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QUARTERLY

Characterization of the oligosaccharide component of arylsulfatase B from rat liver*

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A glycan chain analysis of the total oligosaccharide pool derived from rat liver arylsulfatase B was carried out by P4 Gel Permeation Chromatography and sequential exoglycosidase digestion. It was found that 71% of rat liver arylsulfatase B oligosaccharides were sialylated. The relative contribution of particular structures in the total glycan pool was as follows: sialylated biantennary complex type glycans with terminal galactose — 65%, high-mannose type glycans — 15%, biantennary complex type glycans with core fucose and terminal *N*-acetylglucosamine — 5%, O-linked oligosaccharides — 3.5%.

Arylsulfatase B (*N*-acetylgalactosamine-4-sulfatase, EC 3.1.6.12) is a lysosomal hydrolase which is widely distributed in eukaryotic and prokaryotic cells. *In vivo*, it removes a sulfate ester from *N*-acetylgalactosamine 4-sulfate at non-reducing termini of galactosaminoglycans, dermatan and chondroitin-4 sulfates [1]. Since the time when the enzyme was first studied by Derrien in 1911, a number of studies concerning its purification and function have appeared. A defect in the enzyme activity causes mucopolysaccharidosis VI (the Marotaux-Lamy syndrome, MPS VI), a severe neurological disease which is inherited as an autosomal recessive trait [2]. The availability of a rat model of human MPS VI [3, 4] should render possible development and evaluation of various strategies of treating this human disease. Arylsulfatase B is considered in the main to be the basic form, but apparent monomeric and dimeric anionic species have also been detected in rat liver [5–7]. The rat enzyme shows considerable heterogeneity with respect to its charge, as it was found using isoelectric focusing [8]. It is widely known that most lysosomal hydrolases, including arylsulfatase B, are glycoproteins which contain asparagine-linked oligosaccharide chains. The glycoprotein nature of the enzyme in question is reflected in its powerful binding to Concanavalin A-Sepharose. Despite numerous studies with arylsulfatase B purified from various sources, the knowledge of the structure of the rat liver sulfohydrolase glycans is still scant. Therefore the present study was aimed at

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Abbreviations: GPC, gel permeation chromatography; GU, glucose unit.

characterizing the carbohydrate structure of this enzyme.

MATERIALS AND METHODS

Materials. The Signal Labelling Kit, dextran, neuraminidase from *Arthrobacter ureafaciens*, α-mannosidase, β-galactosidase and β-*N*-acetylhexosaminidase from Jack bean were obtained from Oxford GlycoSystems Ltd.; Dowex AG-50 W-X12 (H^+ form) and AG-3-X4A (OH^- form) were purchased from Bio-Rad, other chemicals were of analytical grade.

Purification of arylsulfatase B. Arylsulfatase B was purified from a lysosomal fraction of three-month-old male Wistar rat livers. The lysosomal fraction was prepared by differential centrifugation [9, 10]. A crude lysosomal extract was applied to an immunoaffinity chromatography IgG-Sepharose column, prepared by coupling the polyclonal antibody raised against the purified arylsulfatase B, to CNBr-Sepharose in 20 mM Tris/HCl buffer, pH 7.4, containing 0.2 M NaCl. The enzyme was eluted with 0.2 M glycine/HCl buffer, pH 3.0, and immediately neutralized to pH 7.0 with 1% Tris solution. After dialysis against 5 mM Tris/glycine buffer, pH 8.6, the concentrated enzyme was subjected to PAGE in a 7.5% gel with 5 mM Tris/glycine buffer, pH 8.6, as an electrophoretic buffer [11]. Fragments of the gel containing the molecules of interest, detected by their enzymatic activity (reaction with *p*-nitrocatechol sulfate) [12], were cut out, covered with water and stirred until arylsulfatase B passed into the fluid. The enzymatic activity and protein concentration were determined according to the routinely used procedures [12, 13].

Glycan chain analysis. The total glycan pool of arylsulfatase B was obtained by hydrazinolysis of 250 μg of glycoprotein using the GlycoPrep 1000 (Oxford GlycoSystems Ltd.) in the N- and O-mode. The oligosaccharide fraction was labelled with 2-aminobenzamide (2-AB) by reductive amination in dimethylsulfoxide using the Signal Labelling Kit. After 2-hour incubation at 65°C, the labelled oligosaccharides were purified by paper chromatography on a 3MM Whatman strip in n-butanol/ethanol/water solution (4:1:1, by vol.), followed by scanning the strip with the GlycoScan V-1.0 and by eluting oligosaccharides with water. The resultant oligosaccharides were separated by P4 Gel Permeation Chromatography (GPC) on the GlycoMap 1000 in the high resolution mode. The calibration standard was a dextran homopolymer standard ladder of 1 to 25 glucose residues. The column temperature was maintained at 55°C. Glycans were eluted from the column with water. Then the glycan pool was sequentially exposed to a series of highly purified exoglycosidases: neuraminidase from *A. ureafaciens* in 100 mM sodium acetate buffer, pH 5.0 (0.1 unit per 10 μl of sample); α-mannosidase in 100 mM sodium acetate buffer, pH 5.0, containing 2 mM Zn^{2+} (1 unit per 20 μl of sample); β-galactosidase in 100 mM sodium acetate buffer, pH 3.5 (0.2 unit per 20 μl of sample); β-*N*-acetylhexosaminidase in 100 mM sodium citrate/phosphate buffer, pH 5.0 (0.12 unit per 20 μl of sample). After 20-hour incubation at 37°C, the oligosaccharide products were desalted by being passed through a mixed bed column of Dowex 50 W - X12 and AG 3 - X4A, prior to being applied to the GlycoMap 1000.

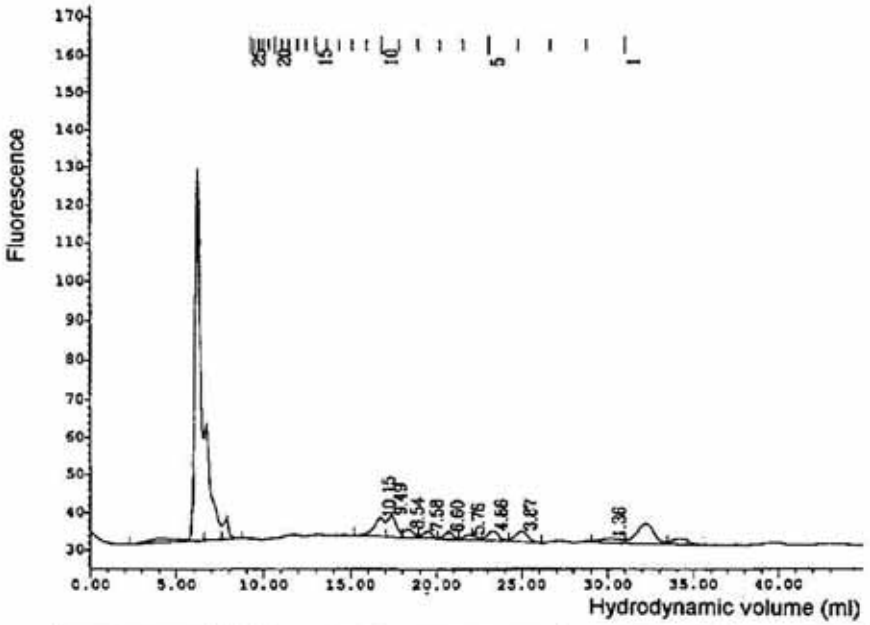
The GlycoMap 1000 automates an optimized process of Gel Permeation Chromatography [14, 15] in which neutral glycan species are separated on the basis of their hydrodynamic volume. The hydrodynamic volume of a glycan is determined by its monosaccharide composition (relative molecular mass), anomericity and position of glycosidic bonds, and degree of its branching. The separated glycans are eluted from the column in order of decreasing hydrodynamic volume and are detected by a fluorescence detector. The data output from the GlycoMap 1000 is a gel permeation chromatogram of a calibration standard (dextran) and the glycan sample. For each peak, the GU (glucose units) value is automatically determined on the basis of its hydrodynamic volume by intrapolation from the calibration curve. The area of each peak is calculated and used to estimate the relative molar concentration of the respective glycan in the glycan mixture.

Size fractionation of glycans offers advantage of providing direct structural information as the size described in GU of each glycan is related to its structure in a predictable manner [14]. From an extensive study of reference compounds over many years by many groups, the general rules which relate the GU contribution of some relevant monosaccharides to the parent glycan structure have been established. By matching the experimentally determined GU to a database of structures of many standard oligosaccharides and oligosaccharides derived from glycoprotein of known glycosylation, or to a relatively large computer generated database of 'theoretical' structures, a list of possible structures corresponding to this size is prepared [15].

These structures can be easily distinguished by numerous techniques, including compositional analysis, relative molecular mass determination, lectin-affinity chromatography, or exoglycosidase digestion. Sequential use of exoglycosidases is the classical method for obtaining structural information on a glycan. A change in elution position in a gel permeation chromatogram due to the action of an exoglycosidase gives the precise number of monosaccharide units removed.

RESULTS

The separation pattern of the arylsulfatase B glycans fractionated by GPC is shown in Fig. 1. A prediction of the oligosaccharide structure related to a particular peak was done by searching the databases for the profile matching the experimentally obtained index of glucose units. Peak No. 1 corre-



Peak No.	Hydrodynamic volume (ml)	GU index	Area (%)
1	6.23	***	74.5
2	16.72	10.15	4.9
3	17.37	9.49	4.9
4	18.37	8.54	2
5	19.48	7.58	1.7
6	20.73	6.6	1.6
7	21.19	5.76	1.6
8	23.29	4.66	2.9
9	24.99	3.87	3.4
10	30.15	1.36	2.5

Figure 1. Separation profile of 2-aminobenzamide-labelled neutral arylsulfatase B glycans on Gel Permeation Chromatography accompanied by the profile raport showing glucose units (GU) index and area % of each peak.

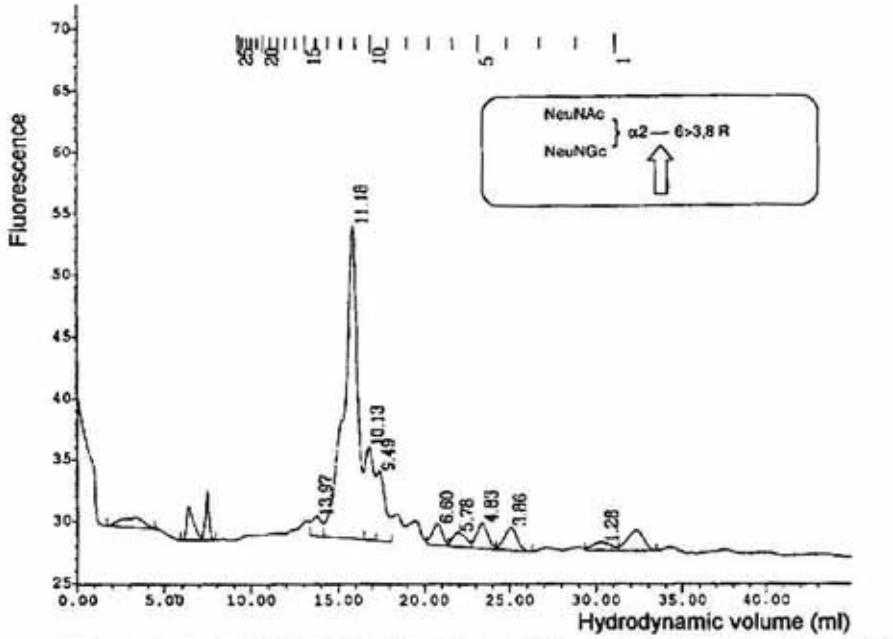
sponds to the material non separated and eluted in the void volume (charged oligosaccharides and/or contamination); peak No. 2 (10.15 GU) represents the biantennary complex type oligosaccharides with the core fucose, and/or the hybrid type oligosaccharides with the bisecting *N*-acetylglucosamine and core fucose. However, the second structure seemed scarcely possible, as all the mammalian liver glycoproteins studied so far have non-bisected N-linked oligosaccharide chains [16].

Peaks No. 3 (9.49 GU) to No. 8 (4.86 GU) are oligosaccharides of the high-mannose type, with eight to three mannose residues, respectively. Peak No. 9 (3.87 GU) represents the O-linked glycans, which are Gal-GalNAc sequences; finally peak No. 10 (1.36 GU) shows the presence of the free fluorescent dye.

The results obtained by GPC fractionation indicate that the neutral fraction of arylsulfatase B glycans consists of 64% of the high-mannose type oligosaccharides, 21% of the biantennary complex type oligosaccharides with the core fucose and/or the hybrid type oligosaccharides with the bisecting *N*-acetylglucosamine and core fucose, and 15% of the O-linked glycans.

In order to examine the nature of the material which was non separated and eluted in the void volume (charged oligosaccharides and/or contamination), the total arylsulfatase B glycan pool was treated with neuraminidase from *A. ureafaciens*, specific for non-reducing terminal sialic acids in α 2-3, 6 or 8 linkages; that step was followed by examination of the digestion products by GPC. The results obtained are shown in Fig. 2. Desialylation of the glycans resulted in the recovery of 93% of the non separated and eluted in the void volume material. The above results indicate that 71% of the oligosaccharides of the total glycan pool are charged, and that the acidic forms of arylsulfatase B glycans result from sialylation of the carbohydrate moietes of the enzyme.

Digestion with neuraminidase revealed two new peaks No. 2 (13.97 GU, 3.5% of the sialylated pool) and No. 3 (11.18 GU, 91.5% of the sialylated pool) which may be N-glycans of the sialylated biantennary complex



Peak No.	Hydrodynamic volume (ml)	GU index	Area of charged form (%)
1		***	7
2	13.75	13.97	3
3	15.84	11.18	85
4	16.77	10.13	5

Figure 2. Separation profile of 2-aminobenzamide-labelled neutral arylsulfatase B glycans desialylated with neuraminidase from *A. ureafaciens* on GPC. The profile raport shows glucose units (GU) index and area % of sialylated forms.

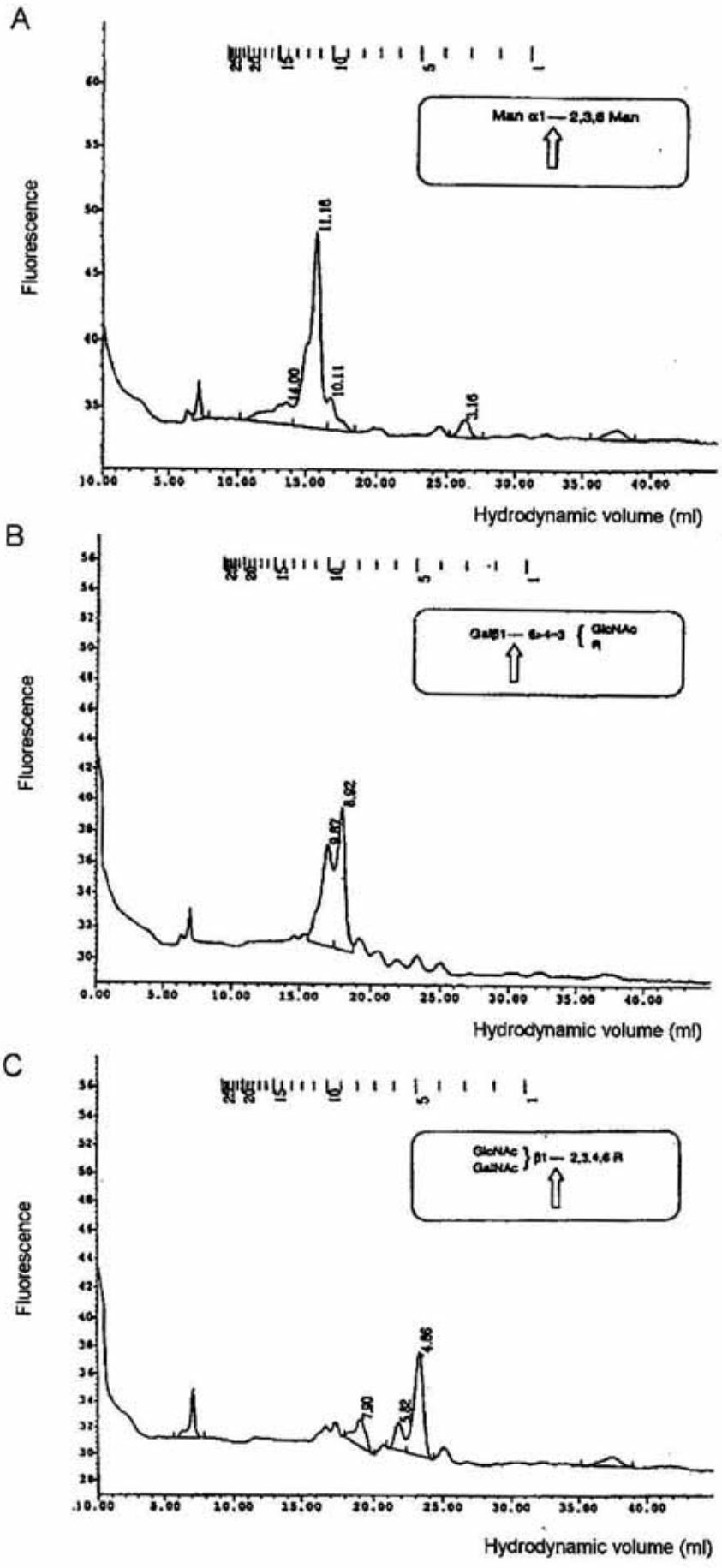


Figure 3. Separation profile of 2-aminobenzamide-labelled desialylated arylsulfatase B glycans on GPC digested with enzymes: Jack bean α-mannosidase (A), β-galactosidase (B) and β-*N*-acetylhexosaminidase from the same source (C).

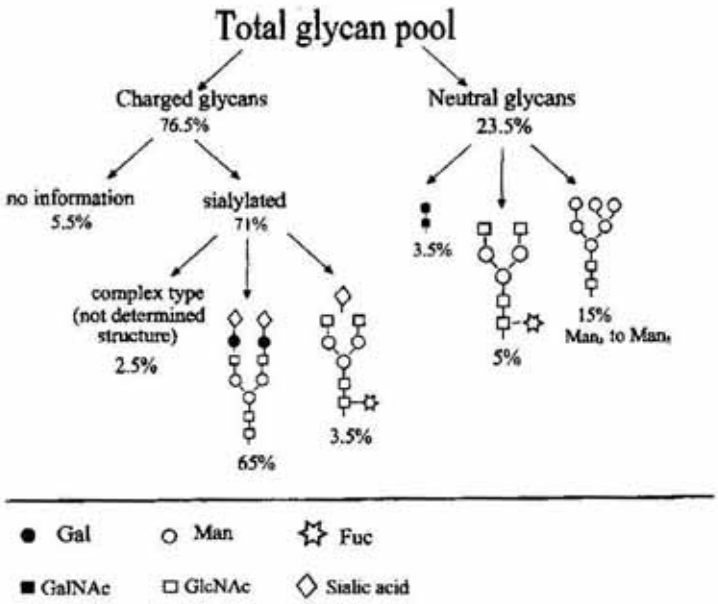


Figure 4. Relative contribution of the particular structures of rat liver arylsulfatase B oligosaccharides to the total pool.

type and/or the sialylated hybrid type. Futhermore, peak No. 4 (10.13 GU) was increased by 76% compared to its value before desialylation.

To investigate the nature of the pool of the desialylated oligosaccharides and to distinguish between N-glycans of complex type and hybrid type, the total arylsulfatase B desialylated glycan pool was treated with α-mannosidase from Jack bean (Fig. 3A) and with β-galactosidase from Jack bean (Fig. 3B). Apart from N-glycans of the high-mannose type, which disappeared upon treatment with α-mannosidase (cf. Figs. 2 and 3A; the peaks with GU index ranging from 9.49 to 4.83, in Fig. 2), no shift in peaks No. 2, No. 3 and No. 4 was observed. This finding excludes the possibility that the glycans in question are of hybrid type, as they are not sensitive to the exoglycosidase which acts with broad specificity on mannose α 1-2, 3 and 6 linkages.

When the mixture of arylsulfatase B oligosaccharides was incubated with β-galactosidase, the apparent molecular mass of the peaks, expressed in GU, was shifted downwards, which indicates liberation of the nonreducing terminal β-linked galactose residues linked to a GlcNAc residue with the 6- and/or 4-linkage. Further digestion with β-*N*-acetylhexosaminidase from Jack bean caused a parallel shift of the apparent molecular mass (Fig. 3C).

DISCUSSION

The present study indicates that oligosaccharides of the complex type are predominant among rat liver arylsulfatase B oligosaccharides. Complex type oligosaccharides have also been found in other lysosomal hydrolases such as human spleen β-glucuronidase, mouse spleen β-glucuronidase, rat liver acid phosphatase [17], human fibroblast cathepsin D, β-hexosaminidase and arylsulfatase B [18], Chang liver α-galactosidase [19], rat liver β-glucuronidase [20], bovine testes β-galactosidase [21] and frog liver acid phosphatase [22]. Indirect evidence for the presence of complex and hybrid type oligosaccharides in lysosomal enzymes was chiefly based on resistance to endo-β-*N*-acetylglucosaminidase H treatment, sensivity to neuraminidase treatment, binding to immobilized lectins, and monocarbohydrate analysis by which fucose, galactose and sialic acid were detected. All those findings indicate that those enzymes must pass through the entire Golgi complex, as the transferases responsible for the terminal glycosylation are present in the *trans*-Golgi elements [23].

Glycans are secondary gene products. Glycosylation of any protein is species, cell or tissue specific, and a polypeptide encodes information that establishes its own pattern of glycosylation. The only studies on rat liver

arylsulfatase B oligosaccharides have been raported by Wójczyk *et al.* [24]. Preliminary analysis of the structure of arylsulfatase B glycans was carried out by assessing the interaction between the whole enzyme and different lectins using two dimensional and rocket immunoelectrophoreses. It was found that arylsulfatase B glycans were N-glycans of the high-mannose, hybrid and/or complex biantennary type (reaction with Concanavalin A). Glycans of the triantennary and tetraantennary complex type, or of the biantennary complex type with bisecting *N*-acetylglucosamine structures were not detected. A part of the complex type oligosaccharides could be fucosylated. Moreover, a weak reaction between arylsulfatase B and *Dolichus biflorus* agglutinin, lectin specific for terminal GalNAc residue, was observed.

In comparison with the study described above, the present study was carried out on labelled oligosaccharides (not on the whole enzyme) using much more precise methods. This allowed us to determine the structures of all arylsulfatase B glycans, estimate their relative molar concentration in the total glycan pool, whereas the previous study determined only the types of glycans present on the enzyme. Moreover, our study pointed to the presence of charged oligosaccharides and revealed their nature.

As has been mentioned elsewhere, arylsulfatase B exists in multiple forms; such forms are found at least in ox tissues, human placenta, brain and liver, rodent liver and kidney [25–29]. However, reports on the effect of neuraminidase treatment on the multiple isoenzymes are contradictory. Some investigators have reported that sialic acids are responsible for the heterogeneity of this enzyme [25–30], and conversion of multiple rodent arylsulfatase B isoenzymes to a single species by bacterial neuraminidase treatment has been demonstrated [27]. However, treatment of anionic rodent arylsulfatase B with neuraminidase from *Clostridium perfringers* did not change the elution pattern of the enzyme from DEAE-Sephacel [5]. The latter results were probably due to the fact that rodents possess mainly O-acetyl forms of sialic acids which reduce the rate of action of *C. perfringers* sialidase on these substrates [31].

The targeting of newly synthesised lysosomal enzymes to their final destination involves at some point their segregation in the common area of the secretory pathway. For most lysosomal enzymes, a key step in the sorting process is generation of the phosphomannosyl residues on their N-linked oligosaccharides, which provide specificity for subsequent segregation [19, 32–35]. As rat arylsulfatase B is transported to lysosomes in the mannose-6-phosphate dependent manner, and since the mannose-6-phosphate residue has been found in its carbohydrates [19], at least some of anionic rat liver arylsulfatase B oligosaccharides should be phosphorylated. Unfortunately, the nature of the material still remaining non separated and eluted in the void volume (5.5% of the total pool) has not been investigated. However, in the lysosomal enzymes isolated from tissues, only a small part of oligosaccharides are phosphorylated. In β-glucuronidase from human spleen only 10% of the total glycans were phosphorylated, compared to less than 4% of the total oligosaccharides in cathepsin D from porcine spleen. On the other hand, in β-glucuronidase from rat liver, no phosphorylated oligosaccharides were detected [19].

Our study has shown that 15% of arylsulfatase B glycans were of the high-mannose type. The data on contribution of particular forms are shown in Fig. 1. The size of oligosaccharides of the high-mannose type depends mainly on the source of the enzyme [19]. The high-mannose oligosaccharides with nine and eight mannose residues are most frequent in human spleen and mouse lymphoma β-glucuronidase, but forms with five mannose residues are prevalent in rat liver β-glucuronidase. However, in human placental arylsulfatase B, 98% of the oligomannoside glycans possess not more than five mannose residues [36]. A series of high-mannose oligosaccharide chains is regarded as an intermediate product of the trimming process [16].

The obtained results suggest that another 3.5% of glycans, which are the biantennary complex type oligosaccharides with the core

fucose and terminal *N*-acetylglucosamine, were sialylated as can be concluded from the observed increase of peak No. 4 (Fig. 2) after neuraminidase treatment. This may seem surprising with respect to the glycosylation scheme [16, 23, 37], however possible explanation of the results obtained may be that an unusual sialic acid substitution took place. In $P388D_1$ murine macrophage cells the oligosaccharides of the hybrid type containing sialic acid as well as phosphomannosyl residues were found [38]. Those glycans contained up to three sialic acid residues. One of them was linked to the penultimate galactose, however the location of the others was not determined due to the shortage of the available material. The authors suggested that the two other residues could be linked directly to the galactose-substituted *N*-acetylglucosamine residue. However, this seems rather unlikely, as an appropriate sialyltransferase has not been found so far.

O-Linked oligosaccharides represented 3.5% of total arylsulfatase B glycans. The presence of O-linked oligosaccharide chains, which are Gal-GalNAc sequences, has also been evidenced in other lysosomal enzymes such as rat liver β-glucuronidase (Hoja-Łukowicz, D., personal communication), the higher relative molecular mass acid phosphatase of frog liver [22], and rat liver acid phosphatase [17].

Summing up, 71% of rat liver arylsulfatase B oligosaccharides were found to possess sialic acid residues. Contribution of the particular structures of rat liver arylsulfatase B in the total glycan pool was as follows (Fig. 4): sialylated biantennary complex type glycans with terminal galactose — 65%, high-mannose type glycans — 15%, biantennary complex type glycans with core fucose and terminal *N*-acetyloglucosamine — 5%, O-linked oligosaccharides — 3.5%.

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