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Effect and Limitation of Excess Ammonium on the Release of O-Glycans in Reducing Forms from Glycoproteins under Mild Alkaline Conditions for Glycomic and Functional Analysis

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Ammonium-based alkali-catalyzed β-elimination under nonreducing conditions was investigated in detail for the stability of the released mucin-type O-glycan chains with β1,3-linked cores. In contrast to the previously studied β1,4-linkage of the N-glycan-type, which was shown to be stable under the ammonium-based alkaline conditions, the β1,3-linkage is labile toward alkaline treatment and considerable peeling was observed with both model heptasaccharides and standard glycoproteins. The former include eight reducing glucoheptasaccharides with different and commonly occurring linkages (α1,2-, β1,2-, α1,3-, β1,3-, α1,4-, β1,4-, α1,6-, and β1,6-linkages), and the latter include mucin-type bovine submaxillary mucin and bovine fetuin, which contains both O- and N-glycans. The results indicated that complete prevention of peeling under nonreducing alkali-catalyzed hydrolysis conditions remains difficult. The yields of released O- and N-glycans were also assessed by use of the two glycoproteins as models. Compared with conventional procedures, Carlson degradation for O-glycan release and PNGase F digestion for N-glycan release, the nonreducing ammonium-based alkaline hydrolysis gave lower yields. Great care has to be taken when employing such nonreducing alkaline conditions in glycomic analysis and in obtaining glycoprotein glycans for functional studies.

Carbohydrate chains (glycans) of glycoproteins can mediate important biological processes through their interactions with carbohydrate-binding proteins. Detailed analysis of the roles of the glycans as recognition structures has been a challenging area due to the diversity of their sequences and the small amounts that can be released and isolated. This has prompted attempts at refinements of methods of glycan release and development of sensitive methods for structural and functional analyses of oligosaccharides.

Typically O-glycosylated proteins are those of mucin type carrying clustered O-glycans linked to proteins via −3GalNAc-*O*-Ser/Thr. The structural complexities of the O-glycans exceed by far those of N-linked chains via −4GlcNAc-*N*-Asn and present major difficulties for their separation and sequencing. The functional aspects of O-glycans appear as complex as their structures: for example, their potential roles in sorting and secretion of glycoproteins, their influence on protein conformation, and their involvement in cell adhesion and immunological and pathogenic processes.[1]

For structure and function studies[2,3] of glycoprotein N- and O-glycans, the ideal is to liberate the oligosaccharide chains from the protein backbone in an intact form with minimal degradation.[2] N-linked glycans typically contain 4-linked GlcNAc cores at the reducing termini that are relatively stable[4] and can be released by chemical methods such as hydrazinolysis[5] or by enzyme digestion.[6] However, O-glycan chains are generally elongated through the 3-position of the core GalNAc and are susceptible to alkali-catalyzed degradation.[3] For O-glycan release, partial acid hydrolysis was used in early studies;[7−9] this yielded oligosaccharide fragments for hapten inhibition studies. However, most methods, early and current, have used alkaline conditions for releasing O-glycans by a β-elimination reaction to cleave the glycosidic bond between the GalNAc and Ser/Thr. In early methods, alkaline reagents, such as tertiary amines,[10] polymeric quaternary amines[11] and NaOH in the presence of $NaBH_4$[12] or

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(1) Hanisch, F. G. *Biol. Chem.* **2001**, *382*, 143–149.
(2) Wada, Y.; Dell, A.; Haslam, S. M.; Tissot, B.; Canis, K.; Azadi, P.; Backstrom, M.; Costello, C. E.; Hansson, G. C.; Hiki, Y.; Ishihara, M.; Ito, H.; Kakehi, K.; Karlsson, N.; Hayes, C. E.; Kato, K.; Kawasaki, N.; Khoo, K. H.; Kobayashi, K.; Kolarich, D.; Kondo, A.; Lebrilla, C.; Nakano, M.; Narimatsu, H.; Novak, J.; Novotny, M. V.; Ohno, E.; Packer, N. H.; Palaima, E.; Renfrow, M. B.; Tajiri, M.; Thomsson, K. A.; Yagi, H.; Yu, S. Y.; Taniguchi, N. *Mol. Cell. Proteomics* **2010**, *9*, 719–727.
(3) Jensen, P. H.; Kolarich, D.; Packer, N. H. *FEBS J.* **2010**, *277*, 81–94.
(4) Lloyd, K. O.; Kabat, E. A. *Carbohydr. Res.* **1969**, *9*, 41–48.
(5) Takasaki, S.; Mizuochi, T.; Kobata, A. *Methods Enzymol.* **1982**, *83*, 263–268.
(6) Plummer, T. H., Jr.; Elder, J. H.; Alexander, S.; Phelan, A. W.; Tarentino, A. L. *J. Biol. Chem.* **1984**, *259*, 10700–10704.
(7) Kabat, E. A.; Baer, H.; Bezer, A. E.; Knaub, V. *J. Exp. Med.* **1948**, *88*, 43–57.
(8) Cote, R. H.; Morgan, W. T. J. *Nature* **1956**, *178*, 1171–1172.
(9) Painter, T. J.; Morgan, W. T. J. *Nature* **1961**, *191*, 39–40.
(10) Rege, V. P.; Painter, T. J.; Watkins, W. M.; Morgan, W. T. J. *Nature* **1964**, *203*, 360–363.
(11) Marr, A. M.; Donald, A. S.; Watkins, W. M.; Morgan, W. T. *Nature* **1967**, *215*, 1345–1349.

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$NaBD_4$,[13] were used to release O-glycans under various conditions. It was later established that immediate reduction of the glycans released under alkaline conditions can effectively prevent the alkali-catalyzed degradation, by eliminating the aldehyde functionality formed at the reducing end. Thus, the alkaline borohydride treatment established by Iyer and Carlson,[14] that is, 1.0 M borohydride and 0.05 M NaOH, has been the most successful means of releasing O-glycans with little "peeling", that is, serious carbohydrate chain degradation and monosaccharide destruction.

Several mild alkali conditions to release O-glycans without reduction have been reported recently, as there has been a pressing need for their use in functional studies and for O-glycan profiling;[2] these allow derivatization of reducing ends in order to increase detection sensitivity in HPLC and mass spectrometric analysis.[2,15,16] Attempts have also been made to prevent further degradation through β-elimination by forming, for example, glycosylamine intermediates with excess ethylamine[17] or hydrazone intermediates with hydrazine,[18,19] but peeling has not been prevented completely.[2,17,20]

Ammonium hydroxide has been used for release of O-glycans. Thomas-Oates and colleagues[21] employed ammonium hydroxide to remove O-glycans and to investigate the stability of the peptide backbones rather than the stability of the glycans.

In a more recent report, ammonium hydroxide saturated with ammonium carbonate was used to release both O- and N-glycans for glycomic profiling by mass spectrometry and capillary electrophoresis following reducing-terminal tagging.[22] The integrity and stabilities of released O- and N-glycans were investigated by use of maltoheptaose as a model reducing oligosaccharide and bovine fetuin as a model glycoprotein. The principle was to prevent peeling by formation of glycosylamine intermediates in ammonium hydroxide solution saturated with ammonium carbonate, in accord with earlier reports that, in aqueous ammonium bicarbonate[23] or ammonium hydroxide in the presence of ammonium bicarbonate,[24] reducing oligosaccharides can be efficiently converted into glycosylamines.

The ammonium hydroxide−ammonium carbonate method,[22] referred to hereinafter as the "$NH_4OH-(NH_4)_2CO_3$" method, has attracted considerable interest as a means of releasing O- and N-linked glycans from glycoproteins in reducing form for glycomic analysis[15,25] and for obtaining intact glycans to investigate their functional activities.[26,27] The yields of released glycans were reported to be quantitative for both O- and N-glycans, although no data were presented, and peeling was considered to be completely prevented[22] or significantly reduced.[28] A major focus of the aforementioned investigation[22] was, however, the stability of 1,4-linked glycan cores, as a maltoheptaose standard with the reducing-terminal sequence Glcα1,4Glc and the fetuin N-glycans with GlcNAcβ1,4GlcNAc cores were used, but the 1,3-linked cores typically present in O-glycans were not included in the study.

A recent study[2] and our preliminary work have indicated that the yield of O-glycan release and the integrity of the released O-glycans under nonreducing conditions could still be an issue. This has been exemplified by the inconsistency of O-glycan profiling of the same glycoprotein by different methodologies, and it has been speculated that peeling is the main reason for the discrepancy of data produced by nonreducing alkaline hydrolysis and reducing-terminal tagging compared with other methodologies.[2]

Here we investigate the stability of mucin-type O-glycans, as this is important not only for obtaining O-glycans for functional study but also for O-glycan profiling via the reducing-terminal tagging methodology. We compare in detail the stabilities in amine-based alkaline solutions of eight standard heptasaccharides with all possible linkages: α1,2-, β1,2-, α1,3-, β1,3-, α1,4-, β1,4-, α1,6- and β1,6-linked cores. We also use bovine submaxillary mucin (BSM) as a model glycoprotein with a high content of short, sialylated mucin-type O-glycans and bovine fetuin, which contains both N- and O-glycans. Detailed information available on the glycans of BSM[29−31] and fetuin[32−35] has helped to distinguish any degradation products resulting from alkaline treatment. As the standard methods for measuring yields and analyzing integrities of glycans released from glycoproteins, we use reductive Carlson degradation[14] for O-glycans and peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (PNGase) digestion[6] for N-glycans.

EXPERIMENTAL SECTION

Materials. Maltoheptaose (Malto-7; α1,4-linkage) was purchased from Sigma (Gillingham, England). Heptasaccharides derived from a cyanobacterial oligosaccharide fructoside[36] (Cyano-

(12) Schiffman, G.; Kabat, E. A.; Thompson, W. *Biochemistry* **1964**, *3*, 113–120.
(13) Kabat, E. A.; Bassett, E. W.; Pryzansky, K.; Lloyd, K. O.; Kaplan, M. E.; Layug, E. J. *Biochemistry* **1965**, *4*, 1632–1638.
(14) Iyer, R. N.; Carlson, D. M. *Arch. Biochem. Biophys.* **1971**, *142*, 101–105.
(15) Karlsson, N. G.; Packer, N. H. *Anal. Biochem.* **2002**, *305*, 173–185.
(16) Tarelli, E. *Carbohydr. Res.* **2007**, *342*, 2322–2325.
(17) Chai, W.; Feizi, T.; Yuen, C.-T.; Lawson, A. M. *Glycobiology* **1997**, *7*, 861–872.
(18) Patel, T.; Bruce, J.; Merry, A.; Bigge, C.; Wormald, M.; Jaques, A.; Parekh, R. *Biochemistry* **1993**, *32*, 679–693.
(19) Cooper, C. A.; Packer, N. H.; Redmond, J. W. *Glycoconjugate J.* **1994**, *11*, 163–167.
(20) Merry, A. H.; Neville, D. C.; Royle, L.; Matthews, B.; Harvey, D. J.; Dwek, R. A.; Rudd, P. M. *Anal. Biochem.* **2002**, *304*, 91–99.
(21) Rademaker, G. J.; Pergantis, S. A.; Blok-Tip, L.; Langridge, J. I.; Kleen, A.; Thomas-Oates, J. E. *Anal. Biochem.* **1998**, *257*, 149–160.
(22) Huang, Y.; Mechref, Y.; Novotny, M. V. *Anal. Chem.* **2001**, *73*, 6063–6069.
(23) Likhosherstov, L. M.; Novikova, O. S.; Derevitskaja, V. A.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *146*, C1–C5.
(24) Lubineau, A.; Auge, J.; Drouillat, B. *Carbohydr. Res.* **1995**, *266*, 211–219.
(25) Schulz, B. L.; Packer, N. H.; Karlsson, N. G. *Anal. Chem.* **2002**, *74*, 6088–6097.
(26) Xia, B. Y.; Royall, J. A.; Damera, G.; Sachdev, G. P.; Cummings, R. D. *Glycobiology* **2005**, *15*, 747–775.
(27) Song, X.; Lasanajak, Y.; Rivera-Marrero, C.; Luyai, A.; Willard, M.; Smith, D. F.; Cummings, R. D. *Anal. Biochem.* **2009**, *395*, 151–160.
(28) Goetz, J. A.; Novotny, M. V.; Mechref, Y. *Anal. Chem.* **2009**, *81*, 9546–9552.
(29) Chai, W.; Hounsell, E. F.; Cashmore, G. C.; Rosankiewicz, J. R.; Bauer, C. J.; Feeney, J.; Feizi, T.; Lawson, A. M. *Eur. J. Biochem.* **1992**, *203*, 257–268.
(30) Chai, W.; Hounsell, E. F.; Cashmore, G. C.; Rosankiewicz, J. R.; Feeney, J.; Lawson, A. M. *Eur. J. Biochem.* **1992**, *207*, 973–980.
(31) Savage, A. V.; Donohue, J. J.; Koeleman, C. A.; Van den Eijnden, D. H. *Eur. J. Biochem.* **1990**, *193*, 837–843.
(32) Bendiak, B.; Harrisbrandts, M.; Michnick, S. W.; Carver, J. P.; Cumming, D. A. *Biochemistry* **1989**, *28*, 6491–6499.
(33) Cumming, D. A.; Hellerqvist, C. G.; Harrisbrandts, M.; Michnick, S. W.; Carver, J. P.; Bendiak, B. *Biochemistry* **1989**, *28*, 6500–6512.
(34) Edge, A. S. B.; Spiro, R. G. *J. Biol.Chem.* **1987**, *262*, 16135–16141.
(35) Spiro, R. G.; Bhoyroo, V. D. *J. Biol. Chem.* **1974**, *249*, 5704–5717.
(36) Fischer, D.; Geyer, A.; Loos, E. *FEBS J* **2006**, *273*, 137–149.

7; α1,2-linkage), *Brucella abortus* cyclic β-glucan[37] (CβG-7; β1,2-linkage), an insoluble fraction of lentinan[38] (Poriaco-7; α1,3-linkage), Curdlan (Curd-7; β1,3-linkage), cellulose (Cello-7; β1,4-linkage), and dextran (Dextra-7; α1,6-linkage) were prepared from the respective polysaccharides after mild acid hydrolysis[39] and from pustulan (Pust-7; β1,6-linkage) by acetolysis as described.[39] Cello-oligosaccharide mixture was obtained from V-Lab (Covington, LA) and Curdlan oligosaccharide mixture was from Megazyme (Wicklow, Ireland).

The hydrolysates and acetolysate were fractionated by gel filtration on a Bio-Gel P4 column (1.6 × 90 cm). Elution was with water, and detection was by refractive index. The fractions were analyzed by matrix-assisted laser desorption/ionization mass spectrometry for oligosaccharide chain size, GC/MS methylation analysis for monosaccharide composition and linkage, and ^{1}H NMR for anomeric configuration.

Bovine submaxillary mucin (type I-S) and bovine fetuin were from Sigma. Ammonium carbonate, aqueous ammonium hydroxide (28%), and aqueous ethylamine (70%) were from Aldrich.

Alkaline Conditions for Treatment of Reducing Heptasaccharides. The following five alkaline conditions were used for treatment of the eight reducing heptasaccharides. Typically 5−25 μg of heptasaccharide, depending on availability, was treated in a reaction volume of 100 μL.

For reductive alkaline treatment (Carlson degradation, "NaOH−$NaBH_4$" condition), conditions used were as described:[14] heptasaccharides were incubated in 0.05 M NaOH−1 M $NaBH_4$ at 45 °C for 16 h. Thereafter, AcOH/H_2O (1:1) was used to destroy $NaBH_4$ and a minicolumn of AG50-X8 (H form) was used to remove sodium. The boric acid generated was removed by repeated coevaporation with MeOH.

Treatment with aqueous ammonium hydroxide saturated with ammonium carbonate [$NH_4OH-(NH_4)_2CO_3$ condition] was essentially as described.[22] In brief, aqueous ammonium hydroxide (28%) was previously saturated with ammonium carbonate at room temperature. After addition of heptasaccharide and a further 10 mg of ammonium carbonate, the reaction mixture was incubated at 60 °C for 40 h. Ammonium hydroxide and carbonate were removed by repeated coevaporation with water by lyophilization. The glycosylamine obtained was then converted into reducing sugars by hydrolysis in water solution at room temperature overnight or in water solution with addition of a few drops of 1 M AcOH.[17] This we found more convenient than the boric acid method.[22] Conversion of glycosylamine into reducing sugars was confirmed by mass spectrometric analysis.

Ammonium hydroxide alone ("NH_4OH" condition) under similar conditions was used for comparison and evaluation of the role of excess ammonium. In this case treatment was carried out in 28% aqueous solution at 60 °C for 40 h. Ammonium hydroxide was removed by repeated coevaporation with water by lyophilization. The glycosylamine obtained was converted into reducing sugars as described above.

Aqueous ethylamine under nonreducing conditions was also used for treatment of the heptasaccharides. The reaction was carried out at either 22 °C for 48 h ("$EtNH_2$-22" condition) or 60 °C for 6 h ("$EtNH_2$-60" condition) in an aqueous solution of ethylamine (70%) as described.[17] After reaction, ethylamine was removed by repeated coevaporation with H_2O by lyophilization. The glycosylamine obtained was converted into reducing sugars as described above.

The oligosaccharides obtained after alkaline treatment were redissolved in water for analysis.

Release of Glycoprotein Glycans with Aqueous Ammonium Hydroxide Saturated with Ammonium Carbonate. Glycoprotein BSM or fetuin were treated similarly as described in the literature and under the $NH_4OH-(NH_4)_2CO_3$ conditions described above. In brief, glycoprotein (5 mg) and ammonium carbonate (500 mg) were added to 5 mL of aqueous ammonium hydroxide solution presaturated with ammonium carbonate. The reaction mixture was incubated at 60 °C for 40 h, and the ammonium hydroxide and ammonium carbonate were removed by repeated coevaporation with water by lyophilization. Water (1 mL) was then added to the residue and the suspension was centrifuged. Aqueous AcOH (50%, 10 μL) was added to the supernatant and the solution was left at room temperature overnight for glycosylamine conversion. The solution was then passed through a minicolumn of cation exchange (AG50-X8) to remove protein/glycoprotein. The column was washed with water (3 bed volumes) and the fallthrough and washings were combined and lyophilized. A further chromatography was carried out with a short column (1.6 × 35 cm) of Bio-Gel P6 eluted with ammonium acetate (0.05 M) to remove further escaped protein/glycoprotein. The eluate in the included volume was pooled and lyophilized and was redissolved in water for analysis.

Release of Glycoprotein Glycans by Carlson Degradation and by PNGase Digestion. O-Glycans were released from glycoprotein BSM or fetuin (5 mg) as oligosaccharide alditols[14] under the reductive alkaline NaOH−$NaBH_4$ conditions described above with 1 mL of 0.05 M NaOH−1 M $NaBH_4$ solution. Borohydride was destroyed by addition of acetic acid (50%). The mixture was passed through a minicolumn of AG50-X8, and boric acid was removed by repeated coevaporation with MeOH. N-Glycan release by PNGase digestion was essentially as described by the manufacturer (New England Biolabs, Hitchin, U.K.) and the released N-glycans were recovered by a short column of Bio-Gel P6 as described above.

Permethylation of Oligosaccharides. Permethylation was carried out by the NaOH method essentially as described.[40] In brief, to the dried oligosaccharide was added dimethyl sulfoxide (DMSO), sodium hydroxide slurry, which was freshly prepared by crushing and grinding a few pellets of sodium hydroxide in DMSO with a homogenizer, and methyl iodide. After reaction at room temperature for 15 min, water was added and the methylated product was extracted with chloroform.

Mass Spectrometry. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in the positive-ion mode was carried out on a Tof Spec-2E instrument (Waters, Manchester, U.K.) for analysis of neutral heptasaccharides before and after

(37) Ciocchini, A. E.; Guidolin, L. S.; Casabuono, A. C.; Couto, A. S.; de Iannino, N. I.; Ugalde, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16492–16497.
(38) Zhang, P. Y.; Zhang, L. N.; Cheng, S. Y. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 1197–1202.
(39) Palma, A. S.; Feizi, T.; Zhang, Y.; Stoll, M. S.; Lawson, A. M.; Diaz-Rodríguez, E.; Campanero-Rhodes, A. S.; Costa, J.; Brown, G. D.; Chai, W. *J. Biol. Chem.* **2006**, *281*, 5771–5779.
(40) Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.

treatment with alkaline reagents. After removal of alkaline reagents, oligosaccharides were dissolved in methanol at a concentration of 50 pmol/μL, and 1 μL was deposited on the sample target together with a matrix of 2-(4-hydroxyphenylazo)benzoic acid. Laser energy was 20% (coarse) and 60% (fine), and resolution was at 1000.

Negative-ion electrospray ionization mass spectrometry (ESI-MS) was carried out on a Micromass Q-Tof for analysis of glycoprotein hydrolysates. Nitrogen was used as the desolvation and nebulizer gas at flow rates of 250 and 15 L/h, respectively. Source temperature was 80 °C and the desolvation temperature was 150 °C. Released oligosaccharide mixtures were dissolved in acetonitrile/1 mM NH_4HCO_3 (1:1 v/v), typically at a concentration of 5−10 pmol/μL, of which 5 μL was loop-injected. Mobile phase (acetonitrile/5 mM NH_4HCO_3, 1:1 v/v) was delivered by a syringe pump at a flow rate of 10 μL/min. Cone voltage was maintained at 50 eV while capillary voltage was at 3 kV.

Gel-Filtration Chromatography of Released Glycans. Gel-filtration chromatography was carried out with a Superdex Peptide column (PC 3.2/30, GE Healthcare, Uppsala, Sweden). Elution was with 0.05 M ammonium acetate at a flow rate of 0.3 mL/min, and detection was by refractive index. The pooled peaks were analyzed by mass spectrometry after removal of the volatile buffer by repeated coevaporation with water by lyophilization.

Other Analytical Methods. The concentration of ammonium cations was measured according to Nessler's method.[41] Concentrations of NH_4^+ were 12.2 M for 28% aqueous ammonia solution, 16.8 M for ammonia solution with saturated $(NH_4)_2CO_3$ at room temperature, and 18.2 M for ammonia solution with saturated $(NH_4)_2CO_3$ at 60 °C, after addition of further 100 mg/mL $(NH_4)_2CO_3$. Hexose assay was based on the orcinol−sulfuric acid method.[42]

RESULTS AND DISCUSSION

Stabilities of Reducing Heptasaccharides with Different Linkages under Different Alkaline Conditions. An α1,4-linked maltohepatose standard was previously chosen for studying the stability of reducing sugars under the $NH_4OH-(NH_4)_2CO_3$ condition.[22] However, it is well-known that reducing sugars with different linkages have different labilities toward alkaline conditions[43] and α1−4 does not represent typical N- or O-glycan cores, the former having a β1,4-linked and the latter having a β1,3-linked core. Generally, a 1,4-linkage with either an α- or β-configuration is relatively stable whereas 1,3-linkage is labile under alkaline conditions. We here selected eight homogluco-heptasaccharide standards, Cyano-7 (α1,2), CβG-7 (β1,2), Poriaco-7 (α1,3), Curd-7 (β1,3), Malto-7 (α1,4), Cello-7 (β1,4), Dext-7 (α1,6) and Pust-7 (β1,6) to investigate the stabilities of oligosaccharides with different linkages under different alkaline conditions. The alkaline conditions selected included the $NH_4OH-(NH_4)_2CO_3$, Carlson[14] ($NaOH-NaBH_4$), and the low (22 °C) and high temperature (60 °C) ethylamine conditions[17] ($EtNH_2$-22 and $EtNH_2$-60, respectively). To assess the effect of the excess of ammonium carbonate under the $NH_4OH-(NH_4)_2CO_3$, ammonium hydroxide alone (NH_4OH) under identical conditions was also included (for detailed conditions, see the Experimental Section).

Table 1. Stability Analysis of Reducing Glucoheptasaccharides under Mild Alkaline Conditions by Positive-Ion MALDI-MS[a]

samples	linkages	intact[b]	−Glc[c]	−2Glc[c]
		$NaOH-NaBH_4$		
Malto-7	α1,4	1177	nd	nd
Cello-7	β1,4	1177	nd	nd
Poriaco-7	α1,3	1177	1015 (5%)	nd
Curd-7	β1,3	1177	1015 (9%)	nd
Cyano-7	α1,2	1177	nd	nd
CβG-7	β1,2	1177	nd	nd
Dextra-7	α1,6	1177	nd	nd
Pust-7	β1,6	1177	1015 (3%)	nd
		$NH_4OH-(NH_4)_2CO_3$		
Malto-7	α1,4	1175	1013 (27%)	851 (3%)
Cello-7	β1,4	1175	1013 (35%)	851 (11%)
Poriaco-7	α1,3	1175	1013 (54%)	851 (18%)
Curd-7	β1,3	1175	1013 (122%)	851 (77%)
Cyano-7	α1,2	1175	nd	nd
CβG-7	β1,2	1175	nd	nd
Dextra-7	α1,6	1175	1013 (42%)	851 (12%)
Pust-7	β1,6	1175	1013 (50%)	851 (24%)
		NH_4OH		
Malto-7	α1,4	1175	1013 (106%)	851 (41%)
Cello-7	β1,4	1175	1013 (115%)	851 (20%)
Poriaco-7	α1,3	1175	1013 (29%)	851 (22%)
Curd-7	β1,3	1175	1013 (133%)	851 (150%)
Cyano-7	α1,2	1175	nd	nd
CβG-7	β1,2	1175	nd	nd
Dextra-7	α1,6	1175	1013 (4%)	nd
Pust-7	β1,6	1175	1013 (6%)	nd
		$EtNH_2$-22		
Malto-7	α1,4	1175	nd	nd
Cello-7	β1,4	1175	nd	nd
Poriaco-7	α1,3	1175	1013 (3%)	nd
Curd-7	β1,3	1175	1013 (6%)	nd
Cyano-7	α1,2	1175	nd	nd
CβG-7	β1,2	1175	nd	nd
Dextra-7	α1,6	1175	1013 (2%)	nd
Pust-7	β1,6	1175	1013 (3%)	nd
		$EtNH_2$-60		
Malto-7	α1,4	1175	1013 (4%)	nd
Cello-7	β1,4	1175	nd	nd
Poriaco-7	α1,3	1175	1013 (21%)	nd
Curd-7	β1,3	1175	1013 (21%)	nd
Cyano-7	α1,2	1175	nd	nd
CβG-7	β1,2	1175	nd	nd
Dextra-7	α1,6	1175	1013 (5%)	nd
Pust-7	β1,6	1175	1013(10%)	nd

[a] Reaction conditions are described in the Experimental Section. [b] The nominal masses of MNa^+ of intact heptasaccharides detected are listed. [c] Nominal masses of MNa^+ of the degradation products, glucohexa- and pentasaccharides (−Glc and −2Glc, respectively), together with the intensities relative to the intact molecules (as 100%) are listed; nd, not detected (below 2%).

After alkaline treatments, the products were analyzed by positive-ion MALDI-MS, and the results were compared with respect to the degree of oligosaccharide degradation (Table 1). As expected, Malto-7 with α1,4-linked Glc, which was used in the previous study,[22] was relatively stable: no or very little degradation product was found in the reaction mixture under Carlson ($NaOH-NaBH_4$) (Table 1 and Figure 1B) and the two ethylamine ($EtNH_2$-22 and $EtNH_2$-60) conditions. Some ap-

(41) Svehla, G. *Vogel's Textbook of Macro and Semimicro Qualitative Inorganic Analysis*; Longman: London, 1979.
(42) Chai, W.; Stoll, M. S.; Galustian, C.; Lawson, A. M.; Feizi, T. *Methods Enzymol.* **2003**, *362*, 160–195.
(43) Lindberg, B.; Lonngren, J.; Svensson, S. *Adv. Carbohydr. Chem. Biochem.* **1975**, *31*, 185–240.

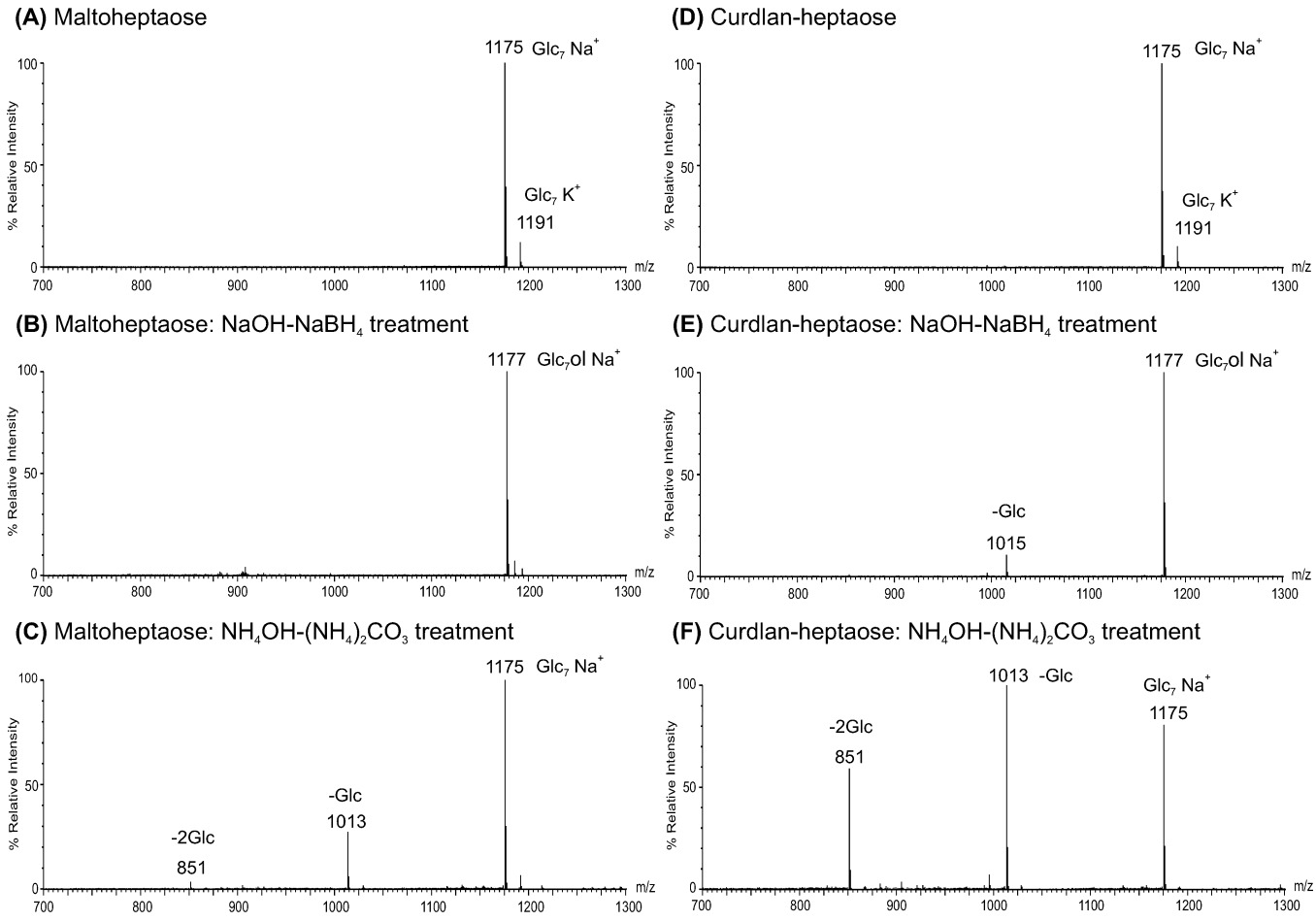

Figure 1. Positive-ion MALDI mass spectra of Malto-7 and Curd-7 after treatment with alkaline reagents: (A) Malto-7, (B) Malto-7 treated with $NaOH-NaBH_4$, (C) Malto-7 treated with $NH_4OH-(NH_4)_2CO_3$, (D) Curd-7, (E) Curd-7 treated with $NaOH-NaBH_4$, and (F) Curd-7 treated with $NH_4OH-(NH_4)_2CO_3$. Detailed reaction conditions are described in the Experimental Section.

preciable amounts of degradation products (loss of one and two Glc residues with relative intensities of 27% and 3%, respectively; Table 1 and Figure 1C) in the product obtained under the $NH_4OH-(NH_4)_2CO_3$ condition.[22] Cello-7 having β1,4-linkages gave similar results as Malto-7 (Table 1).

Not surprisingly, Curd-7 and Poriaco-7 with β1,3- and α1,3-linked Glc, respectively, gave serious peeling under $NH_4OH-(NH_4)_2CO_3$, NH_4OH, and $EtNH_2$-60 conditions (Table 1). With $NH_4OH-(NH_4)_2CO_3$ treatment, the base-induced degradation led to loss of two Glc residues (Figure 1F and Table 1). The relative intensity of the degraded product, −Glc (m/z 1013, 122%), was higher than that of the parent Curd-7 (m/z 1175). Further degraded product by losing two Glc residues was also significant (−2Glc, m/z 851, 77%). Even under Carlson $NaOH-NaBH_4$ (Figure 1E) and the mild $EtNH_2$-22 conditions, some minor degradation products can still be detected (Table 1).

Glucoheptasaccharides with α1,2- (Cyano-7) and β1,2-linkages (CβG-7) were the most stable and no degradation products were detected under all conditions. The 6-linked heptasaccharides Dextra-7 and Pust-7 gave complicated results. Generally they were more stable than the 3-linked and less stable than the 2- and 4-linked. However, under the NH_4OH condition the 6-linked were more stable than the 4-linked sugars.

Among the alkaline conditions tested, Carlson ($NaOH-NaBH_4$) and the low-temperature ethylamine ($EtNH_2$-22) produced less degradation product whereas the $NH_4OH-(NH_4)_2CO_3$ and NH_4OH conditions led to serious peeling. However, the effect of excess ammonium carbonate was apparent as the treatment with ammonium hydroxide alone (NH_4OH) gave much more degraded product than the $NH_4OH-(NH_4)_2CO_3$ treatment. In the case of β1,3-linked Curd-7, peeling products −Glc and −2Glc produced by the NH_4OH treatment had relative intensities of 133% and 150%, respectively, whereas those obtained from the $NH_4OH-(NH_4)_2CO_3$ treatment had the intensities of 122% and 77%, respectively (Table 1). The protecting effect can also be observed in the data obtained with the 4-linked heptasaccharides Dextra-7 and Pust-7 (Table 1).

Release of Mucin-type O-Glycans from Bovine Submixillary Mucin. BSM contains mucin-type short but complex O-glycan chains and has been used extensively for studies of hydrolysis of mucin-type oligosaccharide chains, as the structures of the O-glycans in BSM have been well documented.[29−31] The main feature of BSM O-glycans is the presence of dominant disaccharides NeuAcα2−6GalNAc and NeuGcα2−6GalNAc, together with abundant trisaccharides with the sequences of NeuAcα2−6(GlcNAcβ1−3)GalNAc and NeuGcα2−6(GlcNAcβ1−3)GalNAc. The presence of a GlcNAc at the 3-position of the core GalNAc in the trisaccharides is useful for assessment of the stability of the typical 3-linkage in core GalNAc of mucin glycoproteins.

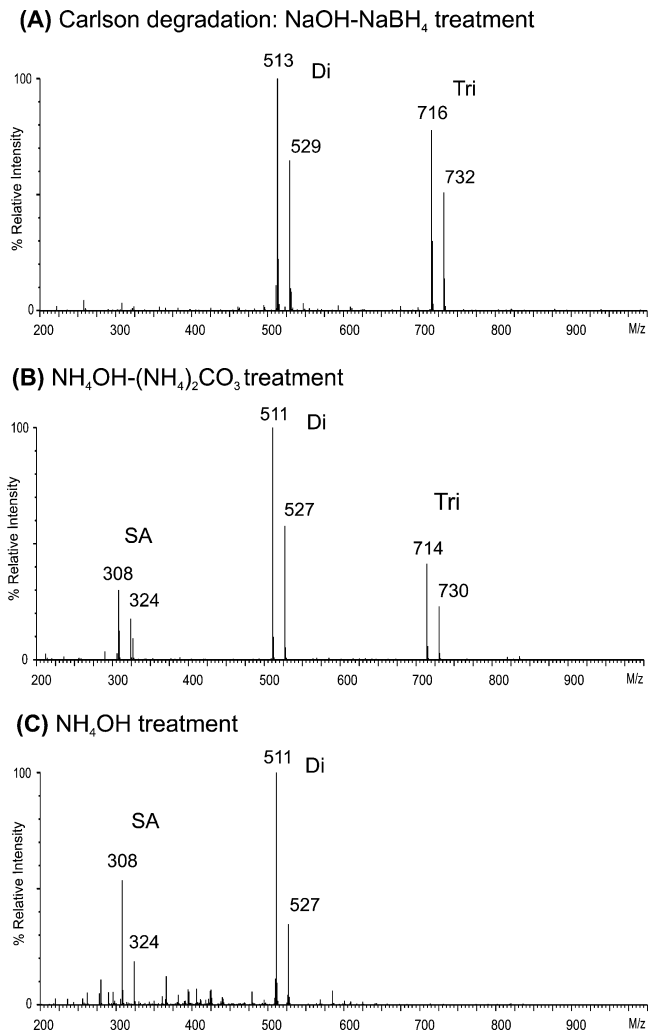

Figure 2. Negative-ion ESI mass spectra of O-glycans released from bovine submaxillary mucin: (A) by Carlson degradation, $NaOH-NaBH_4$, (B) by treatment with $NH_4OH-(NH_4)_2CO_3$, and (C) by treatment with NH_4OH. Detailed reaction conditions are described in the Experimental Section.

By use of reductive alkaline hydrolysis, Carlson degradation ($NaOH-NaBH_4$), as the standard condition, the released glycan mixtures were analyzed by negative-ion ESI-MS and compared with the products obtained from hydrolysis with $NH_4OH-(NH_4)_2CO_3$. In the spectrum of Carlson degradation products (Figure 2A), the reduced sialylated di- and trisaccharide alditols were dominant: NeuAc/NeuGcα2−6GalNAcol with $[M - H]^-$ at m/z 513 and 529 and NeuAc/Gcα2−6(GlcNAcβ1−3)GalNAcol $[M - H]^-$ at m/z 716 and 732, respectively. The intensity ratios of tri- to disaccharide alditols were ∼75%. Under the $NH_4OH-(NH_4)_2CO_3$ condition (Figure 2B), the ratios of the reducing trisaccharides ($[M - H]^-$: m/z 714 and 730) to disaccharides ($[M - H]^-$: m/z 511 and 527) was reduced to ∼45%. In addition, some monosaccharide NeuAc ($[M - H]^-$at m/z 308) and NeuGc ($[M - H]^-$ at m/z 324) were also observed in the spectrum. Clearly a proportion of 3-linked GlcNAc was cleaved together with some 6-linked sialic acid residues. In the negative-ion spectrum, the cleaved neutral monosaccharide GlcNAc was not detected while the acidic NeuAc and NeuGc were readily observed.

The results of BSM O-glycan release under the $NH_4OH-(NH_4)_2CO_3$ condition were in agreement with those of the glucoheptasaccharides obtained above. Reducing-terminal 3-linked residues were not stable in alkaline solutions and can be partially cleaved, in contrast to the previous report.[22] In addition, substitution at the 6-position was also cleavable, although to a much lesser extent. Further experiments with NH_4OH alone without excessive $(NH_4)_2CO_3$ gave a spectrum with undetectable trisaccharides (Figure 2C), indicating that indeed a large excess of $(NH_4)_2CO_3$ did give partial protection through formation of a glycosylamine intermediate.

While mass spectrometry was used for analysis of the integrity of the hydrolysis products, gel-filtration chromatography was used for estimation and comparison of the actual yields of release. Released products from hydrolysis of BSM with $NH_4OH-(NH_4)_2CO_3$ (Figure 3B) can be compared with those of Carlson degradation (Figure 3A). As shown in Figure 3A, the minor fraction peak 1 contains tetra- and pentasaccharides,[30] peak 2 contains trisaccharides, and peak 3 contains disaccharides, as identified by ESI-MS. Clearly the overall yield from $NH_4OH-(NH_4)_2CO_3$ hydrolysis was lower than that of Carlson degradation, where oligosaccharide alditols were obtained. Moreover, the intensity of peak 2 (Figure 3B), the 3-linked GlcNAc-containing trisaccharides, is much weaker than the corresponding peak obtained from Carlson degradation (peak 2, Figure 3A). The large peak at the void volume (V_0) in Figure 3B contains the protein/glycoprotein that evaded the preseparation step. In Figure 3A the peak at V_0 is much smaller as under Carlson conditions the protein was cleaved by the reagents and the majority of the resulting peptides were removed by cation exchange prior to gel-filtration chromatography.

Release of O- and N-Glycans from Bovine Fetuin. Bovine fetuin contains mainly sialylated tri- and biantennary N-glycans,[32,33] together with several sialylated short-chain O-glycans.[34,35] To assess the value of the ammonium-based alkaline hydrolysis method[22] for release of N- and O-glycans, bovine fetuin was treated under the $NH_4OH-(NH_4)_2CO_3$ condition[22] and the released glycans were analyzed by negative-ion ESI-MS and compared with those obtained from the well-established methods, PNGase F digestion for N-glycans and Carlson degradation for the O-glycans, for stabilities and yields of N- and/or O-glycans. For presentation and comparison purposes, the ESI mass spectra are shown in Figure 4 as transformed from raw data into mass scale, and the original spectral data are listed in Table 2.

In the spectrum of PNGase F digest (Figure 4A), two main N-glycans, tri- and tetrasialylated triantennary (2879 and 3170 Da),[32,33] and two minor N-glycans, disialylated biantennary (2222 Da) and pentasialylated triantennary (3461 Da),[33] were observed. Carlson degradation (Figure 4B) produced the expected alditols (with increments of 2 Da to the reducing glycans), O-linked sialylated tri- (676 Da), tetra- (967 Da),[35] and hexasaccharides (1332 Da).[34] In addition, not too surprisingly, weak signals derived from the major N-glycans (2881 and 3172 Da) were also observed in the spectrum of Carlson degradation products (Figure 4B).

Both N- and O-glycans were obtained from $NH_4OH-(NH_4)_2CO_3$ treatment[22] (Figure 4C). However, compared with PNGase digestion, the minor N-glycans pentasialylated triantennary (3461 Da) and disialylated biantennary (2222 Da) were

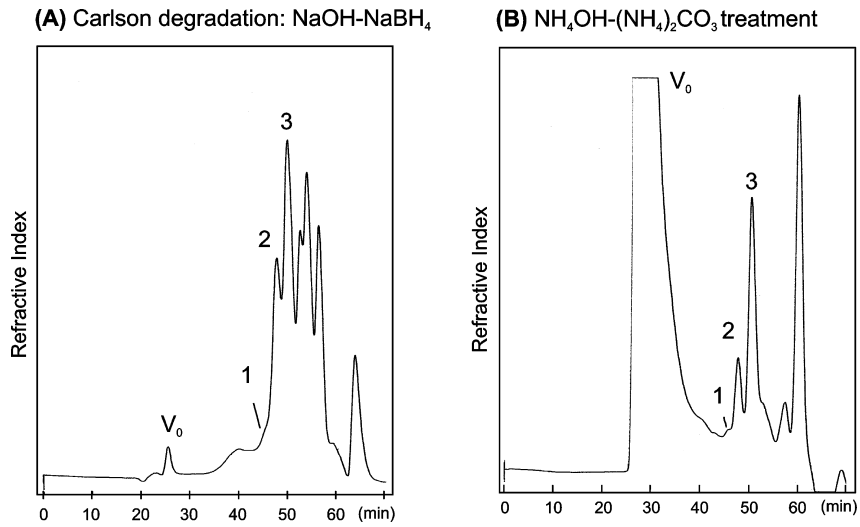

Figure 3. Gel-filtration chromatography of O-glycans released from bovine submaxillary mucin: (A) by Carlson degradation, $NaOH-NaBH_4$, and (B) by treatment with $NH_4OH-(NH_4)_2CO_3$. Detailed reaction conditions are described in the Experimental Section, and identical refractive index scales were used for both chromatograms. Peaks 1 contain the tetra- and pentasaccharides, peaks 2 contain the trisaccharides NeuAc/NeuGc2−6(GlcNAc1−3)GalNAc, and peaks 3 contain the disaccharides NeuAc/NeuGc2−6GalNAc, as analyzed by ESI-MS.

extremely weak or not detectable above the chemical noise background. The O-linked hexasaccharide (1330 Da) was also not detected, whereas a peeled O-glycan, NeuAc-Gal (471 Da), was apparent. The results obtained from negative-ion ESI-MS were corroborated by positive-ion MALDI-MS (spectrum not shown) after permethylation of the glycans released.

The negative-ion ESI spectrum obtained here from $NH_4OH-(NH_4)_2CO_3$ treatment[22] is somewhat different from that reported previously from negative-ion MALDI-MS.[22] As shown in Figure 4C, five major oligosaccharide components were identified: N-linked trisialylated (2879 Da) and tetrasialylated triantennary (3170 Da)[32,33] and O-linked di- (471 Da), tri- (674 Da), and tetrasaccharides (965 Da).[35] The minor N-linked biantennary (2222 Da), pentasialylated triantennary (3461 Da), and O-linked hexasaccharides (1330) were not observed, presumably due to the low quantity of materials used, as evidenced by the higher signal-to-noise ratio (Figure 4C). In the previously published MALDI spectrum,[22] there were several ions resulting from undersialylated bi- and triantennary N-glycans (m/z 1931, 2298, and 2587, respectively), but these were absent in the negative-ion ESI spectrum. These ions present in the previously reported spectrum were presumably produced by the well-known MALDI-induced desialylation-[44−46] and were not authentic glycan sequences present in bovine fetuin.[32,33] Sialylated oligosaccharides, particularly those containing multiple sialic acids, can have problems with MALDI-MS analysis even in the negative-ion mode. MALDI-induced desialylation is particularly serious with DHB matrix and in the reflector mode.[44] For the alkaline labile O-glycans, the peak at m/z 1334 shown in the MALDI spectrum of the previous report[22] was probably incorrectly assigned as the hexasaccharide because the mass for the $[M - H]^-$ of the hexasaccharide NeuAc2−3Gal1−3(NeuAc2−3Gal1−4GlcNAc1−6)GalNAc[34] should have a $[M - H]^-$ ion at m/z 1329 but not 1334. An error of 5 Da in this mass region is unlikely.

Again we used gel-filtration chromatography for comparison of the yields of released N- and O-glycans obtained from $NH_4OH-(NH_4)_2CO_3$ treatment with those derived from the well-established methods. Each fraction was analyzed by ESI-MS for identification. For the same amount of fetuin, PNGase F digestion (Figure 5A) gave a high yield of N-glycans, which were eluted at 37 min in peak 1. The peak at the void volume (V_0) was the de-N-glycosylated glycoprotein, and the minor peaks at 60 min were from various salts used in the digestion. Carlson degradation gave a rather complicated chromatogram (Figure 5B): O-linked hexasaccharide at peak 2, tetrasaccharide at peak 3, trisaccharide at peak 4, and very small amounts of N-glycans coeluted with peptides/glycopeptides at around 38 min. The latter were produced by the high concentration of sodium borohydride and escaped from the prepurification step. The N- and O-glycans obtained from $NH_4OH-(NH_4)_2CO_3$ treatment of the same amount of fetuin are shown in Figure 5C. Clearly the yield of N-glycans (peak 1, Figure 5C) was much reduced compared with that of enzymatic digestion (peak 1, Figure 5A). The peaks for the O-glycan fractions, peaks 2−4, were also reduced. Peak 5 was from the peeled disaccharide. The overall gel-filtration results were consistent with those obtained by direct mass spectrometric analysis (Figure 4).

CONCLUSIONS

Reductive alkaline hydrolysis has been conventionally used for O-glycan release. O-Glycans typically contain a reducing-terminal β1,3-linkage, which is labile toward alkaline treatment. In the present study we have investigated in detail the stabilities of the released O-linked oligosaccharide chains under nonreducing alkaline conditions. Using standard glucoheptasaccharides, we have demonstrated that β1,3-linkage is labile whereas β1,4-linkage is relatively stable under the alkaline conditions among the eight different linkages. It was not surprising that some considerable

(44) Papac, D. I.; Wong, A.; Jones, A. J. S. *Anal. Chem.* **1996**, *68*, 3215–3223.
(45) Powell, A. K.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1027–1032.
(46) O'Connor, P. B.; Mirgorodskaya, E.; Costello, C. E. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 402–407.

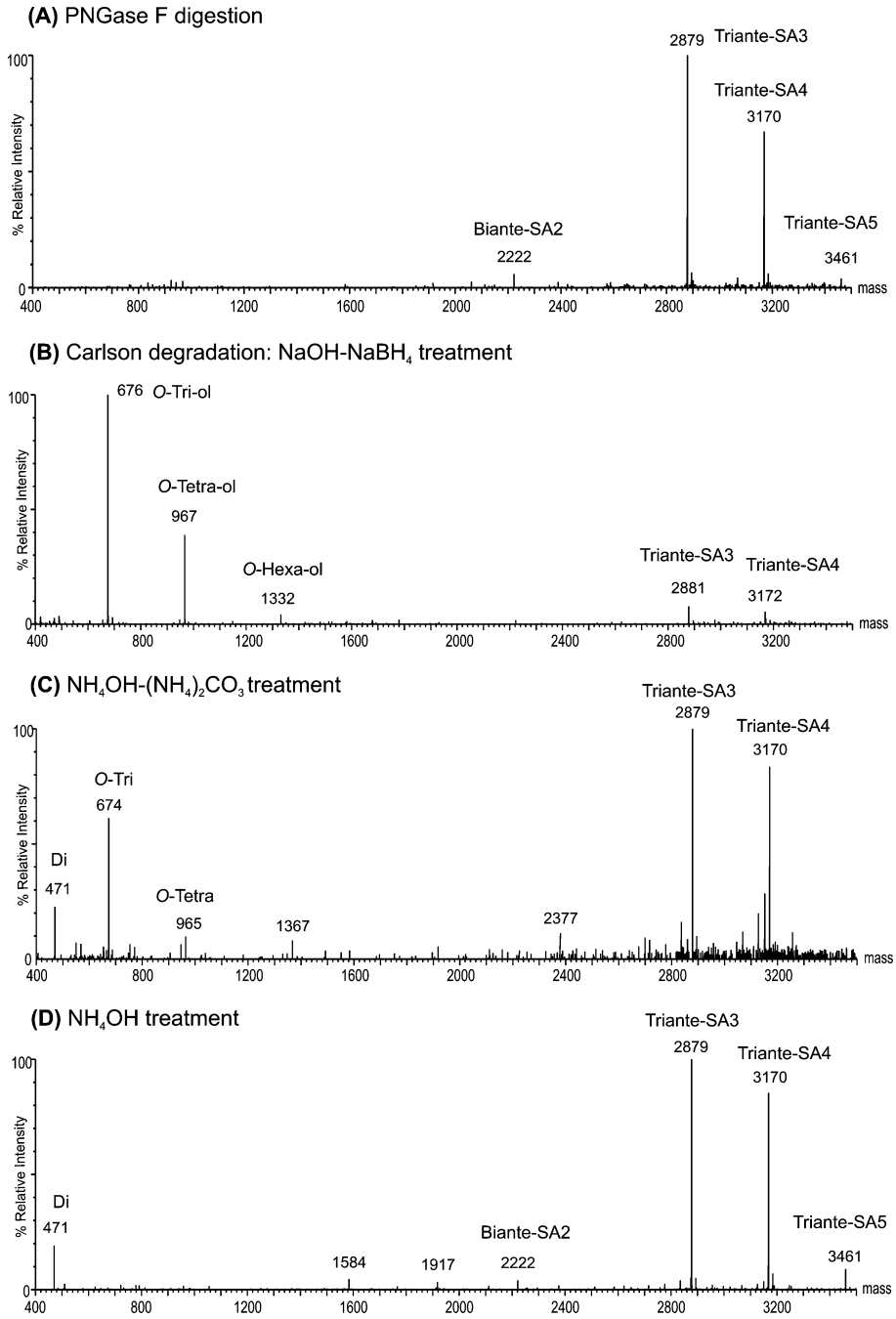

Figure 4. Negative-ion ESI mass spectra of N- and O-glycans released from bovine fetuin: (A) by treatment with PNGase F digestion, (B) by Carlson degradation, NaOH−$NaBH_4$, (C) by treatment with $NH_4OH-(NH_4)_2CO_3$, and (D) by treatment with NH_4OH.

degradation was detected in products of the 3-linked reducing heptasaccharide standards and in the released O-glycans containing the β1,3-linked GalNAc core obtained from both BSM and fetuin. The use of maltoheptaose as a model to validate the ammonium-based method for release of O- and N-glycans in the previous study[22] is questionable. With mucin-type BSM and N- and O-glycan-containing fetuin as model glycoproteins, we have observed that the yields of release of both types of glycans were lower than the standard procedures, O-glycan release by Carlson degradation and N-glycan release by PNGase F digestion. Recovery of the uncleaved glycoproteins for a further alkaline treatment may be necessary for increasing yield.

The role of the large excess of ammonium conferred by the saturated ammonium carbonate was apparent. The concentration of the ammonium increased considerably: 12.2 M for the 28% aqueous ammonium hydroxide solution, 16.8 M for the solution saturated with ammonium carbonate at room temperature, and 18.2 M at 60 °C after addition of further ammonium carbonate. The increased concentration could help formation of a glycosylamine intermediate to limit further degradation, and the increased basicity could help the hydrolysis yield. However, peeling cannot be completely prevented and the hydrolysis yield is still low compared with the standard methods.

N-Linked oligosaccharides have been considered to be resistant to β-elimination under Carlson conditions, and this has been used as a criterion for judging whether a glycoprotein contains N- or O-glycan chains. Our result obtained here is consistent with a number of recent observations indicating that mild alkaline

Table 2. Negative-Ion ESI-MS Analysis of O- and N-Glycans Released from Bovine Fetuin under Different Alkaline Conditions

	observed ions				molecular masses		
glycans[a]	−1	−2	−3	−4	calcd	theor	found under conditions
O-di	470.3				471.3	471.2	$NH_4OH-(NH_4)_2CO_3$; NH_4OH
O-tri	673.4				674.4	674.2	$NaOH-NaBH_4$; $NH_4OH-(NH_4)_2CO_3$
O-tetra	964.6	481.8			965.6	965.3	$NaOH-NaBH_4$; $NH_4OH-(NH_4)_2CO_3$
O-hexa		664.4			1330.8	1330.5	$NaOH-NaBH_4$; $NH_4OH-(NH_4)_2CO_3$
N-biante-NeuAc2		1110.7			2223.4	2222.8	PNGase F; NH_4OH
N-triante-NeuAc3		1438.9	958.9		2879.7	2879.0	all conditions
N-triante-NeuAc4		1584.4	1056.0	791.7	3170.8	3170.1	all conditions
N-triante-NeuAc5			1152.9	864.4	3461.6	3461.2	PNGase F; NH_4OH

[a] O-tri is NeuAc2−3Gal1−3GalNAc; O-tetra is NeuAc2−3Gal1−3(NeuAc2−6)GalNAc; O-hexa is NeuAc2−3Gal1−3(NeuAc2−3Gal1−4GlcNAc1−6)GalNAc; O-di is NeuAc2−3Gal, an O-glycan degradation product.

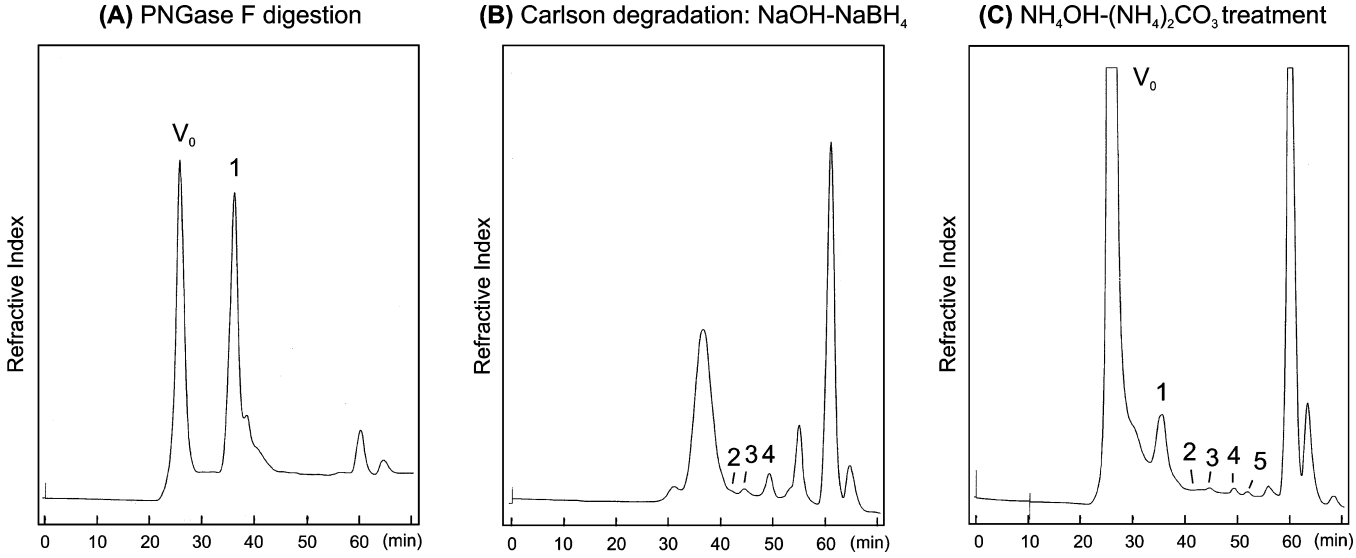

Figure 5. Gel filtration chromatography of N- and O-glycans released from bovine fetuin: (A) by PNGase F digestion, (B) by Carlson degradation, $NaOH-NaBH_4$, and (C) by treatment with $NH_4OH-(NH_4)_2CO_3$. Identical refractive index scales were used for all three chromatograms.

conditions can lead to the release of N-glycans, although with low yield,[15,25] and this once again cautions against conclusions on the nature of glycosylation derived from such treatment.

The data presented here demonstrate that complete prevention of peeling under alkali-catalyzed hydrolysis conditions will remain difficult. Even with the most successful Carlson degradation some 5% peeling has been obtained, as the β-elimination mechanism governing protein−O-linked glycan cleavage is essentially the same as the mechanism of peeling in which further β-elimination along the carbohydrate chain results in progressive chain shortening until alkali-stable linkages are reached. A proportion of peeling product present in nonreducing mild alkaline treatment of O-linked glycoproteins, particularly those of mucin type with clustered glycan chains, may have been the reason for the inconsistency of O-glycan profiling by reducing-terminal tagging strategies of the same glycoprotein. It has been speculated that peeling is the main reason for the discrepancy in data produced by nonreductive alkaline hydrolysis and reducing-terminal tagging compared with other methodologies based on the release by reducing Carlson degradation.[2]

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This paper was published on the Web on October 28, 2010, with a minor error in the trisaccharide sequences. The corrected version was reposted on November 12, 2010.

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