

Hydrolysis, lactonization, and identification of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked tri-, tetra-, and polysialic acids

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Alpha-(2 → 8)/ $\alpha(2 \rightarrow 9)$ alternatively linked polysialic acid (PSA) can be identified by controlled hydrolysis followed by the analysis with capillary electrophoresis (CE). Due to the different stability of $\alpha(2 \rightarrow 8)$ and $\alpha(2 \rightarrow 9)$ linkages in acidic hydrolysis, oligosialic acids (OSAs) from the hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ OSA/PSA could be classified into two groups in the CE profile. The group with an odd numerical degree of polymerization (DP) had two peaks in the CE profile, and the other group, with even number of DP, showed one peak. Each alternating $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ linked OSA contains two isomers: one starts with the $\alpha(2 \rightarrow 8)$ linkage from the nonreducing end and the other starts with the $\alpha(2 \rightarrow 9)$ linkage from the nonreducing end. Trimers and tetramers were isolated by using a Mono Q column with an HPLC system. The two trimer isomers are $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ and $\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)$ linkages and only showed partial separation by CE. After lactonization, sialidase hydrolysis, and alkaline treatment, the two trimer isomers could be separated and identified by CE analysis, but only the $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ trimer could be converted to the dilactone in glacial acetic acid. The two tetramer isomers could be converted to four monolactones and three dilactones. These lactonized species could be identified on the basis of several principles in sialidase hydrolysis and lactonization. In conclusion, regioselectivity on the lactonization of oligosialic acids proceeds under several principles: (1) Lactonization takes place more easily in the $\alpha(2 \rightarrow 8)$ linkage than in the $\alpha(2 \rightarrow 9)$ linkage; (2) all of the positions of $\alpha(2 \rightarrow 8)$ linkages in $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA can be lactonized regardless of external or internal carboxyl groups involved; and (3) for the site of $\alpha(2 \rightarrow 9)$ linkage, only internal carboxyl groups can be lactonized.

Key words: capillary electrophoresis/hydrolysis/
lactonization/oligosialic acid/polysialic acids

Introduction

Sialic acids are widely distributed in nature ranging from bacteria to human (Muhlenoff *et al.*, 1998; Troy, 1992).

This sugar moiety is usually linked to carbohydrate chains of glycolipids and glycoproteins in the nonreducing end and is involved in many important biological events (Schauer *et al.*, 1995). Sialic acids also form linear homopolymers called polysialic acid (PSA). With respect to glycosidic linkage, three kinds of PSA have been isolated from microorganisms (Figure 1a–c) (Muhlenoff *et al.*, 1998; Troy, 1992). The PSA isolated from the capsule of neuroinvasive *Escherichia coli* K1 and *Neisseria meningitidis* serogroup B is composed of $\alpha(2 \rightarrow 8)$ PSA (Neu5Ac), and the PSA from *N. meningitidis* serogroup C is $\alpha(2 \rightarrow 9)$ PSA (Neu5Ac). Alternating $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ linked PSA (Neu5Ac) is from *E. coli* K92 and Bos-12 strains (Bhattacharjee *et al.*, 1975; Egan *et al.*, 1977; Liu *et al.*, 1971a). Alpha-(2 → 8)/ $\alpha(2 \rightarrow 9)$ oligo/polySia does not exist in mammalian systems and therefore could be used as a carbohydrate vaccine to elicit an immunological response (Devi *et al.*, 1991).

The formation of δ -lactone groups in α -2,8 (or α -2,9)-oligosialic acid (OSA)/PSA suggests that the C-2 carboxylic acid of one residue condenses with the hydroxyl groups at C-9 (or C-8) of an adjacent residue to form a six-membered ring between two adjacent sialic acid residues (Figure 1d, e) (Lifely *et al.*, 1981). It is apparent that lactonization in OSA/PSA could alter their charge density, conformation, and probably their antigenicity and biological functions (Ando *et al.*, 1989; Kielczynski *et al.*, 1994). According to the previous report, under acidic conditions, both α -2,8 and α -2,9 PSA can undergo δ -lactonization (Lifely *et al.*, 1984). Due to the steric hindrance in C-8, δ -lactonization in α -2,9 PSA is much slower than that in α -2,8 PSA. It is obvious that the diversity of lactonization becomes more complicated if the degree of polymerization (DP) of OSA increases. The same lactonization also occurs between two sialic acid residues in GD3 and GD1b ganglioside (Fronza *et al.*, 1989; Maggio *et al.*, 1990; Yu *et al.*, 1985). Other types of ganglioside lactones (GM3 and GM4) formed from the carboxyl group of sialic acid and the C-4 (or C-2) hydroxyl group of the adjacent galactosyl residue were found in the whale brain (Terabayashi *et al.*, 1990; Terabayashi and Kawanishi, 1998). Furthermore, GM3 lactone was found to bind to influenza virus hemagglutinin but not to be substrate for virus neuraminidase (Sato *et al.*, 1999). As a consequence, it was proposed that lactone formation might represent an on/off signal of a physiological function.

Recently we have developed a highly sensitive and efficient method for the analysis of oligosialic acids and their lactone species by capillary electrophoresis (CE; Cheng *et al.*, 1998, 1999a). By using this method, the pathway of formation of the fully lactonized $\alpha(2 \rightarrow 8)$ -linked trimer and tetramer in glacial acetic acid, the delactonization of fully

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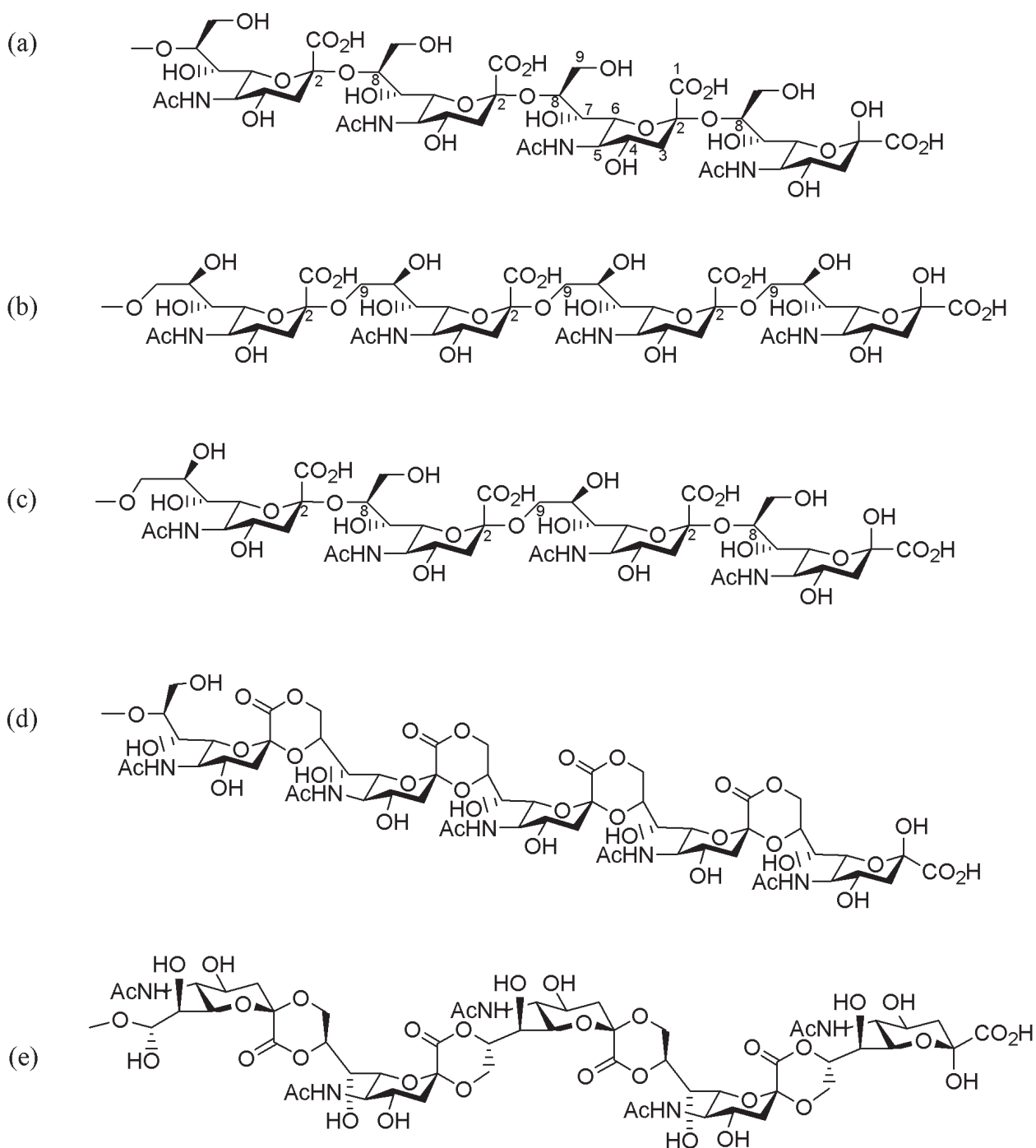


Fig. 1. The three different linkages of polysialic acids: (a) $\alpha(2 \rightarrow 8)$ linkage, (b) $\alpha(2 \rightarrow 9)$ linkage, (c) $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternating linkage, (d) the lactonized structure of $\alpha(2 \rightarrow 8)$ linkage, and (e) the lactonized structure of $\alpha(2 \rightarrow 9)$ linkage.

lactonized trimer and tetramer in basic solution, and simultaneous hydrolysis/lactonization of trimer and tetramer in acidic aqueous solution have been elucidated (Cheng *et al.*, 1999b, 2000; Yu *et al.*, 2001). In this article, we discuss the controlled hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA and the lactonization of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked trimers and tetramers in acidic condition based on the analysis by CE.

Results

Controlled hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA

We have developed a very efficient way to control the hydrolysis of PSA and also a very powerful method to analyze the hydrolysate of PSA by CE (Cheng *et al.*, 1998, 1999a,b). The CE profile of the hydrolysate of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA showed an anomalous pattern (Figure 2a)

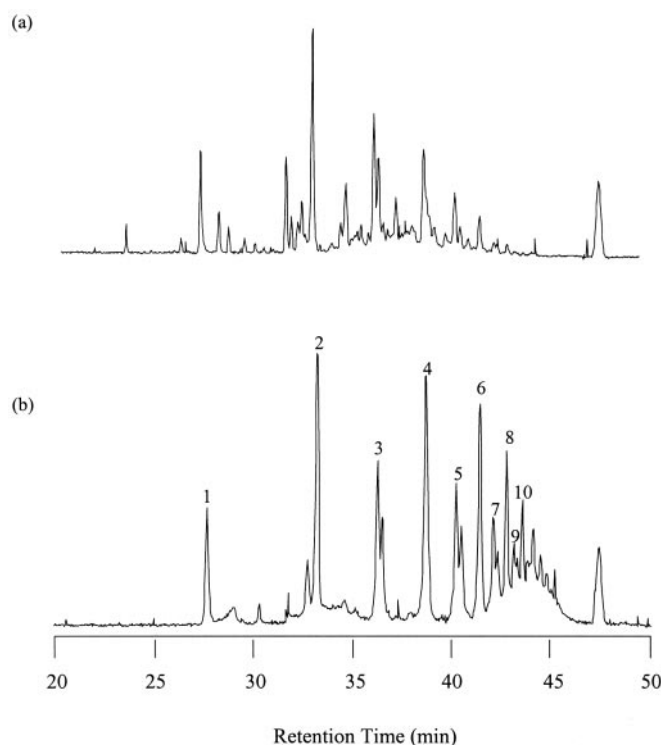


Fig. 2. CE profiles of hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA in 0.1 N acetic acid (a) before and (b) after alkaline treatment. The numerical numbers denote the number of DP.

due to the formation of lactone species. After alkaline treatment, the CE profile was converted into a unique and regular pattern. As shown in Figure 2b, OSA with an odd numerical of DP have two peaks; OSA with an even numerical of DP have a single peak. The results are due to the different hydrolytic rate of the $\alpha(2 \rightarrow 8)$ and $\alpha(2 \rightarrow 9)$ linkages in the $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA. Based on the previous reports and our studies, the $\alpha(2 \rightarrow 9)$ linkage is more easily hydrolyzed in acidic conditions than the $\alpha(2 \rightarrow 8)$ linkage (Liu *et al.*, 1971b). Each $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA has two isomers. The yields of two isomers of oligomers with an even numerical of DP from the hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ polySia were in different quantities. The isomer that started with the $\alpha(2 \rightarrow 8)$ linkage from the nonreducing end generated by the cleavage of two $\alpha(2 \rightarrow 9)$ linkages should be in a higher quantity than the other isomer, which started with the $\alpha(2 \rightarrow 9)$ linkage from the nonreducing end generated by the cleavage of two $\alpha(2 \rightarrow 8)$ linkages. Because of the difficulty in separation, the two isomers showed a single peak in the CE profile. As for the oligomers with an odd numerical of DP, both isomers generated by the cleavage of one $\alpha(2 \rightarrow 8)$ and one $\alpha(2 \rightarrow 9)$ linkage should occur in almost equal amounts and showed two peaks with partial separation in CE analysis.

Separation and identification of two trimer isomers

All of the OSA with a $DP \leq 12$ could be isolated by a Mono Q column (Figure 3), but the two isomers of each

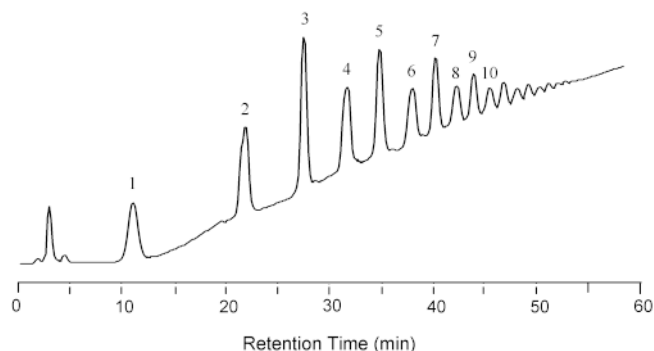


Fig. 3. Isolation and purification of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA by a Mono Q column in a high-performance liquid chromatography system. Nonlactonized OSA peaks are labeled with the number of DP.

OSA could not be separated from each other. Each $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA contains two isomers. For example, the trimer has two isomers, one with $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ linkages (1) and the other with $\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)$ linkages (2). The two trimers, having similar properties and the same charges, are difficult to separate and identify by any separation method. They show only partial separation in CE analysis, but it is not sufficient for identification. To find a way to separate and identify the two isomers, they were dissolved in glacial acetic acid at room temperature, and the progress of lactonization was followed by CE analysis. As shown in Figure 4, in the CE spectra there were four major peaks that should correspond to two monolactones, one dilactone, and the unreacted trimer (Cheng *et al.*, 1999b). According to the previous reports, the intramolecular lactonization of $\alpha(2 \rightarrow 8)$ PSA is much easier than that of $\alpha(2 \rightarrow 9)$ PSA (Lifely *et al.*, 1981, 1984). Therefore, for both of the trimer isomers in glacial acetic acid, lactonization in the position of the $\alpha(2 \rightarrow 8)$ linkage would be easier, and lactonization in the position of the $\alpha(2 \rightarrow 9)$ linkage would be more difficult (Figure 5). Based on this, the two monolactones (1a and 2a) should come from the two trimer isomers with lactonization in the position of the $\alpha(2 \rightarrow 8)$ linkage (Figure 5a).

Because of the difficult lactonization in the $\alpha(2 \rightarrow 9)$ linkage, the two monolactones (1a and 2a) were accumulated and could be separated by CE or Mono Q column. To identify the two monolactones, sialidase was introduced to hydrolyze them. Sialidases recognize and release the sialic acid at the nonreducing end of OSA/PSAs and do not attack the lactonized sialic acid. Figure 5a shows the CE profile for the lactonization of the two trimer isomers in glacial acetic acid at room temperature for 12 h. Peaks A and B at the far right should be two remaining free trimer isomers because they have the highest charge densities. Peaks D and C were two monolactones, and peak E at the far left was a dilactone. The mixture was treated with sialidase for 3 h and analyzed by CE (Figure 5b). The two free trimers (peaks A and B) were hydrolyzed to monomer. The monolactone at peak C (2a), with a lactone in the reducing end, was hydrolyzed to monomer and a lactonized dimer. The monolactone at peak D (1a) and the dilactone at peak E

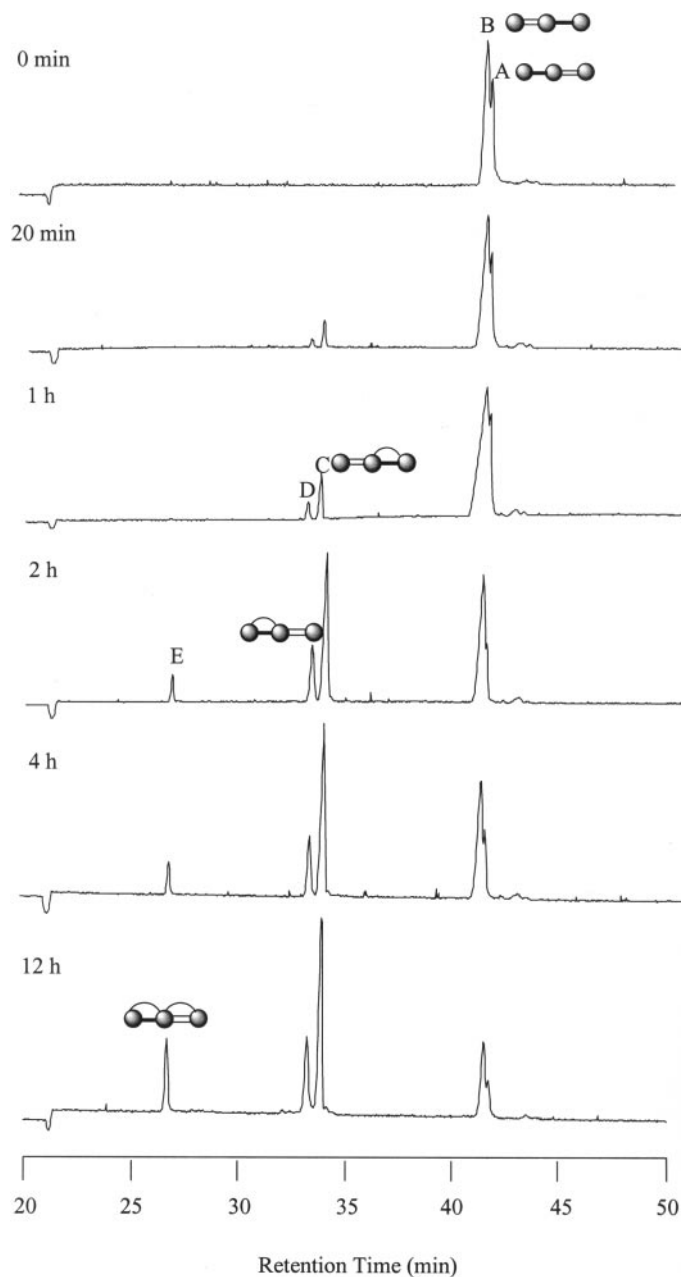


Fig. 4. CE spectra of lactonization of two $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked trimers in glacial acetic acid at different reaction times (0 min, 20 min, 1 h, 2 h, 4 h, and 12 h). The circles represent sialic acid residues where the reducing ends are at the right side. Sites of lactone rings are marked by the curves between circles. Alpha-(2 \rightarrow 8) linkages are shown by single bold lines, and $\alpha(2 \rightarrow 9)$ linkages are by thin double lines.

(1c or 2c) were resistant to hydrolysis and assumed to be 1a and 1c or 2c, respectively.

After alkaline treatment and desalting by gel filtration, the sample of Figure 5b was analyzed by CE with the coinjection of the authentic samples. Three peaks shown in Figure 5c were identified: monomer, $\alpha(2 \rightarrow 8)$ dimer, and $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ trimer. Thus the generation of $\alpha(2 \rightarrow 8)$ dimer, from the cleavage of 2a by sialidase and

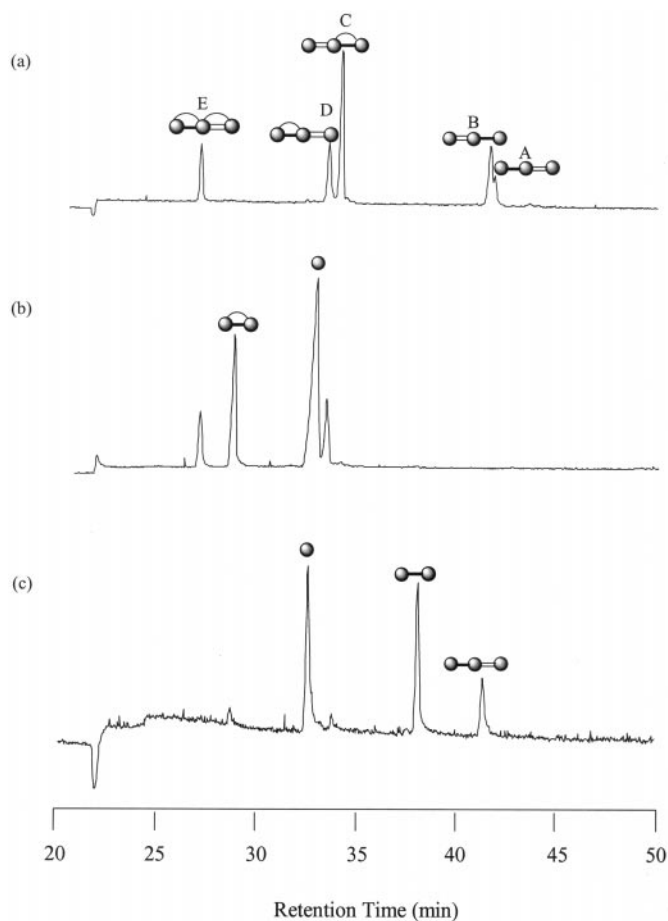


Fig. 5. The CE spectra showed the product mixture obtained from the lactonization of two trimers in glacial acetic acid for 12 h (a). After drying, the lactonized products were hydrolyzed by neuraminidase for 3 h and analyzed by CE (b). The products generated from the hydrolysis of neuraminidase were treated with alkaline solution and then was analyzed by CE (c).

alkaline treatment of a lactonized dimer (peak 2 of Figure 5c), confirms that the lactonization positions of the two monolactones are solely in the $\alpha(2 \rightarrow 8)$ linkage. Also, the generation of 1 in Figure 5c shows that the formation of the dilactone (peak E of Figure 5a) comes only or at least largely from 1 (see *Discussion*) and, finally, the two trimer isomers with partial separation in CE analysis can be identified.

Identification of monolactones from two tetramers

Two tetramers (3, 4) were also obtained by the controlled hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA and purified by a Mono Q column. As described, compound 3 with $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)$ linkages is in much higher quantity than 4 with $\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ linkages, and they could not be separated in the traditional chromatography. The lactonization of 3 and 4 is more complicated than that of 1 and 2. As shown in Figure 6, four monolactone species and three dilactone species were formed from 3 and 4 in glacial acetic acid. In an attempt to identify

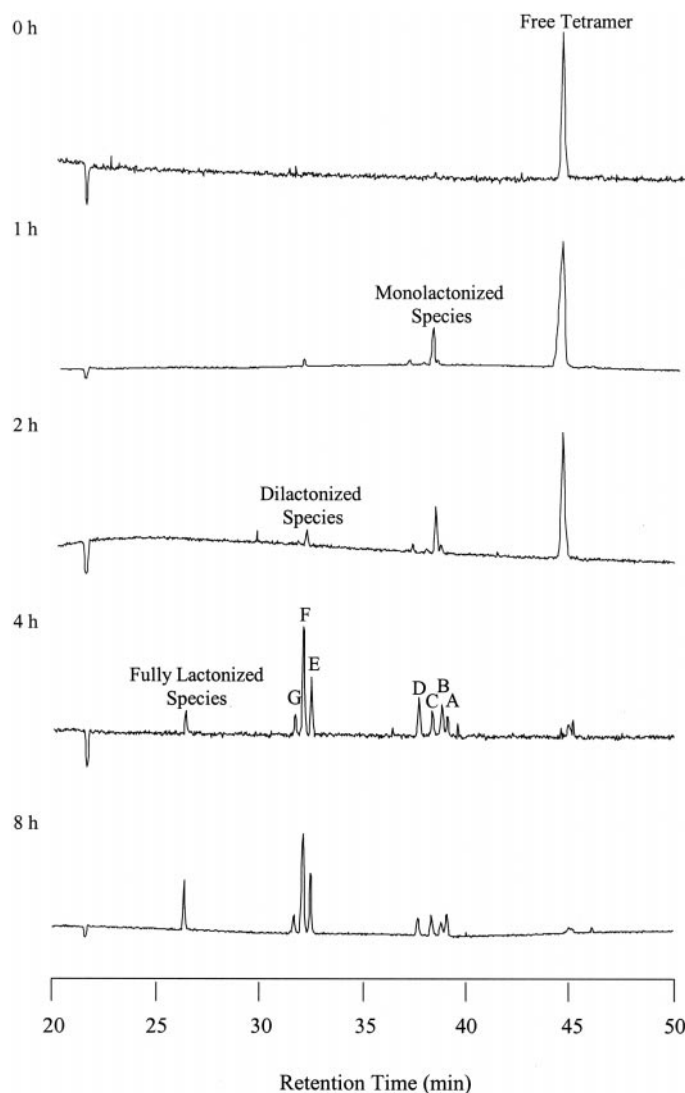


Fig. 6. CE spectra of the lactonization of two $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked tetramers in glacial acetic acid at different reaction times.

each peak shown in the CE profiles, two facts that have been demonstrated in the previous reports were applied: (1) Lactonization in the $\alpha(2 \rightarrow 8)$ linkage is easier than that in the $\alpha(2 \rightarrow 9)$ linkage, and (2) due to the charge repulsion, the carboxyl groups in the middle position are much more easily lactonized than those in both ends. Based on these facts, compound **4a** with the $\alpha(2 \rightarrow 8)$ linkage in the middle position should be the first monolactone formed from **3** and **4**, corresponding to peak **B** in Figure 6. Three other monolactones were identified by the hydrolysis of sialidase.

Several factors in the hydrolysis of sialidases have been demonstrated and were used as principles in the identification of lactone species: (1) Because the hydrolysis of sialidase proceeds solely from the nonreducing end and the free carboxyl group on sialic acid residue in OSA/PSAs is essential for substrate binding by sialidase, the hydrolytic reaction should stop at the lactonized sialic acid residue; (2) the hydrolysis of the $\alpha(2 \rightarrow 8)$ linkage hydrolyzed by sialidase is

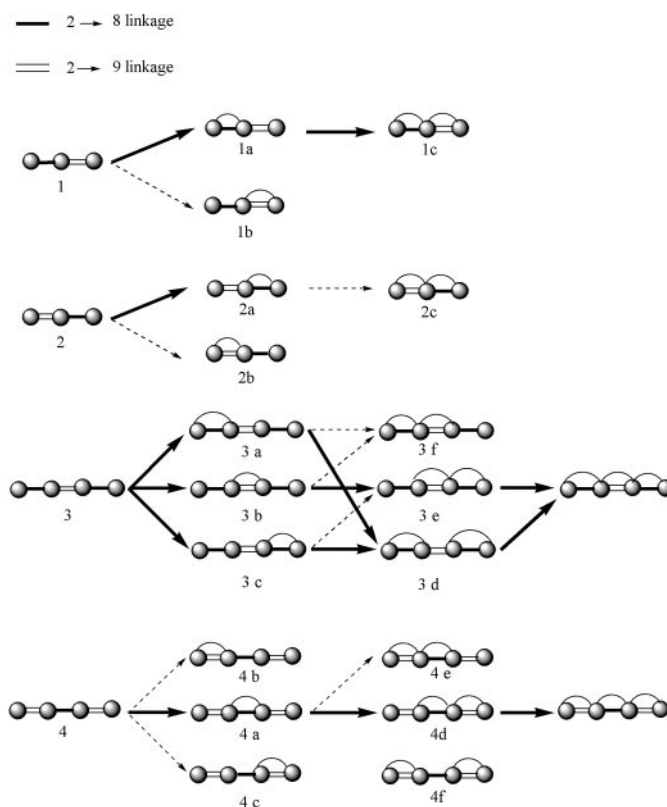


Fig. 7. The pathway of lactonization of two trimers and two tetramers. The circles represent sialic acid residues where the reducing ends are at the right side. Sites of lactone rings are marked by the curves between circles. Alpha-($2 \rightarrow 8$) linkages are shown by single bold lines, and $\alpha(2 \rightarrow 9)$ linkages are by thin double lines. The bold arrows show major routes, and dotted arrows show minor routes or probably no routes.

faster than the $\alpha(2 \rightarrow 9)$ linkage; and (3) the linkage adjacent to the lactonized residue should be cleaved faster than the normal one (unpublished data). As shown in Figure 6, Peak **D** could not be hydrolyzed by sialidase and was identified as compound **3a**, which has a lactonized group at the nonreducing end to resist the hydrolysis by sialidase. Peak **C**, which was hydrolyzed the fastest by sialidase was identified as compound **3b**, which had an $\alpha(2 \rightarrow 8)$ linkage on the nonreducing end adjacent to a lactonized group. Peak **A** could also be hydrolyzed by sialidase and was assumed to be compound **3c**, because only six monolactone species can be formed theoretically, and compound **4b** and **4c** are less likely to be formed from **4** (Figure 7) (see *Discussion*).

Identification of dilactones from two tetramers

Like monolactone species, only six dilactone species can be formed theoretically (Figure 7). Because all the dilactones came from monolactones, the possibility of formation of **4e** and **4f** was ruled out. Among the three peaks of dilactone species in the CE profile, only peak **G**, with the lowest mobility, resisted hydrolysis by sialidase and was reasonably identified as compound **3d**. The other two dilactones could be cleaved by sialidase. Among the dilactones shown in Figure 8, only **3e** and **4d** could be the substrates of

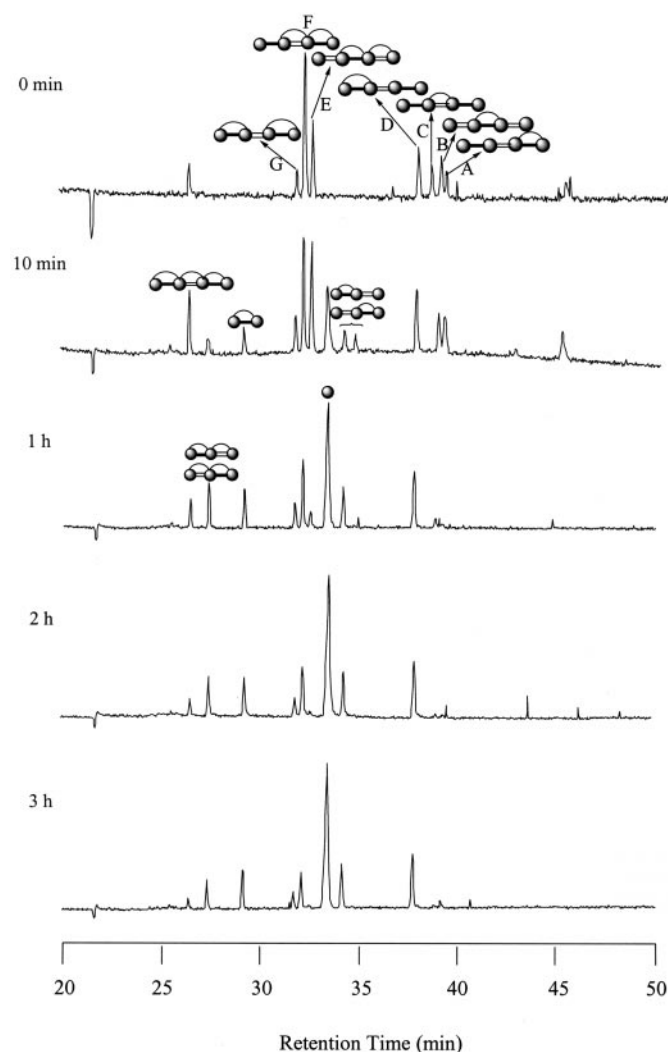


Fig. 8. The product mixture obtained from the lactonization of two tetramers in glacial acetic acid for 2 h was hydrolyzed by neuraminidase and analyzed by CE at different hydrolytic times.

sialidase. From the analysis of sialidase hydrolysis (Figure 8), peak F hydrolyzed faster than peak E and was identified as **3e** and peak E as **4d**. According to the previous report, the carboxyl groups in both ends in OSA/PSA should have lower pKa than the carboxyl groups in the middle. Therefore, the monolactones or dilactones of OSA with the same DP formed lactone moiety in the nonreducing end by losing an external carboxyl group should have the lowest acidity and the least mobility in the analysis by CE among the same species. This conclusion was revealed in the CE profiles in which compound **3a** corresponds to peak D and compound **3d** corresponds to peak G.

Tetramers contain four carboxyl groups and lactone groups can be formed at three different sites: reducing end, middle, and nonreducing end. The first carboxyl group in the reducing end is not involved in the formation of lactone, the second carboxyl group (numbered from the reducing end) can form a lactone in the reducing end, the third carboxyl group (next to the one in the nonreducing

end) forms a lactone in the middle position, and the carboxyl group in the nonreducing end is involved in the formation of lactone in the nonreducing end. The middle position is the easiest site to be lactonized in $\alpha(2 \rightarrow 8)$ -linked tetramer. With the higher pKa and less influence for mobility of internal carboxyl groups, the lactone group in the reducing end formed from the internal carboxyl group is more easily generated than the lactone in the nonreducing end, which is formed the external carboxyl group in reducing end.

Discussion

Identification of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA/PSA by CE

The determination of the type of glycosidic linkages in PSA is a key step in the study of PSAs. Also, a simple way to analyze enzymatic products is necessary in the study of polysialyltransferases. According to the previous reports, several methods, including ^1H , ^{13}C nuclear magnetic resonance (NMR), and periodate oxidation have been developed to differentiate these three types of PSAs (Chao *et al.*, 1999; Egan *et al.*, 1977; Shen *et al.*, 1999). However, a large quantity of PSA samples is needed for the measurement of NMR, and the available NMR data are unable to specify the sequence of the linkages in PSA. Thus there should be a simple and reliable method in microscale to identify the three different PSAs. The unique CE profile of the controlled hydrolysate of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA could be a criterion to identify the linkages of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ PSA. Compared with the previous methods, this way to identify the $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ OSA/PSA is simpler and better.

Analysis of lactonized species of oligomers by CE

Zhang and Lee (1999) have used high-performance anion-exchange chromatography (HPAEC) to analyze lactonized and nonlactonized OSA of DP-6. The separation by HPAEC mainly depends on the negative charges of molecules. Fully lactonized species from dimer to hexamer have approximately the same elution times as the monomer. Multiple forms of partially lactonized species with the same negative charges for each OSA could not be well separated by HPAEC. The separation by CE depends not only on the charges of the molecules but also on the mass of the molecules. Because of high resolution in separation, CE can separate all of the partially lactonized species of OSA having slightly different pKa of the carboxyl groups, even though they have the same charges and masses. In the separation by CE and the hydrolysis of sialidase, all the nonlactonized and lactonized species produced by lactonization and hydrolysis could be identified. Therefore the pathway of lactonization could be clearly drawn.

Selective lactonization in $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA

For OSA, lactonization occurs more easily in the $\alpha(2 \rightarrow 8)$ linkage than in the $\alpha(2 \rightarrow 9)$ linkage. For the lactonization of two trimers, the dilactone is generated solely from $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ trimer, not from $\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)$

trimer. This clearly indicates that the internal carboxyl group of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ trimer can be lactonized in the $\alpha(2 \rightarrow 9)$ linkage, whereas the external carboxyl group at the nonreducing end of $\alpha(2 \rightarrow 9)/\alpha(2 \rightarrow 8)$ trimer has little chance to be lactonized in the $\alpha(2 \rightarrow 9)$ linkage. In principle, all of the positions of $\alpha(2 \rightarrow 8)$ linkages in $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA could be lactonized regardless of external or internal carboxyl groups involved. As for the site of $\alpha(2 \rightarrow 9)$ linkage, only internal carboxyl groups involved can be lactonized. This principle can explain why the two monolactones, **4b** and **4c**, and two dilactones, **4e** and **4f**, of two tetramers could not be found. Lactonization in reducing the end occurs more easily than in the nonreducing end. It is evident, as shown in Figure 6, that **3b** is further lactonized to **3e** but not to **3f** and that **4a** is further converted to **4d**, not to **4e**.

In analysis by CE, negative charges and masses of the molecules are the main factors for mobility. Molecules with the same number of carboxyl groups and DP should have close mobilities as a group. Therefore it is possible that some compounds whose generation we have ruled out may be formed in tiny or minor amount and may merge into the peaks of the major products in the analysis by CE.

Hydrolysis of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA/PSA by sialidase

Although neuraminidase from *Vibrio cholerae* showed a much higher hydrolytic rate on the linkage of $\alpha(2 \rightarrow 8)$ PSA than that of $\alpha(2 \rightarrow 9)$ PSA, there is not much difference in cleaving both $\alpha(2 \rightarrow 8)$ and $\alpha(2 \rightarrow 9)$ linkages in the $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA. That is the reason we cannot use neuraminidase to differentiate two trimers (**1** and **2**) and two tetramers (**3** and **4**). In the previous report, both $\alpha(2 \rightarrow 8)$ PSA and $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA were found to be susceptible to neuraminidase cleavage, whereas $\alpha(2 \rightarrow 9)$ PSA was resistant. The $\alpha(2 \rightarrow 9)$ linkage in both $\alpha(2 \rightarrow 9)$ PSA and $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA showed different accessibility by neuraminidase. However, the antibody produced from the injection of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA as antigen could precipitate $\alpha(2 \rightarrow 9)$ PSA but failed to react with $\alpha(2 \rightarrow 8)$ PSA. This implied that the conformation of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA is similar to that of $\alpha(2 \rightarrow 9)$ PSA but not to that of $\alpha(2 \rightarrow 8)$ PSA. These findings revealed that the chemical properties of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA are similar partly to that of $\alpha(2 \rightarrow 9)$ PSA and partly to that of $\alpha(2 \rightarrow 8)$ PSA.

Conclusion

Both lactonization and hydrolysis in OSA/PSAs are catalyzed by acid in aqueous solution. To simplify the analysis of products, we carry out lactonization in glacial acetic acid to avoid hydrolytic reactions. In our studies, lactonization in glacial acetic acid was faster than in 0.1 N acetic acid, but all lactonized products in both systems were the same (unpublished data). Lactonization can significantly alter the charge density and conformation of OSA/PSA. Therefore, it has been reported that lactonization of PSA *in vivo* might have biological influence in epitope change, interaction between

cell membranes and metabolism of PSA. Natural formation of lactones in ganglioside has been detected. However, it remains unclear whether lactonization *in vivo* occurs only by acid-catalyzed chemical reaction or is controlled by specific enzymes. Our investigation of acid-catalyzed lactonization of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked OSA will not only provide useful information in the identification and analysis of the isomers but also shed light on our understanding of related processes *in vivo* for the three different linkages of PSA.

Materials and methods

Materials

Undeactivated fused silica capillaries for CE were purchased from J&W Scientific (Folsom, CA). *E. coli* K92 was a gift from Dr. Teh-Yung Liu (Department of Biological Chemistry, Academia Sinica, Taipei, Taiwan). Growth medium (medium K): each liter of medium should contain the following: 0.5 g TSB, 1 g NaCl, 1 g K_2SO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 6H_2O$, 0.001 g $FeSO_4 \cdot 7H_2O$, 0.001 g $CuSO_4 \cdot 5H_2O$, 10.8 g NaH_2PO_4 , 0.5 g KH_2PO_4 , 11.3 g L-asparagine $\cdot H_2O$, and 11.3 g D-xylose. The medium was adjusted to pH 8 with 5 M KOH. All other reagents for hydrolysis, lactonization, enzymatic identification, and CE were of the highest grade commercially available.

Chromatographic conditions

CE was performed on a Beckman CE P/ACE system 2100 (Palo Alto, CA) using fused silica capillaries (117 cm \times 75 μ m ID) and applying 20 kV at 25°C. Phosphate buffer (50 mM, pH 8.0) was used as the running buffer. The spectra were monitored by UV absorption at 200 nm. Samples were injected into the capillaries by high-pressure nitrogen (20 psi) for 3 s. The capillaries were regenerated by washing with double-distilled water for 3 min and then 0.1 N NaOH for 5 min.

Preparation of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA

The preparation of $\alpha(2 \rightarrow 8)/\alpha(2 \rightarrow 9)$ alternatively linked PSA was based on the previous report (González-Clemente *et al.*, 1990; Gotschlich *et al.*, 1969; Vann and Freese, 1994). A culture of *E. coli* K92 was inoculated into 2 L medium and allowed to grow at 37°C. Release of the capsular polysaccharide in the medium was maximal (450 μ g/ml) in the stationary phase of growth for 76 h. The culture was separated to cell and medium by centrifugation (7000 \times g at 4°C). The supernatant was then diluted twofold with 0.2% Cetavlon (hexadecyltrimethylammonium bromide) and allowed to stand for at least 2 h. The Cetavlon complex containing polysaccharide was harvested by centrifugation (8000 rpm, 15 min). The pellet containing polysaccharide was dissolved in 1 M $CaCl_2$. The paste was then extracted by homogenizing vigorously in a Sonicator (Sonics 2210, Branson). The viscous mixture was slowly adjusted to 25% ethanol stirred for 1 h at 4°C, and centrifuged to remove cell debris and precipitated nucleic acid and protein. The supernatant was adjusted to 80% ethanol, and the crude

polysaccharide fraction was recovered by centrifugation (16,000 × *g*, 4°C).

The pellet was dissolved in 10% sodium acetate and stirred vigorously with an equal volume of cold buffered phenol for 30 min in an ice bath. The phases were separated by centrifugation at 10,000 rpm. The aqueous layer was removed, adjusted to 25% ethanol, allowed to stand in the cold for 1 h, and then centrifuged at 10,000 rpm. Polysaccharide was recovered from the supernatant by adjusting to 80% ethanol and then centrifuging after standing overnight at 4°C. It was then processed through at least one additional cycle of phenol extraction and ethanol precipitation until the interface was minimal. The final ethanol precipitate was dissolved in 10% sodium acetate and dialyzed extensively against water. The dialyzate was centrifuged at 100,000 × *g* for 2 h to remove lipopolysaccharide. The supernatant was then lyophilized and checked by NMR and CE.

Preparation of α(2 → 8)/α(2 → 9) alternatively linked OSA

Alpha-(2 → 8)/α(2 → 9) alternatively linked PSA was incubated in 0.01 N acetic acid at 40°C for 1 day and evaporated to give a dry residue by lyophilization. Next, 0.1 N NaOH was added to hydrolyze the lactonized mixture at room temperature for 20 min. The hydrolyzed mixture was fractionated by means of a Mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden). The column was applied with a gradient of 0.06 N to 0.5 N NaCl for 60 min and developed at an average flow of 0.5 ml/min. Chromatography was monitored by UV absorption at 214 nm. Fractions were collected, desalted by Superdex PE 7.5/300 (Pharmacia), eluted by distilled water, and identified by CE.

Lactonization in glacial acetic acid

Alpha-(2 → 8)/α(2 → 9) alternatively linked OSA (25 μg) was treated with glacial acetic acid (500 μl) at room temperature for different time intervals and then dried by speed vacuum to remove excess acetic acid. Dried samples were dissolved in distilled water (5 μl) and analyzed by CE.

Neuraminidase hydrolysis

OSAs were lactonized in glacial acetic acid for 10 min, treated with 0.5 mU *Arthrobacter ureafaciens* neuraminidase (Nacalai Tesque, Japan) in 20 μl of 0.1 M ammonia acetate at room temperature, and analyzed directly by CE at different time intervals.

Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectra of the sample were obtained on an Autospec OA-TOF mass spectrometer (Micromass, Manchester, UK) fitted with a cesium ion gun operated at 30 kV. Five milligrams of hydrolysate was dissolved in 5% acetic acid for loading onto the probe tip coated with monothioglycerol as matrix.

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Abbreviations

CE, capillary electrophoresis; DP, degree of polymerization; HPAEC, high-performance anion-exchange chromatography; NMR, nuclear magnetic resonance; OSA, oligosialic acid; PSA, polysialic acid.

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