—Regular Articles—

日本薬局方医薬品各条ヘパリンナトリウム純度試験へのキャピラリー電気泳動法の適用について

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Capillary Electrophoresis Analysis of Contaminants in Heparin Sodium for the Japanese Pharmacopoeia Purity Test

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Heparin is widely used as an anticoagulant for the treatment and prevention of thrombotic disorders. Recently, hundreds of cases of anaphylactic reaction as adverse effects were reported by the presence of contaminating oversulfated chondroitin sulfate (OSCS) in some heparin preparations. In addition, these heparin preparations often contaminated dermatan sulfate (DS). Unfortunately, the Japanese Pharmacopoeia (JP) does not include appropriate purity tests. In the present paper, we show that capillary electrophoresis (CE) is a powerful tool for the analysis of OSCS and DS in heparin preparations. CE method shows high resolution and good quantification of OSCS in heparin preparations. This method (OSCS method) was evaluated for accuracy (93.7 %), repeatability (R.S.D.=2.11), linearity (R^2=0.9996), detection limit (0.1% OSCS) and specificity. In contrast, DS was not able to be detected in high sensitivity by OSCS method. However, a modified CE method (DS method) using the buffer at lower pHs showed good parameters for accuracy (88.1%), repeatability (R.S.D.=1.99), linearity (R^2=0.9998), detection limit (0.25% DS) and specificity. In conclusion, CE will be an alternative to the NMR method which is being adopted for purification test of heparin sodium in the present version of JP.

Key words——capillary electrophoresis; heparin sodium; oversulfated chondroitin sulfate; dermatan sulfate

緒　　言

ヘパリンナトリウムは，ウロン酸（L-イズロン酸またはD-グルクロン酸）とD-グルコサミンの2糖単位の繰り返し構造に，2糖あたり平均2-3個の硫酸基を持つ構造からなる硫酸化グリコサミノグリカンのナトリウム塩である［Fig. 1(a)］．ヘパリンナトリウムは，アンチトロンビンIIIと特異的に結合することにより，第IIa因子や第Xa因子などの血液凝固因子を阻害し血液凝固防止作用を示す.[1,2] そのため，血液透析などの体外循環装置使用時の血液凝固防止剤として世界中で汎用されるなど，臨床上極めて重要な医薬品であり，第15改正日本薬局方に収載されている．また，低分子量ヘパリン製剤の原料としても使用されている.[3]

2007年12月米国において，Baxter社製ヘパリンナトリウム製剤の静脈内急速大量投与を受けた患者に，血圧低下や頻脈等を伴うアレルギー反応が頻発し，80名以上の死亡例が報告された.[4] これまでヘパリン関連製剤に関して，血小板減少症などの副作用が知られていたが，今回発生した副作用はこれまでの報告例とは明らかに異なるものであった．さらに，ドイツでも別メーカーが製造したヘパリンナトリウム製剤の投与を受けた患者に同様のアレルギー反応がみられたことから国際的な問題へと発展した．2008年3月，米国食品医薬品局（FDA）は有害事象が多発したロットにヘパリン様物質が混入してい

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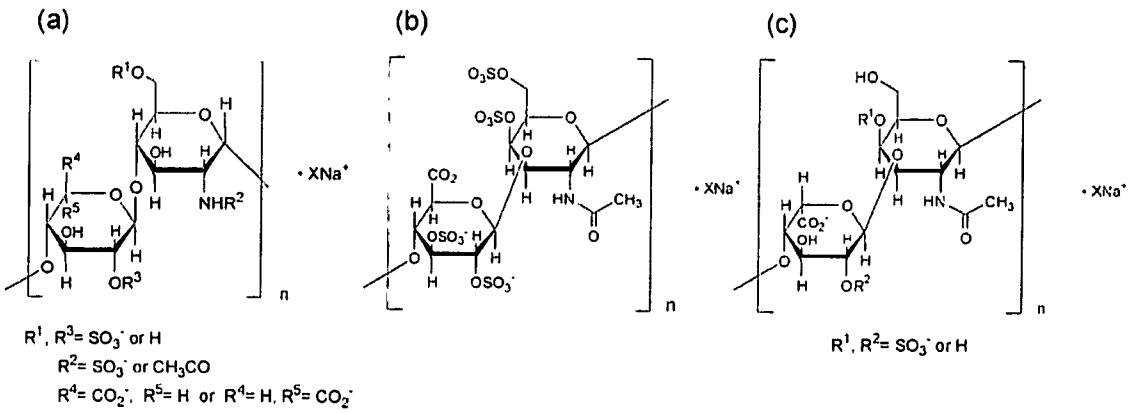

Fig. 1. Structures of the Disaccharide Unit of Glycosaminoglycans Tested in the Present Study
(a) Heparin sodium, (b) oversulfated chondroitin sulfate (OSCS) and (c) dermatan sulfate (DS).

ることを発表し,[5] のちに Guerrini らによる 2 次元 NMR などを用いる構造解析によって，このヘパリン様物質は過硫酸化コンドロイチン硫酸（oversulfated chondroitin sulfate; OSCS）であることが明らかにされた.[6] 通常，天然に存在するコンドロイチン硫酸は，D-グルクロン酸と N-アセチルガラクトサミンの 2 糖単位に，硫酸基が 1—3 個結合している．しかし，問題のヘパリンナトリウム製剤に混入していた OSCS は，コンドロイチン硫酸中のすべての水酸基が硫酸化され，2 糖単位中に硫酸基が 4 個結合した構造であった［Fig. 1 (b)］．また，有害事象を引き起こしたヘパリンナトリウム中には，OSCS に加えて，同じ硫酸化グリコサミノグリカン類の一種であるデルマタン硫酸（dermatan sulfate; DS，別名：コンドロイチン硫酸 B）［Fig. 1 (c)］も，従来の製品よりも多量に含まれていることが明らかにされた．

FDA は，有害事象の原因物質として OSCS を特定したことを発表するとほぼ同時に，^{1}H- 核磁気共鳴スペクトル測定法（^{1}H-NMR）とキャピラリー電気泳動法を用いる OSCS の検出法をインターネット上に公開した.[7,8] ^{1}H-NMR は，ヘパリンナトリウム中の N-アセチルグルコサミンの N-アセチル基と，OSCS 中の N-アセチルガラクトサミンの N-アセチル基の化学シフトが異なることを利用する検出法である．また，キャピラリー電気泳動法はヘパリンナトリウムと OSCS の硫酸基の結合数の違いを利用する分離分析をもとにする検出法である．さらに，陰イオン交換カラムを用いる HPLC 法や，単糖組成分析法，Inhibition of Taq polymerase 法などによる OSCS 検出法が次々に報告された.[9-11] 各国は，これらの検出法を用いてヘパリンナトリウム製剤の分析を行うとともに，OSCS の存在が確認されたヘパリンナトリウム製剤の回収を行う等の対応をとった．わが国でも，問題のヘパリンナトリウム製剤と同じ SPL 社の原薬を使用した国内 3 社が予防的措置として自主回収を行った．そのため，世界的にヘパリン関連医薬品の供給不足への懸念が広がり，ヘパリンナトリウム製剤の安定供給のために，ヘパリンナトリウム原料中の OSCS 及び DS の試験法の整備が緊急課題となった．

問題発生時，第 15 改正日本薬局方医薬品各条ヘパリンナトリウムの項には，バリウムやタンパク質などに関する純度試験が規定されていたが，記載されている試験法では OSCS 混入の有無を評価することができない．そこでわが国においても FDA が公開した試験法を参考に，^{1}H-NMR 及びキャピラリー電気泳動法による日本薬局方への適用を目的とした分析法バリデーションが行われた.[12,13] その結果，官報号外第 166 号（平成 20 年 7 月 31 日）において，「ヘパリンナトリウムに関する日本薬局方の一部改正に伴う取り扱いについて」として，^{1}H-NMR を用いる OSCS の限度試験が導入されることとなった．なお，今回の改正では，キャピラリー電気泳動法による試験の導入は見送られた．その理由として，FDA が公開したキャピラリー電気泳動法の条件は，ヘパリンナトリウムと OSCS のピークの分離が不十分であり，OSCS 検出の特異性や検出感度に問題があったためである.[13] しかし，キャピラリー電気泳動法はルーチン分析に適した分

析法であり，またヘパリンナトリウム中のOSCSやDSを検出できる数少ない分析法の1つであることなどからその価値は高く，米国薬局方ではヘパリンナトリウム確認試験に，また，欧州薬局方ではヘパリンナトリウムの製造部分の試験法に採用されている．

本研究では，ヘパリンナトリウム製剤の品質・安全性確保を目的として，キャピラリー電気泳動法による試験法を確立すると共に，分析法バリデーションを実施し，日本薬局方医薬品各条ヘパリンナトリウム純度試験としての適用可能性を検証した．

実験方法

1. 試料及び試薬　ヘパリンナトリウムは日本薬局方ヘパリンナトリウム標準品を使用した．OSCSは日本薬局方過硫酸化コンドロイチン硫酸標準品を使用した．なお，分析条件の検討には，生化学用ヘパリン（Sigma社製，ブタ腸粘膜由来）を使用した．DS（ブタ皮膚由来）は生化学工業㈱から購入した．OSCSを含有するヘパリンナトリウム製剤原料は日本バルク薬品㈱より供与を受けた．その他の試薬は特級品，あるいはHPLCグレードを使用した．本試験に用いた水は，MILLIPORE Direct-Qにより調製後，直ちに使用した．

2. 試験標準溶液　ヘパリンナトリウムを水に溶解し，ヘパリンナトリウム標準溶液（20 mg/ml）とした．また，OSCS及びDSは水に溶解し，それぞれOSCS標準溶液（4 mg/ml）及びDS標準溶液（4 mg/ml）を調製した．各試験ではこれらの標準溶液を用い，分析能パラメーター評価用試験溶液の濃度に調整後試験に用いた．なお，これらの標準溶液はあらかじめポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターによりろ過した後，試験に供した．

3. 分析条件　キャピラリー電気泳動装置としてBeckman P/ACE MDQ Glycoprotein Systemを用いた．キャピラリーカラムは0.1 M水酸化ナトリウムで10分間，続いて10分間水で洗浄し，3回の空試験を行った後に試験に使用した．キャピラリーカラムは分析毎に，水で4分間，泳動用緩衝液で4分間洗浄した．

1) FDA method：キャピラリーカラムはフューズドシリカキャピラリー（GL Sciences社製，内径50 μm，有効長56 cm）を用いた．電気泳動用緩衝液［36 mM Sodium phosphate buffer (pH3.5)］は，リン酸二水素一ナトリウム一水和物1.0 gを水195 mlに溶解し，リン酸でpHを3.5に調整した後，水を加えて200 mlとし，ポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターでろ過後，脱気して用いた．検出は，紫外部吸収検出（200 nm）により行った．印加電圧は30 kVで，試料導入側を陰極，廃液側を陽極として電気泳動した．分析温度は25℃とした．試料は加圧法（0.7 psi）により30秒間導入した．

2) OSCS method：キャピラリーカラムはフューズドシリカキャピラリー（GL Sciences社製，内径25 μm，有効長20 cm）を用いた．電気泳動用緩衝液［1000 mM Tris-phosphate buffer (pH3.5)］は，トリス30 gを水200 mlに溶解し，リン酸でpHを3.5に調整した後，水を加えて250 mlとし，ポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターでろ過後，脱気して用いた．検出は紫外部吸収検出（200 nm）により行った．電流値を50 μAに設定し，試料導入側を陰極，廃液側を陽極として電気泳動した．分析温度は25℃とした．試料注入は加圧法（3.0 psi）により30秒間導入した．

3) DS method：キャピラリーカラムはフューズドシリカキャピラリー（GL Sciences社製，内径50 μm，有効長20 cm）を用いた．電気泳動用緩衝液［100 mM Tris-phosphate buffer (pH2.5)］は，トリス3.0 gを水200 mlに溶解し，リン酸でpHを2.5に調整した後，水を加えて250 mlとし，ポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターでろ過後，脱気して用いた．検出は紫外部吸収検出（200 nm）により行った．電流値を80 μAに設定し，試料導入側を陰極，廃液側を陽極として電気泳動した．分析温度は25℃とした．試料注入は加圧法（0.7 psi）により30秒間導入した．

4. 分析能パラメーターの評価　キャピラリー電気泳動装置としてBeckman P/ACE MDQ Glycoprotein Systemを用いて試験を実施し，Beckman 32 Karat Gold Software Version 7.0を用いてピーク面積値を算出した．ピーク面積値は最小ピーク幅設定値を2秒とし，OSCS及びDSのピーク開始点とピーク終了点を結ぶ傾斜線をベースラインとして検出されるピークの積算値から求めた．

4-1. OSCS ヘパリンナトリウム標準溶液 (20 mg/ml) 0.8 ml に，OSCS 標準溶液 (4 mg/ml) をそれぞれ 0.004, 0.01, 0.02, 0.04, 0.2 及び 0.4 ml を添加し，ついで水を 0.796, 0.79, 0.78, 0.76, 0.6 及び 0.4 ml 加えて混和し，ヘパリンナトリウムに対して OSCS がそれぞれ 0.1, 0.25, 0.5, 1.0, 5.0 及び 10.0% (w/w) 含む溶液とした．これらの溶液を分析能パラメーター評価用試験溶液とし，キャピラリー電気泳動装置を用いて測定した．

真度，併行精度及び室内再現精度は，5.0% OSCS を含むヘパリンナトリウム試験溶液を用いて各 6 回分析を行い，OSCS のピーク面積値を用いて算出した．特異性は 10% OSCS を含むヘパリンナトリウム試験溶液を用いて求めた．検出限界及び定量限界は 0.1–5.0% の OSCS を含むヘパリンナトリウム試験溶液を，直線性及び範囲は 0.1–10.0% の OSCS を含むヘパリンナトリウム試験溶液を用いて求めた．

4-2. DS ヘパリンナトリウム標準溶液 (20 mg/ml) 0.4 ml に，DS 標準溶液 (4 mg/ml) をそれぞれ 0.005, 0.01, 0.02, 0.05, 0.1 及び 0.2 ml を添加し，ついで水を 0.395, 0.39, 0.38, 0.35, 0.3 及び 0.2 ml 加えて混和し，ヘパリンナトリウムに対して DS がそれぞれ 0.25, 0.5, 1.0, 2.5, 5.0 及び 10.0% (w/w) 含む溶液とした．これらの溶液を分析能パラメーター評価用試験溶液とし，キャピラリー電気泳動装置を用いて測定した．

真度，併行精度及び室内再現精度は，5.0% DS を含むヘパリンナトリウム試験溶液を用いて各 6 回分析を行い，DS のピーク面積値を用いて算出した．特異性は 5.0% DS を含むヘパリンナトリウム試験溶液を用いて求めた．検出限界及び定量限界は 0.25–5.0% の DS を含むヘパリンナトリウム試験溶液を，直線性及び範囲は 0.25–10.0% の DS を含むヘパリンナトリウム試験溶液を用いて求めた．

結　　果

1. OSCS

1-1. OSCS の分析条件 FDA がホームページ上に公開したキャピラリー電気泳動法による OSCS のスクリーニング方法 (FDA method) を参考にして，[8] 10% (w/w) の濃度で OSCS を添加したヘパリンナトリウム溶液を分析した．その結果，ヘパリンナトリウムに由来するピークは 5.8 分に泳動され，OSCS に由来するピークは 5.5 分に観察された [Fig. 2(a)]．両者を識別することは可能であったが，ヘパリンナトリウム由来のピークと OSCS 由来のピークの分離は不完全であり，OSCS 検出に対する特異性は低かった．したがって，FDA method を用いる試験法の分析能パラメーターは，真度 (添加回収率) が 41% (R.S.D.=2.18%) であった．検出限界は 1.5% であり，^{1}H-NMR による OSCS 分析の分析能パラメーターと比べ低い値を示した (Table 1)．[12,13] なお，FDA method の併行精度は 1.36% となり，室内再現精度は 2.17% となったことから，ヘパリンナトリウムと OSCS の分離を改善できれば，日本薬局方の純度試験として十分に採用できると考えられた．そこで，ヘパリンナトリウムと OSCS の分離の向上を目指し分析条件を検討した．

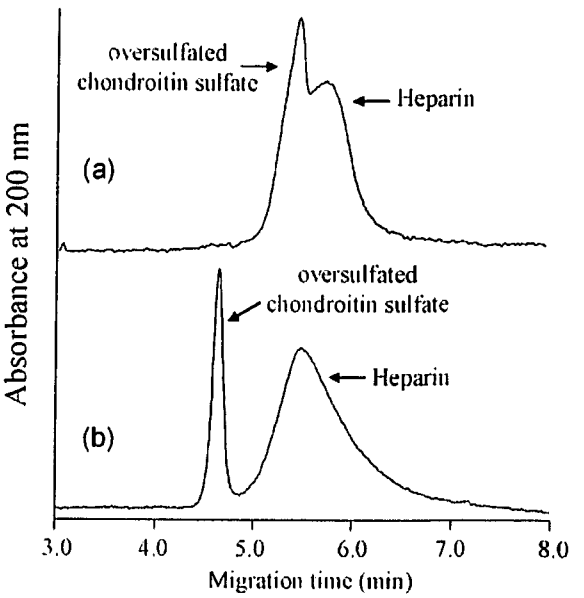

Fig. 2. Capillary Electrophoresis Analysis of 10% (w/w) OSCS Spiked Heparin
(a) FDA method and (b) OSCS method.

最近，Somsen らや Wielgos らは，ヘパリンナトリウムと OSCS の分離の改善例として，高い塩濃度の泳動緩衝液を用いる方法を紹介しており，高濃度緩衝液中では試料イオンがスタッキング効果により濃縮され，ピーク幅が縮小するためヘパリンナトリウムと OSCS の分離が向上することを報告している．[14,15] 一方，緩衝液中の塩濃度が高くなると，泳動時の電流値が高値になり分析が安定せず，発熱

Table 1. Validation Characteristics for OSCS Analysis by Capillary Electrophoresis and ^{1}H-NMR

Validation characteristics	Capillary electrophoresis		^{1}H-NMR*2
	OSCS method	FDA method*1	
Accuracy	93.7% (R.S.D.=3.83%)	41% (R.S.D.=2.18%)	98.3% (R.S.D.=4.63%)
Precision			
Repeatability	2.11%	1.36%	1.6%
Intermed. precision	2.45%	2.17%	—
Specificity	Fig. 2b		High
Detection limit	0.1% (w/w)	1.5% (w/w)	0.35% (w/w)
Quantification limit	0.25% (w/w)	1.5% (w/w)	0.4% (w/w)
Linearity	$y=16992x+3601.6$ ($R^2=0.9996$)	$y=36663x-367.14$ ($R^2=0.9758$)	$y=0.0909x-0.064$ ($R^2=0.9991$)
Range	0.1–10.0% (w/w)	1.5–10.0% (w/w)	0.4–10.0% (w/w)

*1 and *2, from the reports by Hashii *et al.*[12] and Kakehi *et al.*[13] respectively.

により安定した電気泳動が困難となる．これらの問題を解決するために，キャピラリーの内径を 50 μm から 25 μm に変更して電気泳動中に流れる電流量を抑制し，さらに用いる塩を比較的電気伝導度が低いトリスに変更することで電流値を抑制した．本条件では，高濃度塩，低 pH の緩衝液を使用するため，緩衝能の低下の恐れがあるが，繰り返し分析でも高い再現性を与え分析上問題はなかった（data not shown）．加えて，安定に電気泳動するために定電流モード（50 μA）で泳動することとした．また検出に関しては，ヘパリンナトリウムはアセチル基の含有率が低いため紫外部吸収では高い感度を示さないことが知られている．しかし，200 nm の紫外部吸収で検討したところ，ヘパリンナトリウムはオンカラム検出により特に問題はなく検出できた．さらにアセチル基を有する OSCS や DS などの不純物を高感度で検出できるため，純度試験として十分適用できる．以上のことから，本試験では 200 nm の紫外部検出を用いることとした．

以上の検討により設定した条件（OSCS method）を用いて，OSCS を 10%（w/w）添加したヘパリンナトリウム溶液を分析した．その結果，ヘパリンナトリウムに由来するピークは 5.5 分に観察され，OSCS に由来するピークは 4.6 分に観察された［Fig. 2(b)］．FDA method により測定した結果［Fig. 2(a)］と比較すると，ヘパリンナトリウムと OSCS の分離が格段に向上した．分離が向上した要因として，Somsen らや Wielgos らが報告した高濃度緩衝液中での試料イオンのスタッキング効果や，[14,15] 泳動緩衝液に用いたトリスが，カウンターイオンとして試料イオンと良好な相互作用を示したことなどが考えられる．また，キャピラリーの有効長を 20 cm に短縮しても十分な分離を達成でき，Somsen らの報告の半分の時間で分析が完了した．[14]

1-2. OSCS 試験の分析法バリデーション　前項において設定した OSCS method を用いて，日本薬局方医薬品各条ヘパリンナトリウム純度試験への適用を検討した．分析法バリデーションは，日本薬局方「参考情報」に記載されている方法にしたがい，7 つの分析能パラメーター（真度，精度，特異性，検出限界，定量限界，直線性，範囲）を求めることで試験法の妥当性を評価した．

1-2-1. 特異性　OSCS を 10%（w/w）添加したヘパリンナトリウム溶液を試験溶液として測定した結果，ヘパリンナトリウムに由来するピークは 5.5 分をピーク頂点とし 5.0–7.0 分に泳動され，OSCS に由来するピークが 4.6 分をピーク頂点として泳動された［Fig. 2(b)］．ヘパリンナトリウム由来のピークと OSCS 由来のピークは良好な分離を示し（分離度 Rs=1.2），両者を容易に識別できた．

1-2-2. 検出限界及び定量限界　0.1–5.0%（w/w）になるように OSCS の濃度を調製したヘパリンナトリウム試験溶液を用いて，OSCS method の検出限界及び定量限界を求めた．その結果，Fig. 3 に示すように 0.1% の OSCS を含む試験溶液でも，S/N 比 5 以上の感度で OSCS 由来のピークを検出できた．よって OSCS method は，ヘパリンナトリウ

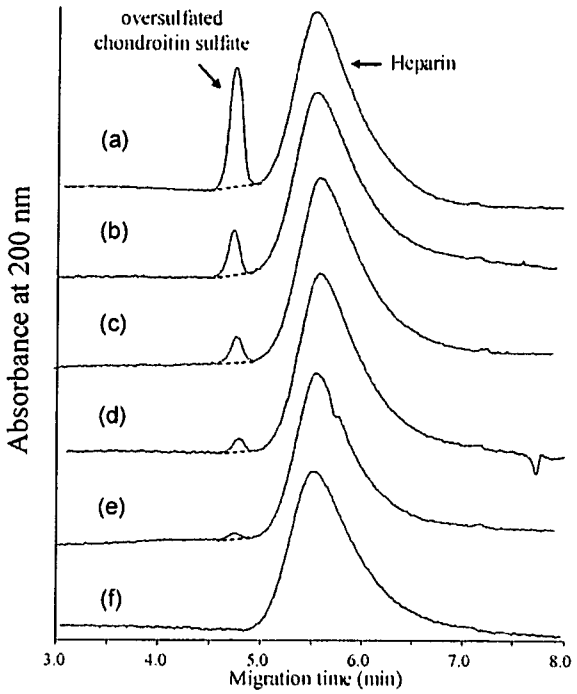

Fig. 3. Lower Limit of Detection of OSCS in the Presence of Heparin

Samples were analyzed at (a) 5.0%, (b) 1.0%, (c) 0.5%, (d) 0.25%, (e) 0.1% and (f) 0% OSCS spiked heparin. OSCS method was used for all analyses.

ム中に混入した 0.1% (w/w) の OSCS を検出できる試験法であることがわかった．また，ヘパリンナトリウム中の 0.25% (w/w) の OSCS を含む試験溶液において，OSCS 由来のピークを S/N 比 10 以上の感度で検出できたため，本試験法の定量限界とした．

1-2-3. 直線性，範囲　0.1–10.0% (w/w) になるように OSCS の濃度を調整したヘパリンナトリウム試験溶液を用いて，OSCS method の直線性を評価した．その結果，OSCS のピーク面積値は，0.1–10.0% (w/w) の範囲で優れた直線性が確認され，その相関係数は 0.9996 であった (Table 1)．

1-2-4. 真度及び精度　5.0% (w/w) の OSCS を含むヘパリンナトリウム試験溶液を用いて，添加回収実験を行い OSCS method における真度を評価した．その結果，添加回収率は 93.7% (R.S.D.＝3.83%) であった．FDA method での真度は，41% (R.S.D.＝2.18%) であったことから (Table 1)，著しい回収率の向上がみられた．よって OSCS method は高い真度を有する試験法であると言える．

また，同じ試験溶液を用いて OSCS method の精度の評価を行った．OSCS ピーク面積値の併行精度 (1 試験日，6 回測定) の R.S.D. は 2.11% であった．一方，室内再現精度 (3 試験日，各 6 回測定) の R.S.D. は 2.45% であった (Table 1)．併行精度並びに室内再現精度ともに，優れた R.S.D. 値を示した．

2. DS

2-1. DS の分析条件　前節で設定した OSCS method を用いて，5.0% (w/w) の DS を添加したヘパリンナトリウム溶液を分析したところ，DS に由来するピークは，6.4 分をピーク頂点とし，6.0–7.0 分にかけてブロードなピークとして観察された [Fig. 4(a)]．DS 由来のピークは広がって観察されるため，検出限界は 1.0% (w/w) と ^{1}H-NMR による試験法や，FDA method を用いるキャピラリー電気泳動法による試験法と比べても，十分な感度を有する試験法とは言えず (Table 2)，OSCS 検出を目的に最適化したキャピラリー電気泳動法の条件では，DS を高感度に検出することは難しい．

そこで，泳動緩衝液の pH について検討したところ，pH2.5 の緩衝液を用いることにより，DS 由来のピークを高い理論段数 (N＝1190) で観察することができ，OSCS method の結果 (N＝470) と比較して良好な分離能を示した．以上の検討により設定した DS method により，DS を 5.0% (w/w) の濃度で添加したヘパリンナトリウム溶液を分析した結果，DS は 4.4 分をピーク頂点とした，シャープなピークとして観察され，3.2 分に観察されたヘパリンナトリウムのピークと良好に分離された [Fig. 4(b)]．

2-2. DS 試験の分析法バリデーション

2-2-1. 特異性　DS を 5.0% (w/w) 添加したヘパリンナトリウム溶液を試験溶液として測定した結果，ヘパリンナトリウムに由来するピークは 3.2 分をピーク頂点とし 2.7–4.3 分に泳動され，DS に由来するピークが 4.4 分をピーク頂点として泳動された [Fig. 4(b)]．ヘパリンナトリウム由来のピークと DS 由来のピークは良好な分離を示し (分離度 Rs＝1.3)，両者を容易に識別できた．

2-2-2. 検出限界及び定量限界　0.25–5.0% (w/w) になるように DS を添加したヘパリンナトリウム試験溶液を用いて，DS method の検出限界及

び定量限界を確認した．その結果，Fig. 5 に示すように 0.25% の DS を含むヘパリンナトリウム試験溶液において S/N 比 5 以上の感度で DS 由来のピークを検出できた．また，ヘパリンナトリウム中の 0.5% (w/w) の DS を含む試験溶液において，DS 由来のピークを S/N 比 10 以上の感度で検出できたため，本試験法の定量限界とした．

2-2-3. 直線性，範囲 0.25–10.0% (w/w) になるように DS を添加したヘパリンナトリウム試験溶液を用いて，DS method の直線性を評価した．その結果，DS のピーク面積は，0.25–10.0% (w/w) の範囲で優れた直線性が確認され，その相関係数は 0.9998 であった (Table 2)．

2-2-4. 真度及び精度 5.0% (w/w) の濃度になるように DS を添加したヘパリンナトリウム試験溶液を用いて，添加回収実験により真度を評価した．その結果，添加回収率は 88.1% (R.S.D.=2.13

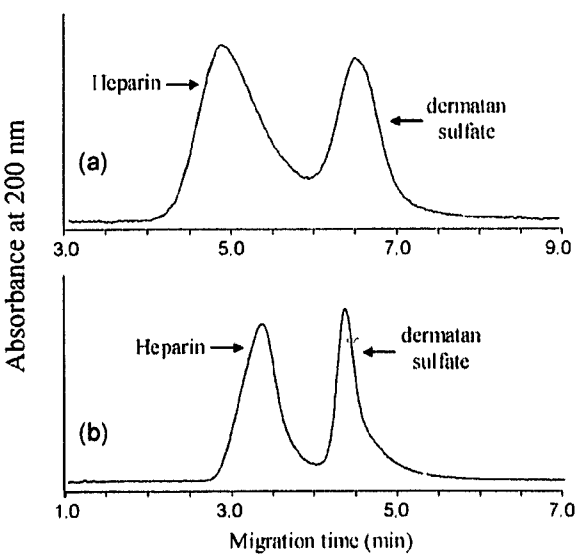

Fig. 4. Capillary Electrophoresis Analysis of 5.0% (w/w) DS Spiked Heparin
(a) OSCS method and (b) DS method.

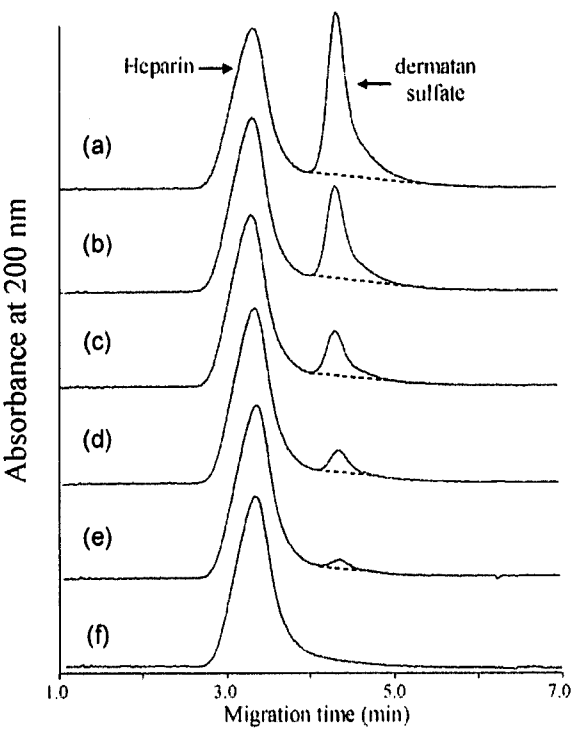

Fig. 5. Lower Limit of Detection of DS in the Presence of Heparin
Samples were analyzed at (a) 5.0%, (b) 2.5%, (c) 1.0%, (d) 0.5%, (e) 0.25% and (f) 0% DS spiked heparin. DS method was used for all analyses.

Table 2. Validation Characteristics for DS Analysis by Capillary Electrophoresis and ^{1}H-NMR

Validation characteristics	Capillary electrophoresis		^{1}H-NMR*2
	DS method	FDA method*1	
Accuracy	88.1% (R.S.D.=2.13%)	82% (R.S.D.=1.78%)	102.6% (R.S.D.=3.99%)
Precision			
Repeatability	1.99%	2.15%	1.5%
Intermed. precision	2.43%	2.48%	—
Specificity	Fig. 4b		fair
Detection limit	0.25% (w/w)	1.0% (w/w)	0.35% (w/w)
Quantification limit	0.5% (w/w)	1.0% (w/w)	0.6% (w/w)
Linearity	y=135944x−2904.9 (R^2=0.9998)	y=16938x−9357.2 (R^2=0.9991)	y=0.08534x−0.0113 (R^2=0.9991)
Range	0.25–10.0% (w/w)	1.0–10.0% (w/w)	0.6–18.7% (w/w)

*1 and *2, from the reports by Hashii *et al.*[12] and Kakehi *et al.*[13] respectively.

%）であった．

また，同じ試験溶液を用いて精度の評価を行った．DSピーク面積値の併行精度（1試験日，6回測定）のR.S.D.は1.99%であった．一方，室内再現精度（3試験日，各6回測定）のR.S.D.は2.43%であった．併行精度並びに室内再現精度ともに，優れたR.S.D.値を示した．

3. 有害ロットのヘパリンナトリウム製剤原料の分析　前節までに検討した3種類の条件で，実際に有害事象を引き起こしたヘパリンナトリウム製剤原料の分析を行った．FDA methodでは，6.2分にヘパリンナトリウムのピークが，6.0分にOSCSのピークが観察された［Fig. 6(a)］．両者の識別は可能であったが分離は不十分であった．次に，OSCS methodにて分析した結果，ヘパリンナトリウムのピークは5.7分付近に，OSCSのピークは4.3分付近に観察され良好な分離を示した［Fig. 6(b)］．また，DS methodで分析した結果，ヘパリンナトリウムのピークは3.2分付近に，OSCSのピークは2.6分付近に観察された［Fig. 6(c)］．このヘパリンナトリウム製剤原料中にOSCSが8.6% (w/w)混入していると算出された．一方，本製剤原料中にはDS由来のピークは観察されなかった．

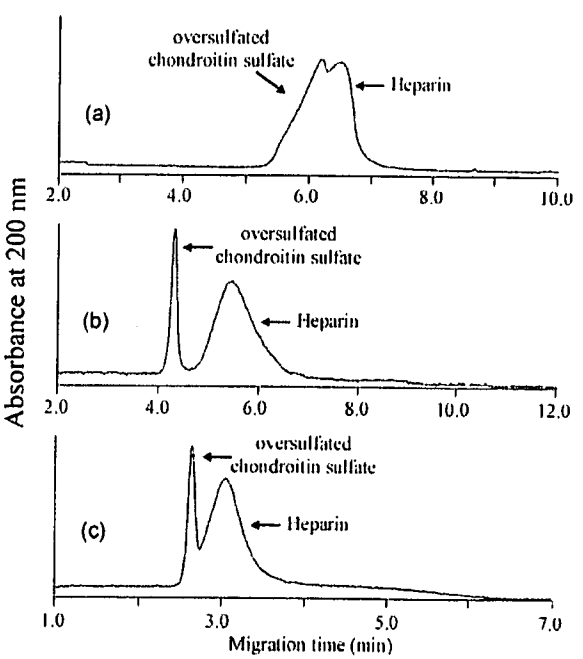

Fig. 6. Capillary Electrophoresis Analysis of Contaminated Heparin Sodium Preparation
(a) FDA method, (b) OSCS method and (c) DS method.

考　　察

1. OSCS　キャピラリー電気泳動法を日本薬局方医薬品各条ヘパリンナトリウム純度試験に用いるためには，OSCSを特異的に検出できる分析条件を設定する必要がある．高濃度の塩を含有する泳動緩衝液（1000 mM Tris-phosphate buffer）を用いる条件を検討し（OSCS method），ヘパリンナトリウムとOSCSを良好に分離できた（Fig. 2）．さらにOSCS methodは，キャピラリーの有効長を短縮しても十分な分離を達成できたことから，これまでに報告されたキャピラリー電気泳動法によるOSCS検出や，[14] Trehyらの報告した陰イオン交換カラムを用いるHPLC法によるOSCS検出と比べても，[9] 高いスループットを備えた分析法といえる．

本分析法はOSCS検出に対して高い特異性を有し［Fig. 2(b)］，ヘパリンナトリウム中に混入した0.1% (w/w)のOSCSを検出できた（Fig. 3）．また，真度（添加回収率：93.7%）や併行精度（R.S.D.=2.11%），室内再現精度（R.S.D.=2.45%）も良好であり，優れた直線性（R^2=0.9996）を示した（Table 1）．これらの結果から，OSCS methodは，ヘパリンナトリウム中のOSCS含量が0.1% (w/w)以下であることを保証する限度試験として，日本薬局方医薬品各条ヘパリンナトリウム純度試験に適用できる試験法であると判断される．

OSCSは今回の有害事象の原因物質であり，また製造工程由来物質や目的物質関連物質として混入する可能性がないことから，ヘパリンナトリウム製剤中に検出されるべきではない．したがって，ヘパリンナトリウム中に混入したOSCSの検出感度ができる限り高い試験法を用いることが望ましい．今回の事件を受けて，日本薬局方に規定された^{1}H-NMRによるOSCS試験の検出限界は0.35% (w/w)である．[12] OSCS methodによるキャピラリー電気泳動法は，^{1}H-NMRによる試験法を上回る検出感度を有している．そのため，キャピラリー電気泳動法によるOSCS試験は日本薬局方各条純度試験として有用であると評価できる．

2. DS　OSCS methodでは，DS由来のピークがブロードに観察されることから，ヘパリンナトリウム中の微量のDSを高い特異性で検出することが難しい［Fig. 4(a)］．そこでDSの感度向上を目

的とした条件(DS method)を検討した．その結果，DSとヘパリンナトリウムを良好に分離でき，併行精度（R.S.D.＝1.99%），室内再現精度（R.S.D.＝2.45%），真度（添加回収率：88.1%）を与える分析条件を設定することができた（Table 2）．DS methodは0.25—10.0%（w/w）の範囲で高い直線性（R^2＝0.9998）が確認されたことから，DS検出に高い特異性と定量性を有した試験法であることがわかった．以上の結果から，キャピラリー電気泳動法によるDS分析は，ヘパリンナトリウム中のDSの混入が0.25%以下であることを保証する限度試験として日本薬局方の純度試験法に採用可能であると判断される．

今回の問題を受けて，米国薬局方に規定された^{1}H-NMRによる試験法では，500 MHzの装置を用いている．この際のDSの検出限界は0.35%である．一方で，国内の多くの製薬企業が設置している400 MHz以下のNMR装置を用いるDS試験では，特異性や検出限界などに問題が残されている．[12] よって，DSに関して高い特異性と検出限界を有するキャピラリー電気泳動法は有用な試験法であり，日本薬局方医薬品各条ヘパリンナトリウム純度試験に適した試験法と言える．

現在，臨床で使用されているヘパリン関連製剤や，同じグリコサミノグリカン類を原料とするヒアルロン酸製剤中に，一定量のDSが混入しているという報告がみられる．[16,17] そのため製剤中へのDS混入の規制の必要性については，DSはヘパリンとは異なる物質であるので，純度試験として適切に規制するべきとする意見と，これまでに毒性等の報告がなく純度試験等により規制する必要はないとする意見があり，国際的にも見解が分かれている．DSはヘパリンを調製する際の原料に含まれるため，ヘパリンの精製の指標として有用であるとも考えられ，今後は国内ヘパリンナトリウム中へのDSの含有量の実態を正確に把握した上で，規制が必要か否か検討していく必要があろう．

結　　論

本研究における分析法バリデーションの結果，キャピラリー電気泳動法は日本薬局方各条ヘパリンナトリウム純度試験として適用可能であることがわかった．

今後は他機関と連携した共同検定を行い，その他の規格を設定していく必要がある．特に検出限界に関しては，他機関においても同程度の値を得るため，分析試料の濃度や試料導入法，試料導入量などを規定しなければならない．

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Notes & Tips

Determination of Tn antigen released from cultured cancer cells by capillary electrophoresis

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ABSTRACT

An incomplete elongation of *O*-glycans in mucins has been found in epithelial cancers, leading to the expression of shorter carbohydrate structures such as Tn antigen (GalNAc-O-Ser/Thr), which has been reported to be one of the most specific human cancer-associated structures. However, there have been no appropriate physicochemical methods for the determination of Tn antigen in biological samples. In the present paper, we developed a capillary electrophoresis method for the determination of Tn antigen, and applied the method to the analysis of the expressed Tn antigen on some leukemia and epithelial cancer cells.

Cancer-associated structural alterations in *O*-glycans such as high level sialylation and low-level sulfation are attracting interest in relation to their biological significance [1]. Especially, incomplete elongation of *O*-glycans in mucins has been found in many cancers, leading to the expression of shorter carbohydrate structures, such as Tn antigen (GalNAc-O-Ser/Thr) [2], which is one of the most specific human cancer-associated structures and a possible early biomarker of cancer [3].

Current analytical methods for characterization of *O*-glycans involve the initial release of the oligosaccharides. However, the release of *O*-glycans has been a difficult task due to lack of the enzyme which shows wide specificity as in the case of *N*-glycoamidase for the release of *N*-glycans. Therefore, *O*-glycans still have to be released from the core proteins by chemical methods, and most of the methods require a lengthy reaction time and cumbersome procedures [4]. Recently, we developed an automated glycan-releasing apparatus (Autoglycocutter: AGC)[1] for *O*-glycosylated proteins [5,6]. The apparatus enables the release of *O*-glycans having the intact reducing end within only 3 min, and was applied to the analysis of *O*-glycans from some leukemia and epithelial cancer cells [7].

The released glycans are often labeled with a fluorescent tag, although we have to remove the excess reagents and the accompanying materials [8–11]. The procedures hitherto reported are effective for purification of higher oligosaccharides. Monosaccharides such as Tn antigen released from the samples are often poorly recovered when the clean-up procedures for higher oligosaccharides are applied.

In the current study, we developed a clean-up method for collection of Tn antigen and the small glycans, and applied the method to the analysis of Tn antigen in some cancer cell lines.

We used human-derived cell lines: Jurkat (acute T cell leukemia), U937 (histiocytic lymphoma), K-562 (chronic myelogenous leukemia), HL-60 (acute promyelocytic leukemia), LS174T and HCT-15 (colorectal adenocarcinoma), BxPC3 (pancreatic adenocarcinoma), PANC1 (pancreatic carcinoma), and MKN7 and MKN45 (gastric adenocarcinoma).

A portion of the glycopeptide fractions obtained from each cancer cell line (1.0×10^7 cells) was injected to the AGC apparatus [6,7], and the collected solution containing the released *O*-glycans was evaporated to dryness followed by labeling with 2-aminobenzoic acid (2-AA) as reported previously [7].

In our previous work using Sephadex LH-20 for clean-up of 2-AA-labeled glycans, we could not recover Tn antigen in good efficiency [7]. We examined several clean-up procedures for the analysis of the 2-AA-labeled GalNAc, and calculated the recovery of GalNAc (Table 1). Most of the reported methods using carbon graphite columns [8], nylon filters [9], polyamide resin (DPA-6S) [10], and acetone precipitation [11] are optimized for the glycans having high molecular masses and not appropriate for clean-up of simple mono and disaccharides. Therefore, we examined an HPLC method using octadecylsilica as the stationary phase. Although a long time (60 min) is required for collection of the fractions containing Tn antigen, we found that higher than 99% of the excess amount of 2-AA was removed and 2-AA-labeled GalNAc was recovered quantitatively.

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[1] *Abbreviations used:* 2-AA, 2-aminobenzoic acid; AGC, Autoglycocutter; CAE, capillary affinity electrophoresis.

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Table 1
Efficiencies in purification of 2-AA-labeled GalNAc using previously reported methods.

Method	Removal of the reagent (%)	Recovery of GalNAc-2-AA (%)	Run time (min)	Reference [Reference No.]
Sehadex LH-20	–	–	180	J. Proteome Res. 8 (2009) 521–537 [7]
ENVI-carb column	10	34	60	Clin. Chem 44 (1998) 2422–2428 [8]
Nylon filter	99	69	60	Anal. Biochem. 373 (2008) 104–111 [9]
Polyamide resin (DPA-6S)	96	78	60	Anal. Biochem. 369 (2007) 202–209 [10]
Acetone precipitation	5	54	30	Anal. Biochem. 384 (2009) 263–273 [11]
COSMOSIL(R) 5C18-PAQ (ID 4.6 × 150 mm)	99	99	60	Present method

In the analysis of Tn antigen, it is necessary to resolve it from other possible monosaccharides and the accompanying materials. In the present study, we employed capillary electrophoresis and achieved excellent resolution of GalNAc (see Supplemental Fig. 1). During the alkali-catalyzed release of glycans from core peptides using the AGC apparatus, epimerization of the glycans is a big problem. We evaluated the epimerization reaction of Tn antigen. After the solution of the standard GalNAc was passed through AGC, the mixture was labeled with 2-AA and analyzed by CE. A small amount of *N*-acetyl-D-talosamine (TalNAc) was observed, but the peak area was 4.2% of GalNAc (see Supplemental Fig. 2). These results indicate that the effect of epimerization during the releasing reaction is negligible.

We applied the method to the analysis of the expressed Tn antigen on Jurkat cells (Fig. 1). After a large peak of the reagent with some artifact peaks, the peak due to Tn antigen was observed at ca. 53 min. It should be noted that a Tn peak at 53 min accompanies some minor components. After collection of this peak, we analyzed it by CE and capillary affinity electrophoresis (CAE), and found that the peak showed the same migration time as that of GalNAc by CE (Fig. 1B). In addition, CAE using the buffer containing $VVAB_4$ lectin (*Vicia villosa* seed) at 10 μM concentration, which specifically recognizes GalNAc [12], clearly caused disappearance of the peak at 8.9 min. In contrast, the peak of 2-AA maltopentaose (internal standard) at 10.3 min was not changed. These results indicate that the peak observed at 8.9 min was due to Tn antigen.

Based on the methods described above, we analyzed the expression of Tn antigen on some cancer cell lines (Fig. 1C). All cancer cell lines examined in this study expressed significantly different amounts of Tn antigen. Of the examined leukemia cell lines, Jurkat cells express Tn antigen most abundantly. Because Jurkat cells lack a molecular chaperone (cosmc) which is necessary for expression of core 1 beta 3 galactosyl transferase activity [13], Jurkat cells cannot establish the core 1 structure, and the truncated *O*-glycan (i.e., Tn antigen) which is a precursor of core 1 type glycans is abundantly observed. Epithelial cancer cells other than HCT-15 cells expressed Tn antigen more abundantly than Jurkat cells. Especially, LS174T cells expressed about 10 times larger amounts of Tn antigen than other epithelial cancer cells. We reported that poorly differentiated pancreatic cancer cells (PANC1) showed simple *O*-glycan profiles, but moderately differentiated pancreatic cancer cells (BxPC3) showed relatively complex *O*-glycan profiles. In sharp contrast with these results, MKN45 cells (poorly differentiated species) and MKN7 cells

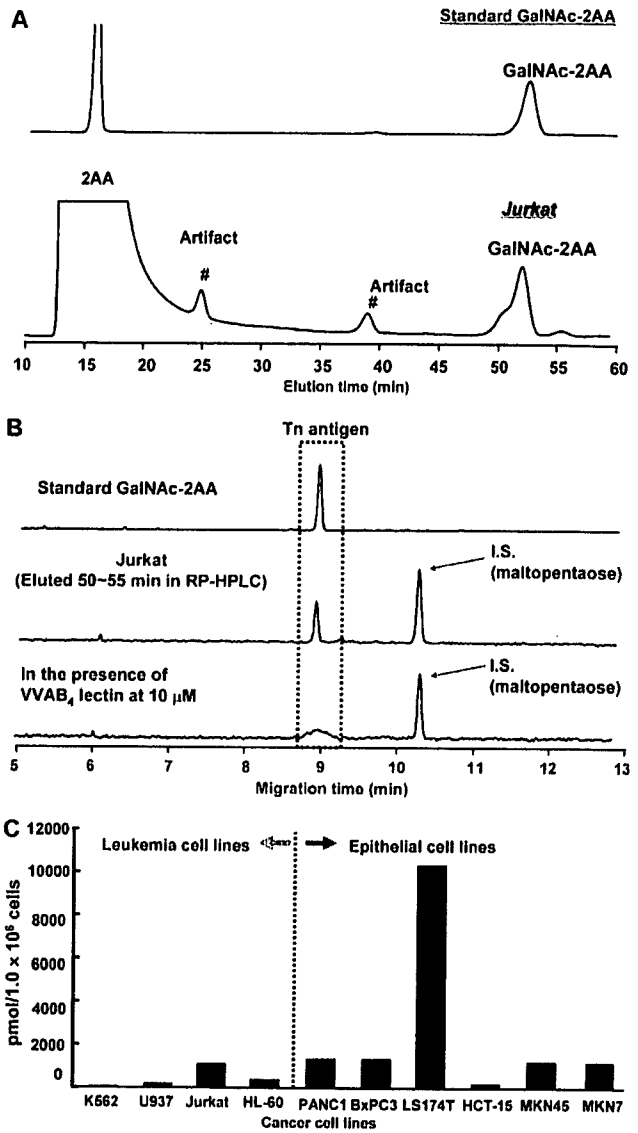

Fig. 1. Determination of Tn antigen in some cancer cells. Purification of 2-AA-labeled Tn antigen derived from Jurkat cells by RP-HPLC (A). Conditions for purification by RP-HPLC. Column, COSMOSIL 5C18-AR-II (4.6 × 150 mm); eluent, solvent A, 0.1% HCOOH in water; solvent B, 20% acetonitrile containing 0.1% HCOOH. Gradient elution; a linear gradient (10–20% solvent B) from 0 to 20 min, then followed by a linear gradient (20–22.5% solvent B) from 20 to 70 min. CE and CAE analysis of 2-AA-labeled Tn antigen form Jurkat cells (B). Analytical conditions for CE and CAE. Capillary, DB-1 capillary (100 μm i.d. × 40 cm); running buffer, 100 mM Tris–borate buffer (pH 8.3) containing 5% PEG70000; applied voltage, 25 kV; injection, pressure method (1.0 psi for 10 s); temperature, 25 °C; detection, helium–cadmium laser-induced fluorescence (excitation 325 nm, emission 405 nm). CAE of the Tn antigen from Jurkat cells in the presence of GalNAc-specific lectin ($VVAB_4$). The peak observed at 10.3 min was due to maltopentaose labeled with 2-AA, which is used as the internal standard for migration times. The migration time of 2-AA-labeled maltopentaose was not changed in the presence/absence of $VVAB_4$. Expression of Tn antigen in 10 cancer cell lines (C). The amount of Tn antigen was calculated by peak area observed by CE.

(well differentiated species) showed the reverse results [7]. Prior to starting this work, we had expected that expressions of Tn antigen are varied with stages of cancer differentiation. However, significant differences of the expression of Tn antigen were not observed in these four epithelial cancer cells. On the other hand, expression of Tn antigen was quite different between HCT-15 and LS174T, which are colon cancer cell lines. LS174T cells, high metastasis cells [14],

expressed the largest amount of Tn antigen of the examined cancer cells. However, HCT-15 cells, low metastasis cells [14], expressed trace amounts of Tn antigen. Hirao et al. indicated that Tn antigen could be used as a marker for the level of metastasis of uterine cervix cancer cells [15]. Our present results are well correlated with their observations.

In the present study, we developed a method for the analysis of Tn antigen, and found that high metastasis cancer cells expressed Tn antigen extremely abundantly. Although further studies using clinical samples are required, the present method will be a useful tool for characterization of clinical significance of cancer cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.08.021.

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Structural characterization of multibranched oligosaccharides from seal milk by a combination of off-line high-performance liquid chromatography–matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry and sequential exoglycosidase digestion

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ABSTRACT

A complex mixture of diverse oligosaccharides related to the carbohydrates in glycoconjugates involved in various biological events is found in animal milk/colostrum and has been challenging targets for separation and structural studies. In the current study, we isolated oligosaccharides having high molecular masses (MW ~ 3800) from the milk samples of bearded and hooded seals and analyzed their structures by off-line normal-phase–high-performance liquid chromatography–matrix-assisted laser desorption/ionization–time-of-flight (NP–HPLC–MALDI–TOF) mass spectrometry (MS) by combination with sequential exoglycosidase digestion. Initially, a mixture of oligosaccharides from the seal milk was reductively aminated with 2-aminobenzoic acid and analyzed by a combination of HPLC and MALDI–TOF MS. From MS data, these oligosaccharides contained different numbers of lactosamine units attached to the nonreducing lactose (Galβ1–4Glc) and fucose residue. The isolated oligosaccharides were sequentially digested with exoglycosidases and characterized by MALDI–TOF MS. The data revealed that oligosaccharides from both seal species were composed from lacto-*N*-neohexaose (LNnH, Galβ1–4GlcNAcβ1–6[Galβ1–4GlcNAcβ1–3]Galβ1–4Glc) as the common core structure, and most of them contained Fucα1–2 residues at the nonreducing ends. Furthermore, the oligosaccharides from both samples contained multibranched oligosaccharides having two Galβ1–4GlcNAc (*N*-acetyllactosamine, LacNAc) residues on the Galβ1–4GlcNAcβ1–3 branch or both branches of LNnH. Elongation of the chains was observed at 3-OH positions of Gal residues, but most of the internal Gal residues were also substituted with an *N*-acetyllactosamine at the 6-OH position.

Specific sequences of monosaccharides occur as important structural elements of oligo- and polysaccharides of glycoproteins and glycolipids, and they comprise recognition motifs for ligand–receptor or cell–cell interactions [1–4]. Oligosaccharides are cooperatively synthesized by actions of various glycosyltransferases and are usually present as a complex mixture of diverse oligosaccharides. In particular, the isomeric/branching structure is the major feature, and their structural determination is essential for understanding the biosynthesis and biological significance.

Mammalian milk/colostrum is a rich source of carbohydrates of diverse structures [5–8]. Although the most dominant carbohydrate in mammalian milk is generally lactose, a small amount of characteristic oligosaccharides are also present [9–13]. The milk oligosaccharides usually have a common lactose (Galβ1–4Glc) core that is extended at the 6- and/or 3-OH positions of the Gal as linear/branched mode [14]. Furthermore, the linear/branched chains are frequently fucosylated and/or sialylated and in a few cases are sulfated.

Due to the similarities and complex structures of milk oligosaccharides, structural determination of them has been a big and challenging work. Urashima and coworkers isolated various oligosaccharides from many mammalian species' milk/colostrum and characterized their structural features by a combination of

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at void volumes, from the HS milk sample was used in the following preparations and characterization of each oligosaccharide.

The fraction from the BS milk sample was further separated by ion exchange chromatography, as indicated in the previous study [18]. The lyophilized material was dissolved in 50 mM Tris–HCl buffer (pH 8.7, 2.0 ml) and subjected to anion exchange chromatography on a DEAE Sephadex A-50 (1.5 × 35 cm). The unadsorbed oligosaccharide fractions were used for structural study of the oligosaccharides in the previous study [18]. The adsorbed oligosaccharides were eluted by linear gradient elution with changing NaCl concentrations from 0 to 0.25 M in the same buffer. Two fractions (BS1 and BS2) obtained by linear gradient elution were pooled and lyophilized to dryness. The lyophilized material was dissolved in water and passed through a Biogel P-2 column (2.5 × 100 cm). The fractions eluted at the void volume were pooled and lyophilized to dryness to yield a mixture of acidic oligosaccharides (2.0 and 2.3 mg of BS1 and BS2, respectively).

Fluorescent labeling of oligosaccharides with 2AA

Fluorescent labeling of oligosaccharides was performed according to the method reported previously [30,31]. Briefly, a solution (250 μl) of 2-aminobenzoic acid (2AA) and $NaBH_3CN$, prepared by dissolution of both reagents (30 mg each) in methanol (1 ml) containing 4% CH_3COONa and 2% boric acid, was added to a mixture of oligosaccharides (100 μg). The mixture was kept at 80 °C for 60 min. After cooling, water (250 μl) was added, and the mixture was applied to a small column (1 × 50 cm) of Sephadex LH-20 previously equilibrated with 50% aqueous methanol. The earlier eluted fluorescent fractions that contained labeled oligosaccharides were collected and evaporated to dryness. The residue was dissolved in water (1 ml), and the solution was stored at −20 °C until analysis.

Preparation of asialo-oligosaccharides

A mixture of 2AA-labeled acidic oligosaccharides (~10 μg) was dissolved in 20 mM acetate buffer (pH 5.0, 50 μl), and neuraminidase (10 mU, 10 μl) was added to the mixture. After incubation at 37 °C for 24 h, the reaction mixture was kept in the boiling water bath for 5 min. After centrifugation of the mixture at 10,000g for 10 min, a portion of the supernatant was used for the analysis.

α-Fucosidase digestion

A mixture of 2AA-labeled asialo-oligosaccharides (~2 μg), as described above, was dissolved in 20 mM phosphate buffer (pH 7.5, 50 μl) for α1–2 fucosidase digestion or in 20 mM phosphate buffer (pH 6.0, 50 μl) for α1–3,4 fucosidase. α1-2 Fucosidase (40 μU, 2 μl) or α1–3,4 fucosidase (10 μU, 10 μl) was added to the mixture. After incubation at 37 °C for 24 h, the reaction mixture was kept in the boiling water bath for 5 min, and centrifuged at 10,000g for 10 min. The supernatant was diluted with water to adjust the volume of 200 μl. A portion of each solution (5 μl) was used for NP–HPLC analysis.

Sequential exoglycosidase digestion of oligosaccharides

Each oligosaccharide isolated by NP–HPLC was dissolved in 20 mM citrate buffer (pH 3.5, 8 μl), and β-galactosidase (1 mU, 2 μl) was added to the mixture. After incubation at 37 °C for 12 h, the reaction mixture was kept in the boiling water bath for 5 min. After centrifugation of the mixture, the supernatant was diluted with water (10 μl). A portion of the solution (2 μl) was analyzed by MALDI–TOF MS. Another portion (5 μl) was mixed with 30 mM citrate buffer (pH 5.0, 5 μl) containing β-*N*-acetylhexosaminidase (5 mU), and the reaction mixture was kept at 37 °C for 12 h. The supernatant was diluted with water (10 μl), and then a portion of the solution (2 μl) was also analyzed by MALDI–TOF MS.

Separation of the 2AA-labeled oligosaccharides

HPLC was performed with a Shimadzu apparatus equipped with two LC-6ADvp pumps and an FP-920 fluorescence detector (Waters). Separation was done with an Amide 80 column (TOSOH, 4.6 mm i.d. × 250 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min. After 2 min, solvent B was increased to 95% over 80 min and kept for further 20 min [32]. Fluorescence detection was performed at 410 nm by irradiating at 325 nm light.

MALDI–TOF MS

MALDI–TOF mass spectra were acquired with a Voyager DE-PRO mass spectrometer (PE Biosystems, Framingham, MA, USA). A nitrogen laser was used to irradiate samples, and an average shot of 50 times was taken. The instrument was operated in a linear mode at an accelerating voltage of 20 kV. An aqueous sample solution (2 μl) was mixed with a matrix solution (2 μl) of 1% 2,5-dihydroxybenzoic acid (DHB) in methanol/water (1:1). The mixture was applied to a polished stainless-steel target and then dried in atmosphere for a few hours.

MALDI–QIT–TOF MS

MALDI–QIT–TOF mass spectra were acquired on an AXIMA–QIT–TOF mass spectrometer (Shimadzu, Kyoto, Japan). A nitrogen laser was used to irradiate samples, and an average shot of 50 times was taken. Argon was used for CID. The instrument was operated in positive and reflectron mode. An aqueous sample solution (2 μl) was mixed with a matrix solution (2 μl) of 1% DHB in ethanol/water (1:1), and the mixture was applied to a polished stainless-steel target and dried in atmosphere for a few hours.

Results

Acidic oligosaccharides having high molecular masses in BS and HS milk samples

The method for the preparation of the oligosaccharide samples used in the current study was reported previously [17,18]. Two fractions (BS1 and BS2, 2.0 and 2.3 mg, respectively) from the BS milk sample (40 ml) and a fraction containing acidic oligosaccharides (HS, 1.7 mg) were used in the current study. Because the oligosaccharides from the HS and BS milk samples contained type II chain (Galβ1–4GlcNAc-R) but not type I chain (Galβ1–3GlcNAc-R), we add "neo" to all core oligosaccharide structures.

Oligosaccharides obtained from BS1 and BS2 were fluorescently labeled with 2AA and analyzed by MALDI–TOF MS. As shown in Fig. 1A, a large number of ion signals were observed at the range from *m/z* 1484.8 to *m/z* 3530.4. In BS1, two major molecular ions were observed at *m/z* 2362.3 and 2653.3, which were due to monofucosyl LNnH with one and two NeuAc residues (NAc1H4N2F1–2AA and NAc2H4N2F1–2AA). Ions at *m/z* 2151.0, 3027.9, and 3318.6 are 80 mass units larger than the *m/z* values of monofucosyl LNnH (theoretical molecular mass [mw] 2071.2), difucosyl lacto-*N*-neotetradecaose (LNnTD, theoretical mw 2946.7), and monosialyl difucosyl LNnTD (theoretical mw 3238.6), respectively. These data indicate that these oligosaccharides are substituted with one SO_3H group. In BS2, we observed two major ions at *m/z* 2337.2 and

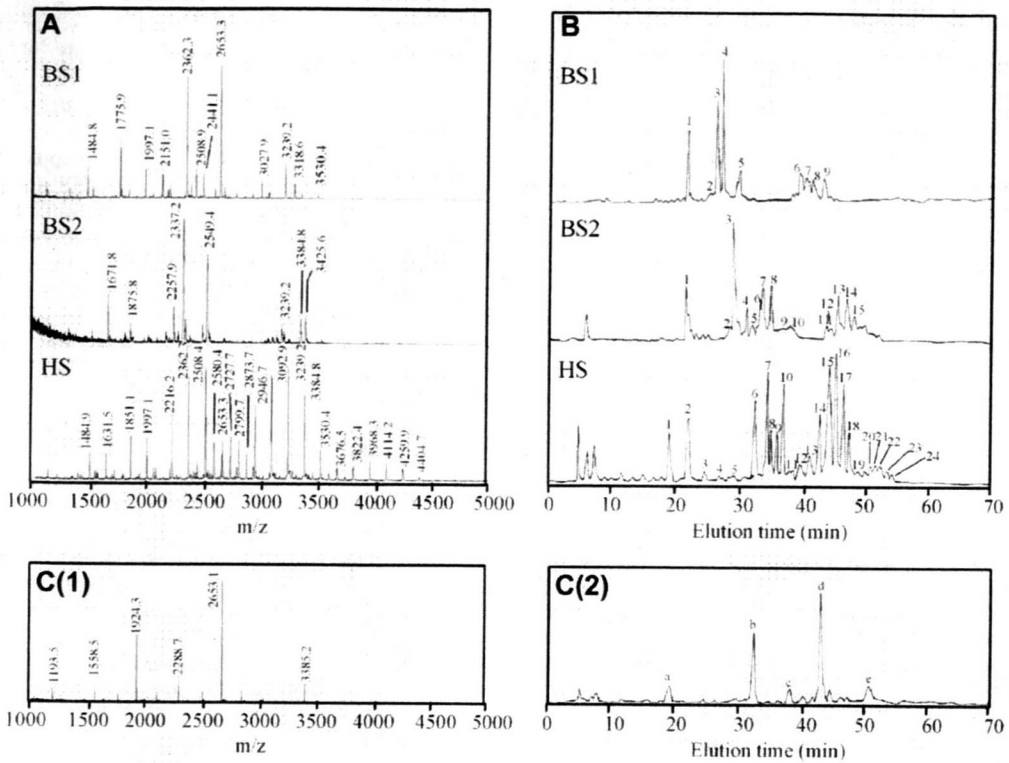

Fig. 1. MALDI–TOF MS and NP–HPLC analysis of higher oligosaccharides from bearded and hooded seal milk. (A) MALDI–TOF MS analysis of sialo-oligosaccharides from bearded and hooded seal milk. (B) NP–HPLC analysis of asialo-oligosaccharides from bearded and hooded seal milk. (C) MALDI–TOF MS and NP–HPLC analysis of defucosylated asialo-oligosaccharides from hooded seal milk. BS1, DEAE-adsorbed fraction 1 in BS milk oligosaccharides; BS2, DEAE-adsorbed fraction 2 in BS milk oligosaccharides; HS, higher oligosaccharide fraction in HS milk oligosaccharides; digestion product of HS with α1–2 fucosidase. The monosaccharide compositions of asialo-oligosaccharides are summarized in Table 1.

2549.4, which were due to difucosyl decaose with one SO_3H group (H5N5F2–SO_3H–2AA) and monosialo–difucosyl neodecaose with one SO_3H group (NAc1H5N5F2–SO_3H–2AA), respectively. The molecular ion observed at *m/z* 3384.8 was due to monosialo LNnTD with three Fuc residues (NAc1H8N6F3–2AA).

HS showed characteristic ladder ions between *m/z* 1400 and *m/z* 4500. These ladder ions were classified into five groups based on the number of lactosamine (Galβ1–4GlcNAc) units. The ions observed at *m/z* 1484.9 and 1631.5 have the composition of NAc1H4N2–2AA and NAc1H4N2F1–2AA, respectively, and are due to mono- and difucosyl LNnH with one NeuAc residue. Molecular ions at *m/z* 1851.1 and 1997.1 are due to the oligosaccharides having compositions of NAc1H5N3–2AA and NAc1H5N3F1–2AA, respectively. A series of the ions at *m/z* 2216.2, 2362.3, 2508.4, and 2653.3 were observed abundantly in HS and are due to the oligosaccharides having monosialo lacto-*N*-neodecaose (LNnD) core (NAc1H6N4–2AA) to which 0, 1, 2, and 3 Fuc residues are attached. Five signals from *m/z* 2946.4 to *m/z* 3530.4 were consistent with oligosaccharides of NAc1H8N6–2AA having 0 to 4 Fuc residues. In addition, we found characteristic glycans having extremely large molecular masses, as observed for the series of ions observed from *m/z* 3676.5 to *m/z* 4404.7. These oligosaccharides are considered to have the core of LNnTD to which 0 to 5 Fuc residues are attached.

To determine linkages of Fuc residues in HS oligosaccharides, we carried out specific fucosidase digestion. A mixture of HS asialo-oligosaccharides was digested with α1–3/4 fucosidase and α1–2 fucosidase, respectively, and the products were analyzed with MALDI–TOF MS. We found that α1–3/4 fucosidase did not act on these oligosaccharides, whereas digestion with α1–2 fucosidase (*Corynebacterium* sp.) caused disappearance of most ions, and six ions were observed at *m/z* 1193.5, 1558.5, 1924.3, 2288.7, 2653.1, and 3385.2 (Fig. 1C(1)). These ions are consistent with the theoretical *m/z* values of H4N2–2AA, H5N3–2AA, H6N4–2AA, H7N5–2AA, H8N6–2AA, and H10N8–2AA, respectively.

The oligosaccharides obtained from milk samples were also analyzed by HPLC using a TSK-Gel Amide-80 column after removing sialic acids with neuraminidase to improve resolution (Fig. 1B) [32]. We collected the major peaks and observed the molecular ions by MALDI–TOF MS. The results are summarized in Table 1.

BS1-1 obtained from the BS1 fraction was assigned as LNnH having two LacNAc units and core Galβ1–4Glc unit from its molecular ion (*m/z* 1193.5) (Fig. 1B, top panel). Molecular ions (*m/z* 2071.8) of BS1-3 and BS1-4 are consistent with the theoretical *m/z* values of H6N4F1–2AA, suggesting the presence of isomers of monofucosyl LNnD. The peak observed at 30 min (BS1-5) gave two molecular ions (*m/z* 2802.6 and 2949.0). The molecular ion at *m/z* 2802.6 is consistent with the theoretical *m/z* value of H8N6F1, suggesting the structure of monofucosyl LNnTD. Likewise, the molecular ion at *m/z* 2949.0 was assigned as difucosyl LNnTD. Minor peaks (BS1-6 to BS1-9) were due to the oligosaccharides having a core structure of LNnD or LNnTD to which a sulfate group is attached (for confirmation of the structure, see the following section).

In the BS2 fraction, BS2-1 and BS2-3 are composed of H3N3F1–2AA and H5N5F2–2AA, respectively. Digestion of BS2-1 and BS2-3 with α1–2 fucosidase caused the loss of one and two fucose residues, respectively. The defucosylated oligosaccharides gave molecular ions corresponding to the theoretical *m/z* values of H3N3–2AA and H5N5–2AA. From the monosaccharide compositions, these oligosaccharides are considered to be hexa- and decasaccharide, having LacNAc units at the reducing end (for confirmation of the structure, see the following section). Oligosaccharides observed be-

Table 1
List of asialo-oligosaccharides observed in bearded and hooded seal milk.

Peak number	Observed mass	Calculated mass	Composition
(a) BS1			
-1	1193.5	1194.1	H4N2-2AA
-2	2110.8	2111.9	H5N5F1-2AA
-3	2071.8	2071.2	H6N4F1-2AA
-4	2071.8	2071.2	H6N4F1-2AA
-5	2802.6	2801.7	H8N6F1-2AA
	2949.0	2947.9	H8N6F2-2AA
-6	2151.0	2151.2	H6N4F1-SO_3H-2AA
	3027.0	3027.9	H8N6F2-SO_3H-2AA
-7	2150.8	2151.2	H6N4F1-SO_3H-2AA
	3026.9	3027.9	H8N6F2-SO_3H-2AA
-8	2150.5	2151.2	H8N6F2-SO_3H-2AA
	3026.6	3027.9	H6N4F1-SO_3H-2AA
-9	2151.2	2151.2	H6N4F1-SO_3H-2AA
(b) BS2			
-1	1381.6	1381.3	H3N3F1-2AA
-2	2111.6	2111.9	H5N5F1-2AA
-3	2259.5	2258.2	H5N5F2-2AA
-4	2217.7	2217.7	H6N4F2-2AA
-5	2217.2	2217.7	H6N4F2-2AA
	2363.5	2363.7	H6N4F3-2AA
-6	2988.7	2988.0	H7N7F2-2AA
	3134.6	3134.1	H7N7F3-2AA
-7	3134.0	3134.1	H7N7F3-2AA
-8	2948.7	2947.9	H8N6F2-2AA
-9	3094.4	3093.5	H8N6F3-2AA
-10	3240.5	3239.2	H8N6F4-2AA
-11	2338.1	2338.2	H5N5F2-SO_3H-2AA
-12	2338.2	2338.2	H5N5F2-SO_3H-2AA
-13	2337.8	2338.2	H5N5F2-SO_3H-2AA
-14	2337.5	2338.2	H5N5F2-SO_3H-2AA
-15	3215.5	3215.1	H7N7F3-SO_3H-2AA
(c) HS			
-1	1193.2	1194.1	H4N2-2AA
-2	1339.3	1340.4	H4N2F1-2AA
-3	1558.6	1559.0	H5N3-2AA
-4	1704.9	1705.4	H5N3F1-2AA
-5	1761.9	1762.8	H5N4-2AA
-6	1923.9	1924.4	H6N4-2AA
-7	2068.7	2070.0	H6N4F1-2AA
-8	2069.5	2070.0	H6N4F1-2AA
-9	2215.2	2216.3	H6N4F2-2AA
-10	2215.5	2216.3	H6N4F2-2AA
-11	2288.8	2289.4	H7N5-2AA
	2363.5	2362.3	H6N4F3-2AA
-12	2435.6	2435.5	H7N5F1-2AA
	2492.7	2492.4	H7N6-2AA
-13	2580.9	2581.5	H7N5F2-2AA
	2637.0	2638.5	H7NF1-2AA
-14	2652.7	2654.0	H8N6-2AA
-15	2798.6	2799.7	H8N6F1-2AA
-16	2944.5	2945.7	H8N6F2-2AA
-17	3089.9	3091.7	H8N6F3-2AA
-18	3236.4	3237.7	H8N6F4-2AA
-19	3236.7	3237.7	H8N6F4-2AA
-20	3382.8	3384.1	H10N8-2AA
-21	3528.8	3530.0	H10N8F1-2AA
-22	3674.6	3676.3	H10N8F2-2AA
-23	3819.8	3822.0	H10N8F3-2AA
-24	3820.0	3822.0	H10N8F3-2AA
(d) Defucosyl HS			
a	1193.5	1194.1	H4N2-2AA (lacto-*N*-neohexaose)
b	1924.2	1924.4	H6N4-2AA (lacto-*N*-neodecaose)
c	2288.4	2289.4	H7N5-2AA (lacto-*N*-neododecaose)
d	2653.1	2654.0	H8N6-2AA (lacto-*N*-neotetradecaose)
e	3382.5	3384.1	H10HN8-2AA (lacto-*N*-neooctadecaose)

tween 30 and 38 min (BS2-4 to BS2-10) are considered to have multi-Fuc residues. BS2-4 and BS2-5 have the core structure of the LNnH unit and contain Fucα1–2 residues at the nonreducing ends because these fucose residues were specifically released by digestion with α1–2 fucosidase (data not shown). BS2-6 and BS2-7 showed molecular ions at *m/z* 2988.7 and 3134.6, respectively, which correspond to the compositions of H7N7F2–2AA and H7N7F3–2AA. Peaks BS2-8 to BS2-10 were LNnTD containing multiple Fucα1–2 residues. As a group of characteristic oligosaccharides in BS2, oligosaccharides having 80 mass units larger than BS2-3 were observed between 42 and 49 min. Oligosaccharides (BS2-11 to BS2-14) showed molecular ions at *m/z* 2338.2, indicating the composition of H5N5F2–SO_3H–2AA. These oligosaccharides are considered to be isomers having both Fuc and sulfate groups at different positions.

We found 24 oligosaccharide peaks in total in the HS milk sample. These oligosaccharides had LNnH, LNnD, lacto-*N*-neododecaose (LNnDD), LNnTD, and lacto-*N*-neooctadecaose (LNnOD) as core structures (Table 1, part c). HS-1 and HS-2 observed at 20.0 and 21.5 min, respectively, gave molecular ions at *m/z* 1193.2 and 1339.3, which correspond to H4N2–2AA and H4N2F1–2AA, respectively. HS-6, -7, -8, -9, and -10 showed molecular ions at *m/z* 1923.9, 2068.7, 2069.5, 2215.2, and 2215.5, respectively. The molecular ion of HS-6 is consistent with the theoretical mw of H6N4–2AA, suggesting the structure of LNnD. HS-7/8 (*m/z* 2068.7/2069.5) and HS-9/10 (*m/z* 2215.2/2215.5) showed larger molecular ions than those of HS-6 by one Fuc (146 mass units) and two Fuc (292 mass units), respectively. From these results, we concluded that these oligosaccharides had the core structure of LNnD to which different numbers of Fuc residues were attached (for confirmation of the structures, see below). The most abundant group of peaks (HS-14 to HS-19) commonly contains LNnTD (HS-14 at *m/z* 2652.7) as the core structure. HS-15, -16, -17, and -18/19 showed *m/z* values larger than LNnTD by one (146 mass units) to four (584 mass units) Fuc residues. These results indicate that HS-14 to HS-19 have LNnTD unit to which different numbers of Fuc residues are attached. The peaks (HS-20 to HS-24) having high molecular weights (*m/z* 3382.8 to 3820.0) were also observed between 50 and 54 min. These ladder peaks contained LNnOD (theoretical mw 3384.1) as the core structure to which one to four fucose residues are attached.

Urashima and coworkers reported that GlcNAc residues of LNnT and LNnH units in BS and HS oligosaccharides are not fucosylated. In contrast, most GlcNAc residues in bear milk oligosaccharides are fucosylated at OH-3 [17,18]. After digestion of asialo-oligosaccharides derived from HS with α1–3,4 fucosidase from *Streptomyces* sp. 142 or α1–2 fucosidase from *Corynebacterium* sp., the digestion products were analyzed by NP–HPLC. α1–3,4 Fucosidase did not act on the HS oligosaccharides, indicating that the oligosaccharides were not substituted at OH-3/4 on GlcNAc residues with fucose residues (data not shown). In contrast, most peaks disappeared on digestion with α1–2 fucosidase, and five peaks were observed at 19 min (peak a), 32 min (peak b), 38 min (peak c), 43 min (peak d), and 51 min (peak e) (Fig. 1C and Table 1, part d). These data indicated that all core oligosaccharides observed in Fig. 1C(2) were composed of one reducing terminal lactose and 2 to 8 LacNAc units. Peaks a and b showed molecular ions at *m/z* 1193.5 and 1924.2, which correspond to the molecular masses of LNnH and LNnD, respectively. Peak c showed a molecular ion at *m/z* 2288.4 of LNnDD. Peak d was the most abundant one in HS and showed the molecular ion of LNnTD at *m/z* 2653.1. In a similar manner, we confirmed that peak e was due to LNnOD.

Characterization of the branching pattern of BS oligosaccharides

The structures of dominant oligosaccharides in BS1 (BS1-1, -3, and -4 in Fig. 1B) were easily assigned. Digestion of BS1-1 with β-galactosidase from jack beans caused loss of two galactose res-

idues ($\Delta m/z$ 324). Further digestion with β-*N*-acetylhexosaminidase gave a molecular ion at m/z 461 corresponding to lactose with 2AA (data not shown). From the data, we concluded that BS1-1 was substituted with two Gal-GlcNAc residues at Gal OH-6 and Gal OH-3 of lactose. The BS1-3 and BS1-4 were digested with α1–2 fucosidase to afford an ion at m/z 1923.5 corresponding to LNnD. Digestion of the defucosylated oligosaccharide with β-galactosidase caused the loss of three galactose residues ($\Delta m/z$ 486), and the product showed a molecular ion at m/z 1435. From these results, we concluded that the core oligosaccharide of BS1-3 and BS1-4 is substituted with two LacNAc units on either branch of LNnH (data not shown). Oligosaccharides observed between 38 and 42 min gave molecular ions H6N4F1–SO_3H–2AA and H8N6F1–SO_3H–2AA (m/z 2151.2 and 3026.6, respectively). Among these oligosaccharides, we obtained BS1-9 as nearly pure state (Fig. 2). Digestion of the BS1-9 with α1–2 fucosidase caused the loss of one fucose residue and gave a molecular ion corresponding to LNnD (m/z 2005.1) with a sulfate group. Serial digestions of the defucosylated oligosaccharide with β-galactosidase and β-*N*-acetylhexosaminidase caused the loss of two LacNAc units and gave a molecular ion corresponding to the composition of H4N2–SO_3H–2AA (m/z 1275.6). These results indicated that the defucosylated oligosaccharide has two nonsubstituted Gal residues at the nonreducing ends. Further digestion of the oligosaccharide with β-galactosidase gave a molecular ion, H3N2–SO_3H–2AA (m/z 1113.3). Urashima and coworkers reported that some oligosaccharides in BS milk were sulfated at the nonreducing terminal Gal OH-3 [18]. From this report and our observations, we concluded that the oligosaccharides from BS1-6 to BS1-9 were due to LNnD and LNnTD substituted with one sulfate group at the OH-3 position of the nonreducing terminal Gal residue.

Structures of dominant oligosaccharides in BS2 (BS2-1 and BS2-3) were confirmed in a similar manner. Digestion of BS2-1 with α1–2 fucosidase caused the loss of one fucose residue (Fig. 3A). Further digestion with β-galactosidase gave a molecular ion (m/z 911.4) corresponding to H3N3–2AA. Finally, digestion with β-*N*-acetylhexosaminidase gave a molecular ion at m/z 505.1 corresponding H1N1–2AA. Accordingly, we concluded that the core disaccharide at the reducing end in BS2-1 was Gal-GlcNAc and that BS2-1 was a hexasaccharide substituted with two LacNAc units at OH-6 and OH-3 of Gal residue of the terminal Gal-GlcNAc. Oligosaccharide BS2–3 was also digested with α1–2 fucosidase to give a glycan showing the molecular ion at m/z 1966.5 corresponding to H5N5–2AA (Fig. 3B). Digestion of the core oligosaccharide with β-galactosidase caused the loss of three galactose residues ($\Delta m/z$ 486). Further digestion with β-*N*-acetylhexosaminidase gave a molecular ion (m/z 869.9) corresponding to H2N2–2AA. Thus, we concluded that one of the branched units on BS2-1 was further substituted with two LacNAc residues. The characteristic oligosaccharides from BS2-11 to BS2-14 showed molecular ions at m/z 2238.1, which are consistent with the composition of H5N5F2–SO_3H–2AA. These oligosaccharides were digested with α1–2 fucosidase to produce a signal at m/z 2046.3 corresponding to H5N5–SO_3H–2AA. Further digestion of the defucosylated oligosaccharide with β-galactosidase caused the loss of two Gal residues. These observations indicated that the oligosaccharides from BS2-11 to BS2-14 have two LacNAc branches substituted with α1–2 Fuc residue (data not shown).

Characterization of the branching pattern of HS oligosaccharides

Digestion of the core oligosaccharide (A, peak a in Fig. 1C(2)) with β-galactosidase caused the loss of two galactose residues

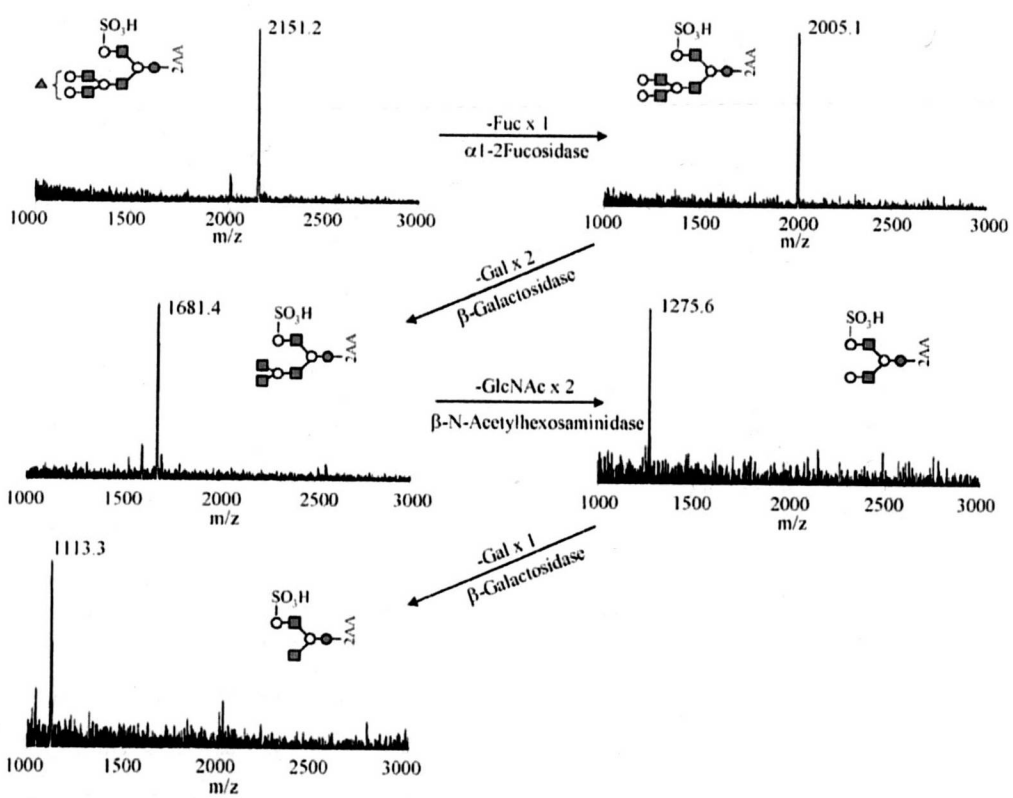

Fig. 2. Stepwise exoglycosidase digestion of characteristic oligosaccharide BS1-9 observed in Fig. 1B. Conditions for the enzymatic reaction with exoglycosidases are shown in Materials and methods. Symbols: open circles; Gal; filled circles, Glc; filled squares, GlcNAc; filled triangles, Fuc. Linkage positions are assigned tentatively.

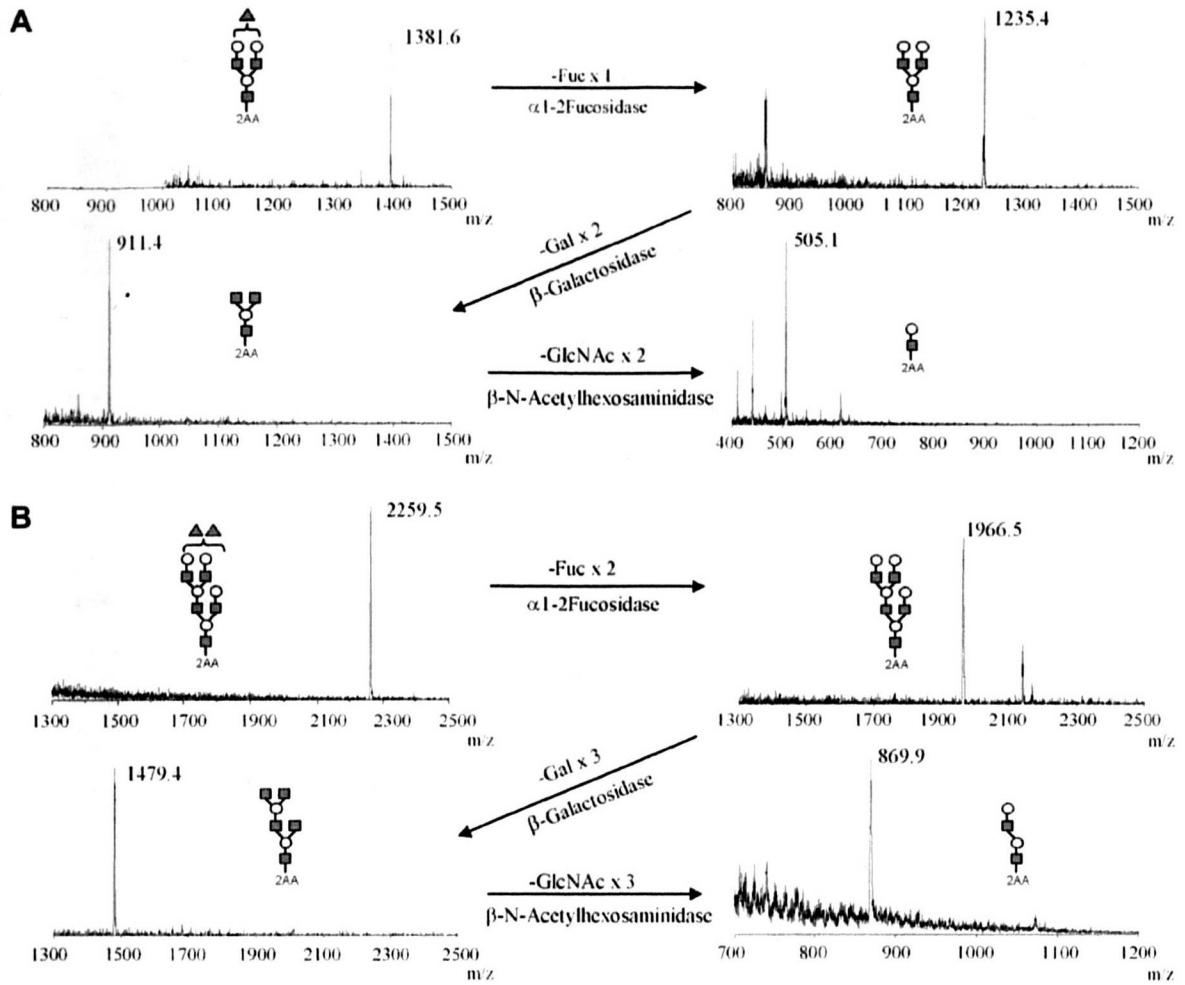

Fig. 3. Stepwise exoglycosidase digestion of characteristic oligosaccharides BS2-1 (A) and BS2-3 (B) observed in Fig. 1B. Symbols: open circles, Gal; filled circles, Glc; filled squares, GlcNAc; filled triangles, Fuc. Linkage positions are assigned tentatively.

(Δ*m*/*z* 324) and gave a product ion at *m*/*z* 868.8 (Fig. 4A). The oligosaccharide at *m*/*z* 868.8 was further digested with β-*N*-acetylhexosaminidase, and a new ion corresponding to Gal-Glc-2AA was observed at *m*/*z* 462.4. From these observations, we concluded that the oligosaccharide (peak a) has the structure of Galβ1–4GlcNAcβ1–6[Galβ1–4GlcNAcβ1–3]Galβ1–4Glc (LNnH). Digestion of the core oligosaccharide (B, peak b in Fig. 1C(2)) with β-galactosidase caused the loss of three galactose residues (Δ*m*/*z* 486), and the product showed a molecular ion at *m*/*z* 1437.5 (Fig. 4B). The oligosaccharide at *m*/*z* 1437.5 was further digested with β-*N*-acetylhexosaminidase to release three GlcNAc residues. These observations indicated that the core oligosaccharide (peak b) has a triantennary structure. The produced oligosaccharide corresponding to Gal-GlcNAc-Gal-Glc-2AA (*m*/*z* 826.7) was again digested with β-galactosidase to produce a peak at *m*/*z* 665.1 (GlcNAc-Gal-Glc-2AA). The structure was confirmed by comparison of the retention time with that of trisaccharide (GlcNAcβ1–3Galβ1–4Glc-2AA) prepared by digestion of lacto-*N*-tetraose (Galβ1–3GlcNAcβ1–3Galβ1–4Glc) with β-galactosidase using HPLC on an ODS column and capillary electrophoresis (data not shown). These observations indicated that the core oligosaccharide (peak b) has two LacNAc units on the α1–3 LacNAc branch of LNnH. Digestion of the core oligosaccharide (C, peak c in Fig. 1C(2)) with β-galactosidase caused the loss of three Gal residues (Δ*m*/*z* 486), and the product showed a molecular ion at *m*/*z* 1801.9. The oligosaccharide at *m*/*z* 1801.9 was further digested with β-*N*-acetylhexosaminidase to produce a molecular ion at *m*/*z* 1193.8. The oligosaccharide has the structure of H6N4–2AA. These results indicated that peak c has a triantennary structure. The oligosaccharide (*m*/*z* 1193.8) was again digested with β-galactosidase to produce a peak at *m*/*z* 869.1 by the loss of two galactose residues. The course of digestion by a combination of exoglycosidases revealed that peak c was a dodecasaccharide having three LacNAc residues at nonreducing ends, and we concluded that the oligosaccharide of peak c has two LacNAc units and one LacNAc unit on both branches of LNnH. Digestion of the core oligosaccharide (D, peak d in Fig. 1C(2)) with β-galactosidase caused the loss of four galactose residues (Δ*m*/*z* 648), and the product showed a molecular ion at *m*/*z* 2004.5. The product was further digested with β-*N*-acetylhexosaminidase to produce an ion at *m*/*z* 1193.8 corresponding to LNnH. These observations indicated that peak d has a tetraantennary structure. The produced oligosaccharide corresponding to LNnH was again digested with β-galactosidase to produce a molecular ion at *m*/*z* 869.1. From these results, we concluded that peak d was a tetradecasaccharide having four LacNAc residues at the nonreducing ends and that both branches of LNnH were substituted with two LacNAc units. The oligosaccharide (E, peak e in Fig. 1C(2)) having the largest molecular mass (*m*/*z* 3382.5) present in HS milk caused

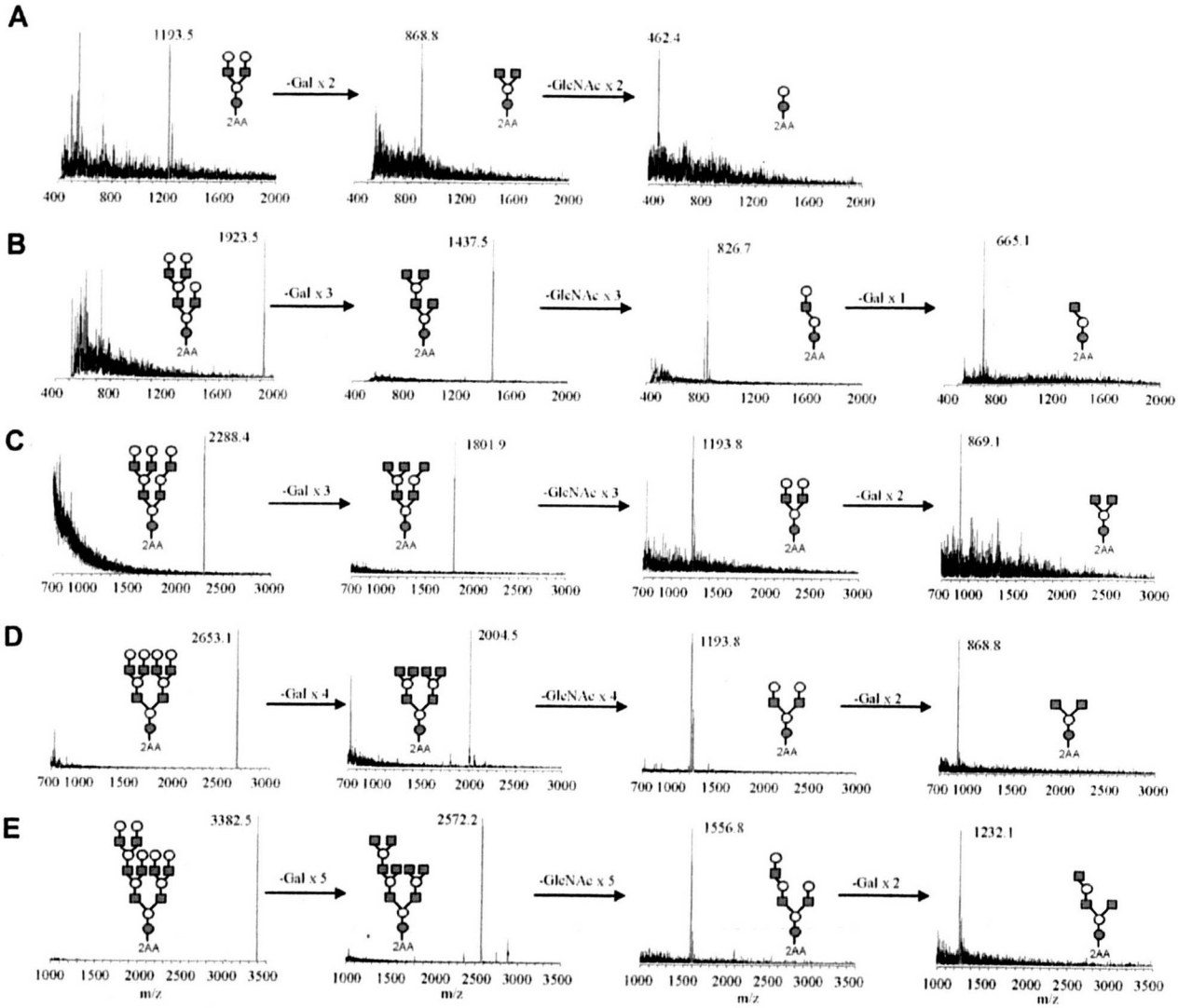

Fig. 4. Stepwise exoglycosidase digestion of core oligosaccharides derived from HS observed in Fig. 1B: (A) lacto-*N*-neohexaose (LNnH); (B) lacto-*N*-neodecaose (LNnD); (C) lacto-*N*-neododecaose (LNnDD); (D) lacto-*N*-neotetradecaose (LNnTD); (E) lacto-*N*-neooctadecaose (LNnOD). Symbols: open circles, Gal; filled circles, Glc; filled squares, GlcNAc.

the loss of five galactose residues ($\Delta m/z$ 810) by digestion with β-galactosidase, and the product showed a molecular ion at m/z 2572.2. The product was further digested with β-*N*-acetylhexosaminidase to produce a molecular ion at m/z 1556.8. The oligosaccharide was again digested with β-galactosidase to produce a peak at m/z 1232.1. From these results, we concluded that the oligosaccharide derived from peak e was an octadecasaccharide, as shown in Fig. 4E.

Structural determination of fucosylated decasaccharides by MALDI–QIT–TOF MS

The core oligosaccharides in HS milk are substituted with a different number of fucose residues, as shown by characteristic ladder patterns (Fig. 1B). We purified monofucosylated LNnD (MFLNnD, HS-7, and HS-8 in Fig. 1B) and difucosylated LNnD (DFLNnD, HS-9, and HS-10 in Fig. 1B) and analyzed them using MALDI–QIT–TOF MS. Fig. 5 shows the MS/MS spectra using $[M + Na]^+$ observed at m/z 2093.1 for the purified MFLNnD (Fig. 5A and B). The Y ion at m/z 1947.5/1947.2 corresponding to the loss of 146 mass units (dHex-18 mass) from $[M + Na]^+$ indicates the presence of a Fuc residue. The Y ions at m/z 1728.0/1728.1 and 1581.9/1582.0 are due to H5N3F1–2AA and H5N3–2AA, respectively. These fragment ions were commonly observed in HS-7 and HS-8. We also found the set of B ion series, $[H2N2]^+$ at m/z 753.5/754.5, $[H3N3]^+$ at m/z 1118.7/1118.8, $[H3N3F1]^+$ at m/z 1264.8/1264.9, and $[H4N3F1]^+$ at m/z 1791. Characteristic ions observed at m/z 1264.8 (Fig. 5A) and m/z 1118.8 (Fig. 5B) suggested the difference in the linkage positions of Fuc residues at the nonreducing Gal residues. The B ion at m/z 1264.8 ($B_{5\alpha}$) indicates that a Fuc residue is linked to the most outer LacNAc residue. The B ion at m/z 1118.8 corresponding to three LacNAc units suggests that one Fuc is attached to the 6-branch side of the reducing terminal lactose. Urashima and coworkers reported that small oligosaccharides in HS milk contained type II LacNAc (Galβ1–4GlcNAc-R) but not type I LacNAc (Galβ1–3GlcNAc-R) [14]. Thus, the oligosaccharides HS-7 and HS-8 are assigned to those as indicated in Fig. 5.

Fig. 6 shows the MS/MS spectra of the ions at m/z 2239.8 for the $[M + Na]^+$ of DFLNnD (HS-9 and HS-10 in Fig. 1B). The Y ions at m/z 2093.3 corresponding to a loss of 146 (dHex-18 mass) from $[M + Na]^+$ indicate the presence of Fuc residue. In a similar manner, in the case of MS/MS of MFLNnD (Fig. 5), the Y ions observed at m/z