3.1/1.4; SM, 3.4/2.3.

187x161mm (72 x 72 DPI)

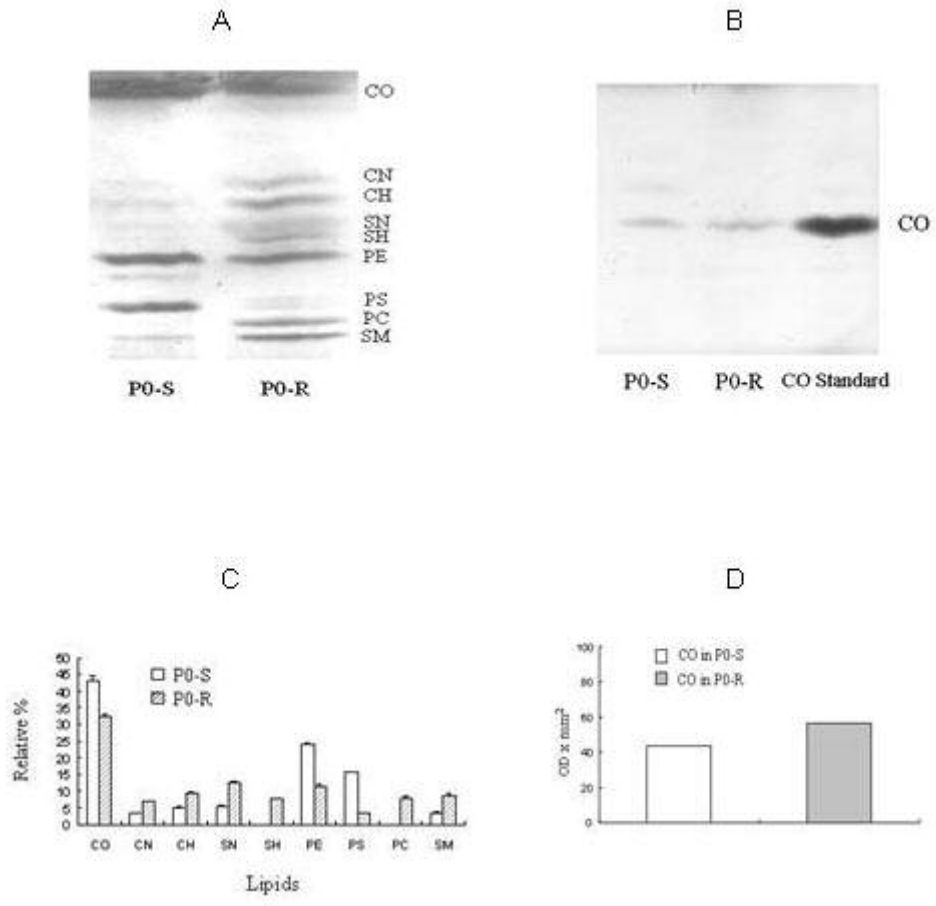

Fig. 3. HPTLC analysis of polar and neutral lipids associated to the purified proteins P0-S and P0-R. Panel A: HPTLC analysis of polar lipids associated to P0-S (lane 1) and P0-R (lane 2). NL, neutral lipids; other abbreviations as in fig. 2. Panel B: HPTLC analysis of cholesterol and neutral lipids in P0-S and P0-R. Panel C: Relative percentage of polar lipids bound to P0-S and P0-R calculated by densitometric analysis. The values for P0-S and P0-R respectively [in brackets, for comparison, the values for non-raft CNS MBP (Haas et al. 2004)], were the following: CO and neutral lipids, 43.1/32.4 (31.5); CN, 3.2/6.9 (2.5); CH, 5.3/9.3 (6.0); SN, 5.5/12.5 (1.5); SH, 0.0/7.6 (0.0); PE, 24.0/11.2 (48.0); PS, 15.5/3.2 (2.5); PC, 0.0/8.0 (7.0); and SM, 3.4/8.9 (1.0). Panel D: Analysis of neutral lipids showing a CO ratio in P0-S/P0-R of 0.75.

171x165mm (72 x 72 DPI)

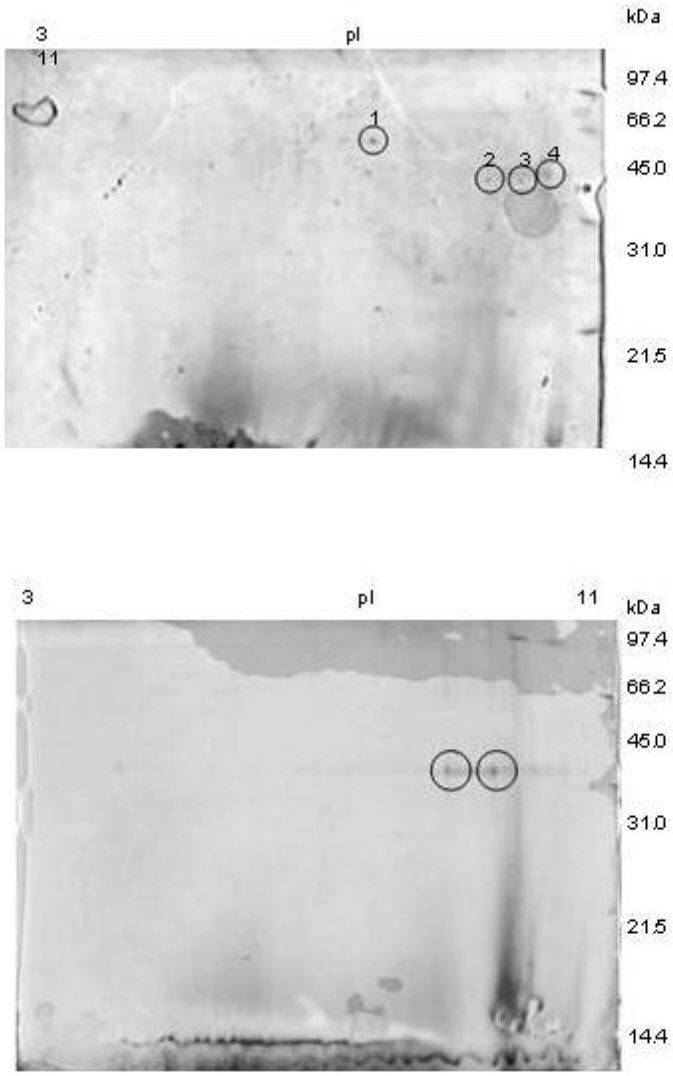

Fig. 4: Two-dimensional gel electrophoresis of purified P0-S (A) and P0-R (B) after gel filtration.
138x212mm (72 x 72 DPI)

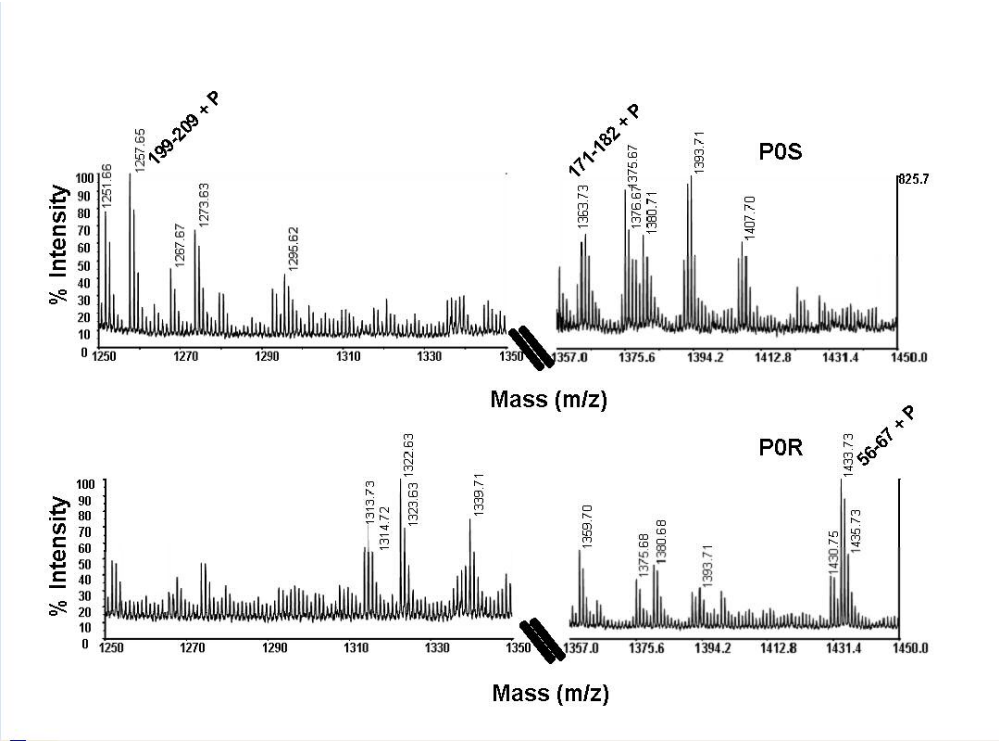

Fig. 5: Partial MALDI MS spectra of peptide mixtures from P0-S (panel A) and P0-R (panel B). Phosphorylated peptides are indicated.
381x281mm (72 x 72 DPI)

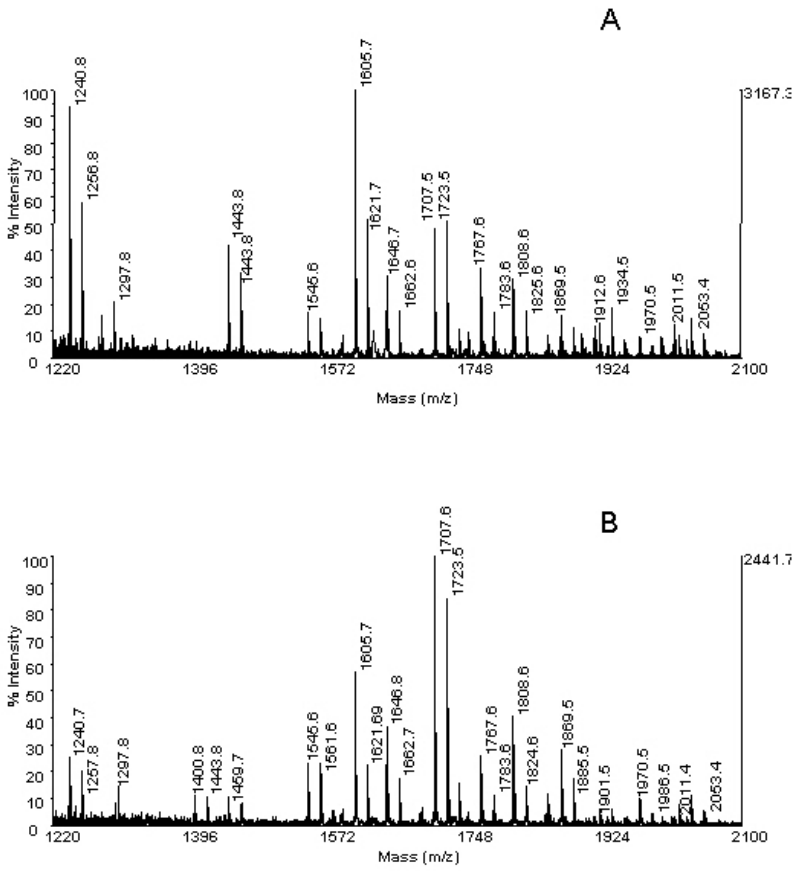

Fig. 6: MALDI-MS analysis of oligosaccharide mixture from P0-S (panel A) and P0-R (panel B).

231x225mm (72 x 72 DPI)

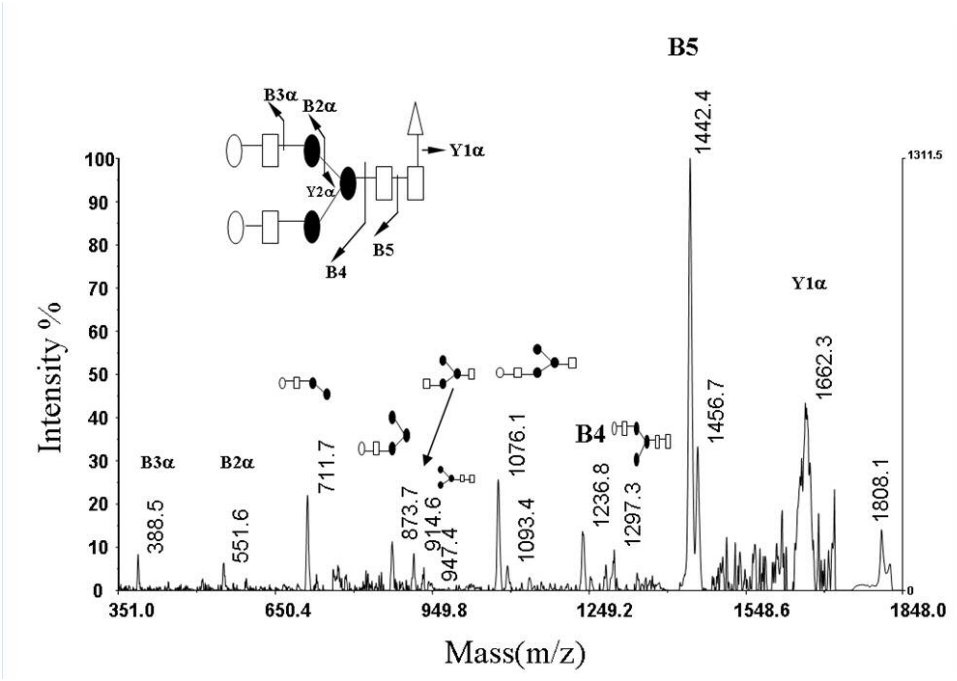

Fig. 7: MALDI PSD spectrum of m/z 1808.9 precursor ion. Y, B series and internal fragments are indicated.
365x247mm (72 x 72 DPI)