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A Cationic Cysteine-Hydrazide as an Enrichment Tool for the Mass Spectrometric Characterization of Bacterial Free Oligosaccharides

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Abstract

In *Campylobacterales* and related ε-proteobacteria with N-linked glycosylation (NLG) pathways, free oligosaccharides (fOS) are released into the periplasmic space from lipid-linked precursors by the bacterial oligosaccharyltransferase (PglB). This hydrolysis results in the same molecular structure as the oligosaccharide that is transferred to a protein to be glycosylated. This allowed for the general elucidation of the fOS branched structures and monosaccharides from a number of species using standard enrichment and mass spectrometry methods. To aid characterization of fOS, hydrazide chemistry has often been used for chemical modification of the reducing part of oligosaccharides resulting in better selectivity and sensitivity in mass spectrometry; however, the removal of the unreacted reagents used for the modification often causes the loss of the sample. Here, we develop a more robust method for fOS purification and characterize glycostructures using complementary tandem mass spectrometry (MS/MS) analysis. A cationic cysteine hydrazide derivative was synthesized to selectively isolate fOS from periplasmic fractions of bacteria. The *cy*steine *h*ydrazide *n*icotinamide (*Cyhn*) probe possesses both thiol and cationic moieties. The former enables reversible conjugation to a thiol-activated solid support, while the latter improves the ionization signal during MS analysis. This enrichment was validated on the well-studied *Campylobacter jejuni* by identifying fOS from the periplasmic extracts. Using complementary MS/MS analysis we approximated data of a known structure of the fOS from *Campylobacter concisus*. This versatile enrichment technique allows for the exploration of a diversity of protein glycosylation pathways.

Keywords

Enrichment; Free oligosaccharide; Campylobacter; Hydrazide; Glycomics

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Introduction

Protein N-linked glycosylation (NLG) is observed in all domains of life. In bacteria, the discovery of a functional NLG pathway in the gram-negative human-pathogen *Campylobacter jejuni* [1] created a novel route for understanding this important basic biological process. In *C. jejuni*, NLG is required for pathogenicity creating many questions about the biological role of NLG in bacteria [2,3]. Moreover, the simpler bacterial system has drawn attention for the potential of engineering glycosylation to generate therapeutic glycoproteins [4,5]. To date, NLG pathways have only been confirmed in ε-proteobacteria, *i.e. Campylobacter* spp. [1,4] and *Helicobacter* spp. [6], where there are significant differences in the glycans produced.

In the periplasmic space of *C. jejuni*, free heptasaccharide, structurally identical to that found as an N-linked glycan counterpart, is present. It is presumed to be the result of an additional hydrolase activity of PglB [7] because the assembly of the glycan to the protein is done in consecutive steps, one sugar unit after another [8,1]. Thus, free oligosaccharides (fOS) would not be expected to be present from the assembly. Previously, fOS were exploited to demonstrate diverse glycostructures across a variety of *Campylobacter* and related species [9]. Given these observations, one would hypothesize that identification and characterization of fOS from the periplasmic extracts of more distant bacteria would allow prediction of the N-linked glycan structure and provide strong evidence for the broad existence of bacterial NLG systems. This is limited by the need for selective enrichment of fOS from periplasmic extracts and then subsequent structural characterization.

The general elucidation of bacterial periplasmic fOS structure has been done by standard enrichment and mass spectrometry methods. Zwitterionic hydrophilic interaction chromatography (ZIC-HILIC), known to have high capability to separate eukaryotic N-glycans or glycopeptides [10,11], has been established for the studies in *Campylobacter* species [9,8]. The enriched glycans and glycoprotein can then be analyzed by mass spectrometry. In addition to that, hydrazide chemistry has often been used for chemical modification of the reducing part of oligosaccharides resulting in better selectivity and sensitivity in mass spectrometry [12,13]; however, the removal of the unreacted reagents used for the modification is sometimes troublesome when their chemical properties are the same as the target molecules and can often cause the loss of the sample.

In this report, we have developed a new chemical probe to take advantage of the hydrazide chemistry. The probe contains thiol and pyridyl moieties in addition to the hydrazide group (Figure 1). The thiol group allows for conjugation to a thiol-activated solid support, which can be released under reducing conditions after oligosaccharide capture, allowing simple cleanup of unreacted reagents and other impurities. The pyridyl group serves as a handle for forming a positive charge that can dramatically improve the ionization signal during MS analysis [14,15]. The cationic cysteine hydrazide functionalized resin was used to selectively capture bacterial free oligosaccharides in periplasmic fractions of *C. jejuni*, which were then analyzed by mass spectrometry. Further methylation resulted in a methylated nicotinamide structure that provides a fixed cationic charged probe producing predominantly singly charged ions during MS analysis, resulting in at least an 8-fold increase in signal-to-noise

ratio (SNR). These tools represent a significant enhancement in sensitivity to assist glycomic studies.

Materials and methods

Chemicals and materials

Nicotinic acid, *N*,*N*-dimethylformamide, *N*,*N*-diisopropylethylamine, L-cysteine methyl ester hydrochloride, hydrazine monohydrate, and iodomethane were purchased from Sigma-Aldrich (St. Louis, MO). Thionyl chloride was obtained from Junsei Chemical Co., Ltd (Tokyo, Japan). Thiopropyl Sepharose™ 6B resins were purchased from GE Healthcare Biosciences (Pittsburgh, PA). *C. jejuni* NCTC11168 (#700819) and *C. concisus* RM5485 (#BAA-1457) were from ATCC (Manassas, VA). All other chemicals were of analytical grade.

Procedures for the synthesis of the *Cyhn* probe

Step 1, Preparation of *cysteine methyl ester nicotinamide* 4—Nicotinic acid (**2**, 7.9 g, 64.2 mmol) was suspended in 100 mL thionyl chloride (512 mmol). *N*,*N*-Dimethylformamide (0.2 mL, 2.6 mmol) was added to the solution as a catalyst. The reaction mixture was stirred for 1 h at room temperature (RT), during which time the slurry became homogeneous. Upon complete consumption of the nicotinic acid, the thionyl chloride was removed *in vacuo*, and the residue was azeotroped with benzene to remove residual thionyl chloride and HCl. The crude off-white solid (**3**) was suspended in 150 mL of acetonitrile and cooled to 0 °C under a nitrogen atmosphere. A heterogeneous mixture containing cysteine methyl ester hydrochloride **1** (10 g, 58.3 mmol) and *N*,*N*-diisopropylethylamine (8.3 mL, 47.2 mmol) in 200 mL of acetonitrile was transferred in one portion to the nicotinic acid chloride. The resulting thick white slurry was stirred vigorously and allowed to warm to RT over 2 hours. The reaction was quenched with saturated aqueous $NaHCO_3$ and the acetonitrile was removed *in vacuo*. The aqueous phase was extracted with dichloromethane (3×100 mL), and the combined organic phase was washed with brine. The organic layer was dried over Na_2SO_4, and then the solvent was removed *in vacuo*. The crude residue was purified using silica column chromatography (50→100% EtOAc:hexanes) affording the cysteine methyl ester nicotinamide **4** as a viscous colorless oil (6.0 g, 25.0 mmol, 43% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.07 (dd, *J* = 2.3, 0.9 Hz, 1H), 8.77 (dd, *J* = 4.9, 1.7 Hz, 1H), 8.15 (ddd, *J* = 7.9, 2.3, 1.7 Hz, 1H), 7.42 (ddd, *J* = 7.9, 4.9, 0.9 Hz, 1H), 7.09 (br d, *J* = 7.1 Hz, 1H), 5.09 (dt, *J* = 7.1, 4.0 Hz, 1H), 3.85 (s, 3H), 3.16 (dd, *J* = 9.0, 4.0 Hz, 2H), 1.40 (t, *J* = 9.0 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$) δ: 170.37, 165.17, 152.69, 148.24, 135.06, 129.22, 123.46, 53.92, 53.02, 26.78; FTIR (NaCl, thin film) 3309, 3035, 2951, 1740, 1653, 1591, 1539, 1351, 1217 cm^{-1}; HRMS (ESI+) calc'd for $C_{10}H_{12}N_2O_3S$ $[M+H]^+$ 241.0641, found 241.0643 (Supplement Figure 1).

Step 2, Preparation of *cystine dihydrazide nicotinamide* 6—Cysteine methyl ester nicotinamide (**4**, 5.9 g, 24.4 mmol) was dissolved in 100 mL of dry methanol, then hydrazine monohydrate (9.5 mL, 195.2 mmol) was added. The solution was stirred at 50 °C for 5 h, and the conversion to cysteine hydrazide nicotimamide (*Cyhn* monomer) was monitored by LC-MS. After the complete consumption of the starting material, the solution

was sparged with oxygen gas for 15 minutes, then maintained under an oxygen atmosphere overnight at 50 °C. After LC-MS analysis indicated complete conversion to the *Cyhn* disulfide dimer, the volatiles were removed *in vacuo*. The white residue was subjected to hot trituration in refluxing methanol (100 mL), and the precipitated product was filtered and washed with cold methanol. Drying under vacuum (0.1 Torr) afforded the cystine dihydrazide nicotinamide (*Cyhn* dimer) **6** as a white solid (2.4 g, 41% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.42 (s, 1H), 8.99 (dd, *J* = 2.3, 0.9 Hz, 1H), 8.87 (d, *J* = 8.2 Hz, 1H), 8.69 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.17 (app dt, *J* = 7.9, 2.0 Hz, 1H), 7.48 (ddd, *J* = 7.9, 4.8, 0.9 Hz, 1H), 4.74 (m, 1H), 4.30 (br s, 2H), 3.22 (dd, *J* = 13.6, 4.9 Hz, 1H), 3.03 (dd, *J* = 13.6, 9.9 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ: 168.90, 165.04, 151.96, 148.72, 135.26, 129.44, 123.30, 51.34, 39.74; IR (NaCl/thin film) 3281, 3035, 2960, 1633, 1537, 1327; HRMS (ESI−) calc'd for $C_{18}H_{22}N_8O_4S_2$ $[M-H]^-$ 477.1133, found 477.1148 (Supplement Figure 2).

Independent preparation of *Cyhn* monomer 5

Cysteine methyl ester nicotinamide (**4**, 80 mg, 0.33 mmol) was dissolved in 400 mL of dry methanol under nitrogen, then hydrazine monohydrate (67 mL, 1.33 mmol) was added. The solution was stirred at RT until conversion to the cysteine hydrazide nicotimamide (*Cyhn* monomer) was achieved by LC-MS. All manipulations thereafter were performed under an argon atmosphere. The volatiles were evaporated *in vacuo*, and the residue was slurried in ethanol, filtered, and washed with cold ethanol. Drying under vacuum (0.1 Torr) afforded the cysteine hydrazide nicotinamide (*Cyhn* monomer) as a white solid (50 mg, 62% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.35 (s, 1H), 9.05 (dd, *J* = 2.3, 0.9 Hz, 1H), 8.77 (d, *J* = 8.1 Hz, 1H), 8.71 (dd, *J* = 4.8, 1.7 Hz, 1H), 8.23 (app dt, *J* = 7.9, 2.0 Hz, 1H), 7.52 (dd, *J* = 8.0, 4.8 Hz, 1H), 4.51 (td, *J* = 9.0, 5.2 Hz, 1H), 4.28 (br s, 2H), 2.90 (dd, *J* = 13.5, 5.2 Hz, 1H), 2.82 (dd, *J* = 13.5, 9.0 Hz, 1H), S*H* not observed; ^{13}C NMR (126 MHz, DMSO-d_6) δ: 169.00, 165.17, 151.98, 148.76, 135.34, 129.55, 123.33, 55.10, 26.00. FTIR (NaCl/thin film) 3290, 3036, 2960, 1630, 1537, 1328; HRMS (ESI+) calc'd for $C_9H_{12}N_4O_2S$ $[M+H]^+$ 241.0754, found 241.0755 (Supplement Figure 3).

Preparation of cationic cysteine hydrazide-functionalized resins

Cyhn dimer **6** (86 mg, 180 μmol) was suspended in 50% methanol (1 mL) and one equivalent of dithiothreitol was added to the solution. Thiopropyl Sepharose™ 6B resins (1 mL, 30 μmol) were washed with deionized water and suspended in 50% methanol. Five hundred milliliters of the *Cyhn* solution was added to the suspension. The suspensions were placed on a rocking incubator at RT overnight then washed with 50% methanol, followed by water and 20% ethanol. The resulting resin was stored in 20% ethanol at 4 °C prior to use.

Preparation of bacterial periplasmic fraction

C. jejuni was grown with brain heart infusion (BHI; Difco Laboratories Inc, Detroit, MI) medium as in [16] and *C. concisus* were cultured with Brucella Broth (Difco) with 2% yeast extract under microaerobic condition (1% O_2, 10% CO_2, 10% H_2 and balanced with N_2) at 37 °C overnight. Each 20 mL of cell culture was inoculated to 400 mL culture medium and incubated for 24 h. Cells were harvested from cell cultures using centrifugation at 3,500 rpm

for 10 min. Periplasmic extraction was performed using an osmotic shock method. Briefly, cell pellets were resuspended with 20 mL of 30 mM Tris-HCl (pH 8.0) containing 20% sucrose and 1 mM Na_2EDTA then incubated at RT for 10 min on a rocking platform as in [17]. After centrifugation at 10,000 rpm for 10 min, the pellets were resuspended with 5 mL of cold 5 mM $MgSO_4$ solution then incubated on ice for 10 min. Periplasmic supernatants were collected using centrifugation at 13,000 rpm for 10 min, then stored at 4 °C prior to use. For free glycan enrichment experiments, those (each 0.5 mL) were then applied on an Amicon ultra centrifugal filter units (10K molecular weight cutoff, Millipore, Billerica, MA) to remove periplasmic proteins. The flow-through was stored at 4 °C prior to use.

Solid-phase extraction of free oligosaccharides

Free oligosaccharides (fOS) were extracted from bacterial periplasmic samples using a Carbograph Extract-Clean™ cartridge (150 mg, 4 mL, Grace, Deerfield, IL). The solvent system was as follows: 0.1% (w/v) trifluoroacetic acid (TFA) in 50% acetonitrile/50% water (solvent A) and 0.1% (w/v) TFA in 5% acetonitrile/95% water (solvent B). The cartridge was washed with 30% acetic acid in water, washed with HPLC-grade water, and then primed with 3 mL of solvent A followed by 6 mL of solvent B. The periplasmic sample was applied to the column and then washed with water and solvent B. The fOS were eluted with 2 × 0.5 mL of solvent A and then dried under vacuum. The extracted fOS were reconstituted in 100 μL of deionized water for MALDI-TOF MS analysis.

Enrichment of free glycans and post-methylation of the *Cyhn*-conjugate

To capture free glycans, reducing glycans (each 50 nmole) were incubated with 10 μL of *Cyhn*-6B resin (ca. 300 nmole) at 100 °C for 20 min in 2% (v/v) acetic acid in acetonitrile. After conjugation, the resin was subsequently incubated with 20% of methyl iodide in acetonitrile at RT for 10 h with gentle shaking. The resin was washed with acetonitrile, followed by deionized water. The enriched fOS were released by 100 μL of 10 mM dithiothreitol in 50% methanol for further MS analysis.

MALDI-MS analysis

The extracted fOS solution (0.5 μL) was applied to a MALDI target followed by 2,5-dihydroxybenzoic acid (DHB, Sigma-Aldrich) matrix solution (i.e.; 30 mg/mL in 70% acetonitrile/30% water [v/v], 0.5 μL). The fOS were analyzed on a Voyager DE PRO MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) operating in reflector and positive ion modes. For all experiments, the accelerating voltage was held at +20 kV, grid voltage at 75%, and guide wire at 0.004%; delay was 100 ns. Mass spectra were acquired from 300 laser shots.

LTQ FT-ICR analysis

To obtain structural information of enriched fOS, MS/MS analysis was performed on a 7T LTQ FT-ICR Ultra mass spectrometer (Thermo Fisher Scientific., Bremen, Germany) with positive ion mode at spray voltage of 2.2 kV [18]. Samples dissolved in 50% methanol including 2% acetic acid were directly infused into the mass spectrometer at a flow rate of 1 μL/min using a syringe pump. The heated capillary was maintained at a temperature of

200°C. Precursor ions were isolated in the linear ion trap, with an isolation window of 5–10 Da. Collision-induced dissociation (CID) was performed in the linear ion trap with helium as the collision gas. Infrared multiphoton dissociation (IRMPD) and electron-induced dissociation (EID) were performed in the ICR analyzer. For CID the automatic gain control (AGC) target value was set at 5×10^4 ions and for IRMPD and EID experiments at 1×10^5 ions. The maximum ion injection times were 1500 ms for CID, and 2000–2500 ms for IRMPD and EID. In order to achieve optimal fragmentation the collision energy, the irradiation times, the laser power and the cathode voltages were adjusted for each precursor ion. CID was carried out with a normalized collision energy of 15%–20% and 30 ms activation time. IRMPD experiments were performed with a continuous 20 W, 10.6 μm, CO_2 laser (Synrad, Mukilteo, WA) at 15–20% laser power and photon irradiation times of 50 ms. EID experiments were carried out with an indirectly heated dispenser cathode (Heatwave, Watsonville, CA) at a cathode potential of −20 to −60 V and irradiation time of 50–100 ms.

Results and Discussion

Preparation of the cationic cysteine hydrazide derivative and functionalized resins

A straightforward procedure was developed to prepare the required *cy*steine *h*ydrazide *n*icotinamide (*Cyhn*, **5**, Figure 1a). Racemic cysteine methyl ester (**1**) was coupled with nicotinoyl chloride to provide cysteine methyl ester nicotinamide (**4**). Unreacted cysteine methyl ester was removed using solution-phase extraction, and the major impurity of the reaction (acylation of the thiol group of the product) was separated by silica column chromatography. Reaction of **4** with excess hydrazine hydrate in methanol delivered the *Cyhn* compound (**5**). The reaction was monitored by LC/MS, and upon complete consumption of the starting material the solution was sparged with oxygen gas to effect dimerization of the *Cyhn* monomer. Precipitation of *Cyhn* dimer (**6**) resulted during this process, and after concentration and trituration with methanol, **6** was obtained in >95% purity. The structures of the intermediates and final product were confirmed by ^{1}H and ^{13}C NMR, FTIR, and HRMS (Supporting Information).

During these studies it was observed that ester **4** is stable as the free thiol; however, the *Cyhn* monomer **5** was susceptible to oxidation to the disulfide. This feature hampered the initially attempted isolation and purification of this compound. Alternatively, the *Cyhn* dimer (**6**) existed as a stable solid that could be reliably produced in high purity. The stability and ease of purification of the *Cyhn* dimer was exploited to obtain gram quantities of this material, which serves as precursor to **5**. Prior to the conjugation, the disulfide moiety of **6** was reduced with dithiothreitol (DTT) and the liberated monomer **5** was used *in situ* for conjugation to the activated thiol resin (Figure 1b). The *Cyhn* monomer **5** was conjugated to a commercially available thiol-activated solid support (thiopropyl Sepharose™ 6B resin) resulting in the *Cyhn*-6B resin **8** (Figure 1b). The prepared *Cyhn* resin was employed to selectively conjugate free oligosaccharides as well as oxidized glycoproteins from mixtures.

Enrichment of free reducing sugars using *Cyhn* resins

As a first test for the functionality of the *Cyhn* moiety, commercially available free oligosaccharides, *i.e.* maltopentose (M5) and maltohexose (M6), were incubated with the *Cyhn*-6B resins resulting in selective isolation of the reduced glycans; however, it was observed that the *Cyhn*-conjugated glycans ionized as the sodium adduct, not the protonated pyridinium (Figure 2a upper panel), even though the pyridine ring is expected to be protonated in solution at low pH. It is presumed that the glycan-*Cyhn* prefers the sodium ion due to the chelating effect of the hydroxyl groups of the glycans. In order to enhance the ionization signals for mass spectrometry (MS) analysis, we sought to generate a discrete, permanent positive charge on the *Cyhn* resin by *N*-methylation of the pyridine nitrogen (Figure 2b). This was accomplished by a mild post-methylation of the glycan-*Cyhn* conjugates with 20% [v/v] methyl iodide in acetonitrile (Figure 2a). The reaction optimization was undertaken and a condition using a lower reaction temperature (i.e., 24°C) and a longer reaction time (i.e., 10 hours) was chosen. Under this condition, the pyridine nitrogen of *Cyhn* moiety was almost completely methylated, resulting in the 8-fold improved signal-to-noise ratio (SNR) during MS analysis (Figure 2a).

To validate this enrichment method, a bacterial periplasmic extract, fractionated from osmotically shocked *Campylobacter jejuni* NCTC11168, was used to selectively capture free oligosaccharides (fOS). Initially, fOS were enriched using a standard method [19] involving binding to a solid-phase Carbograph™ extraction cartridge followed by elution. This eluate was subjected to MALDI-TOF MS analysis, which identified a peak that corresponded to the expected heptasaccharide (Figure 3a); however, the noise associated with this purification included a number of additional significant peaks. Thus, to obtain a superior sample the fOS from the enriched fraction were covalently bound to a resin through the hydrazide moiety generating fOS-*Cyhn* conjugate and subsequently released under reducing conditions. This sample was analyzed by MALDI-TOF and the resulting spectrum contained a single significant peak at the observed mass-to-charge (*m/z*) value at 1660.4 correlating well with the predicted mass of the *C. jejuni* heptasaccharide-*Cyhn* conjugate (Figure 3b). The mass difference of 214 Da represents the fOS_{Cj} covalently conjugated *via* hydrazone formation to a methylated *Cyhn* moiety. This doubled the SNR of the MS analysis and then also confirmed the presence of a reducing end sugar. Structural information for the enriched fOS_{Cj}-*Cyhn* was obtained from MS/MS analysis using collision-induced dissociation (CID). For this, fragment ions of the abundant precursor ion at *m/z* 1660.60 were identified by tandem mass spectrometry (Figure 3c). As expected [20,21], CID spectra resulted predominantly in glycosidic bond cleavages revealing the monosaccharide composition of the fOS_{Cj}, which comprises the branched heptasaccharide (GalNAc-GalNAc-[Glc]-GalNAc-GalNAc-GalNAc-diNAcBac) as previously seen [22,1], where diNAcBac (2,4-diacetamido-2,4,6-trideoxyglucose) is a diacetylated bacillosamine. These results confirm the robustness of the *Cyhn* conjugation as a tool for enrichment and identification of free oligosaccharides.

Structural characterization of fOS of *C. concisus* by complementary MS/MS analyses

For additional validation, the *Cyhn* resin was used to resolve the different oligosaccharides reported in the literature for the bacteria *Campylobacter concisus* [23,9]. Two recent

publications identified different N-linked glycan structures for this species highlighting the complexities associated with bacterial glycan structure determination. Nothaft *et al.* proposed the unresolved branched structure of 234-HexNAc-[Hex]-$HexNAc_3$-diNAcBac for *C. concisus* [9], while Jervis *et al.* proposed the structure to be HexNAc-[Hex]-HexNAc-HexNAc-217-HexNAc-228-Asn [23]. Here, this new independent method is used to clarify these incompatible results. As done for *C. jejuni*, a solid phase extracted periplasmic fOS_{Cc} from *C. concisus* RM5485 was identified by MS at *m/z* 1460.1 (Figure 4a). Further enrichment by *Cyhn* conjugation resulted in a peak at *m/z* 1674.7 consistent with a heptasaccharide conjugated to a *Cyhn* moiety (Figure 4b). This abundant and fully-resolved precursor ion was subjected to CID MS/MS analysis that generated predominantly free oligosaccharides consistent with a branched heptasaccharide (i.e. HexNAc-[Hex]-HexNAc-HexNAc-217-HexNAc-228) (Suppl. Figure 4a). The mass of 228 was consistent with diNAcBac that had previously been identified for *C. jejuni* and, based on the operon structure, is expected for *C. concisus*. To resolve the specific glycans, further MS/MS analysis using additional fragmentation techniques were required. Infrared multiphoton dissociation (IRMPD) [24,25] and electron-induced dissociation (EID) [26–29] along with CID were used for the structural elucidation of fOS_{Cc}. Our studies support the characterization by Jervis et al. as shown in Suppl. Figure 4. Particularly, IRMPD and EID MS/MS analysis identified the existence of the enrichment tag. Typical tag fragmentations of the *Cyhn*-conjugated reduced sugar were detected at *m/z* 255.09, 240.08 and 223.05 in both IRMPD and EID spectra (Figure 4c and Suppl. Figures). These fragment ions can be attributed to labile amide and hydrazide bond fragmentations providing diagnostic ions to identify the enriched glycans and differentiate them from other molecules. In CID we were unable to observe the tag fragment ions due to the low-mass cutoff (LMCO) of the ion trap, also known as the one-third rule, in which product ions with masses below 28% of the precursor ions mass cannot be trapped during CID.

Based on the applied methods, the EID spectra resulted in the most fragmentation compared to CID and IRMPD. Cross-ring fragmentations (i.e. $^{1,5}X$ ions) were more abundant along with the common glycosidic cleavages (Y and Z ions). Upon CID and IRMPD, the sugar at the third position from the reducing end gave a mass of 217 Da, 14 Da higher than a hexosamine sugar. While CID and IRMPD did not provide more information to characterize this sugar, the EID spectrum of fOS_{Cc} (Figure 4c) contained a fragment ion at *m/z* 841.3412 that is consistent with the loss of 43.9888 Da from the Z_3 ion (*m/z* 885.3300). This appears to be the loss of a CO_2 (theoretical mass 43.9898), which supports the presence of N-acetyl hexuronic acid (HexNAcA) moiety at this third position. Taken together, the evidence supports a fOS_{Cc} branched heptasaccharide structure with the following sequence, HexNAc-[Hex]-HexNAc-HexNAcA-HexNAc-diNAcBac. The similarity of the *pgl* operon of *C. concisus* with *C. jejuni* (see Suppl. Figure 5) suggests that the fOS_{Cc} would be composed of GalNAc units and a diNAcBac like fOS_{Cj}. The high-resolution MS/MS analysis revealed that the third sugar from the reducing end contains a carboxylic acid moiety like N-acetyl galacturonic acid (GalNacA) previously identified in the LPS O-antigen structure of *Aeromonas salmonicida* 80204-1 strain [30]. In agreement with the previous report,[31] we propose that the more likely structure of fOS_{Cc} is GalNAc-[Glc]-GalNAc-GalNAc-GalNacA-GalNAc-diNAcBac.

Conclusions

In this study, we demonstrate a robust method for fOS purification that additionally provides a convenient signature ion for MS characterization. The method enables the novel use of diverse fragmentation techniques provides a rapid tool for accurately characterizing the individual glycan moieties. While this does not distinguish between isomers, it provides a significant improvement over traditional CID without the need for costly NMR studies. This technique is versatile and should enabled efficient isolation of bacterial periplasmic free oligosaccharides for MS structure determination.

It was reported that free reducing sugars exist in bacterial periplasm perhaps due to the hydrolysis activity of oligosaccharyltransferases, so the structure of the free sugars is expected to reflect the structure of the protein N-linked saccharide [19,7]. Conversely, it might be possible to elucidate the existence of NLG system from certain bacteria of interest, whose NLG pathway have yet to be investigated, and identify their N-glycan structures based on the information of their periplasmic free oligosaccharides. We applied the *Cyhn*-resin to identify free oligosaccharides of *Campylobacter* species. Having demonstrated that free oligosaccharides were selectively detected from the periplasmic extracts of *C. jejuni* and *C. concisus*, this enrichment tool can be further applied to investigate free oligosaccharides from a diverse set of bacterial species in order to profile their periplasmic free oligosaccharides and obtain information about novel NLG pathways.

The *Cyhn*-based enrichment technique, developed in this study, showed highly efficient capture of bacterial free glycans. As the pyridine moiety on the *Cyhn* molecule can be used as a UV-chromophore, the *Cyhn*-conjugation could additionally be developed for quantitative glycomics in combination with conventional HPLC. This enrichment strategy should also be applicable for eukaryotic samples; for example, PNGase F-digest of eukaryotic N-glycans could be used as baits. The versatility of these tools suggests that they can be applied to a broad set of problems in glycobiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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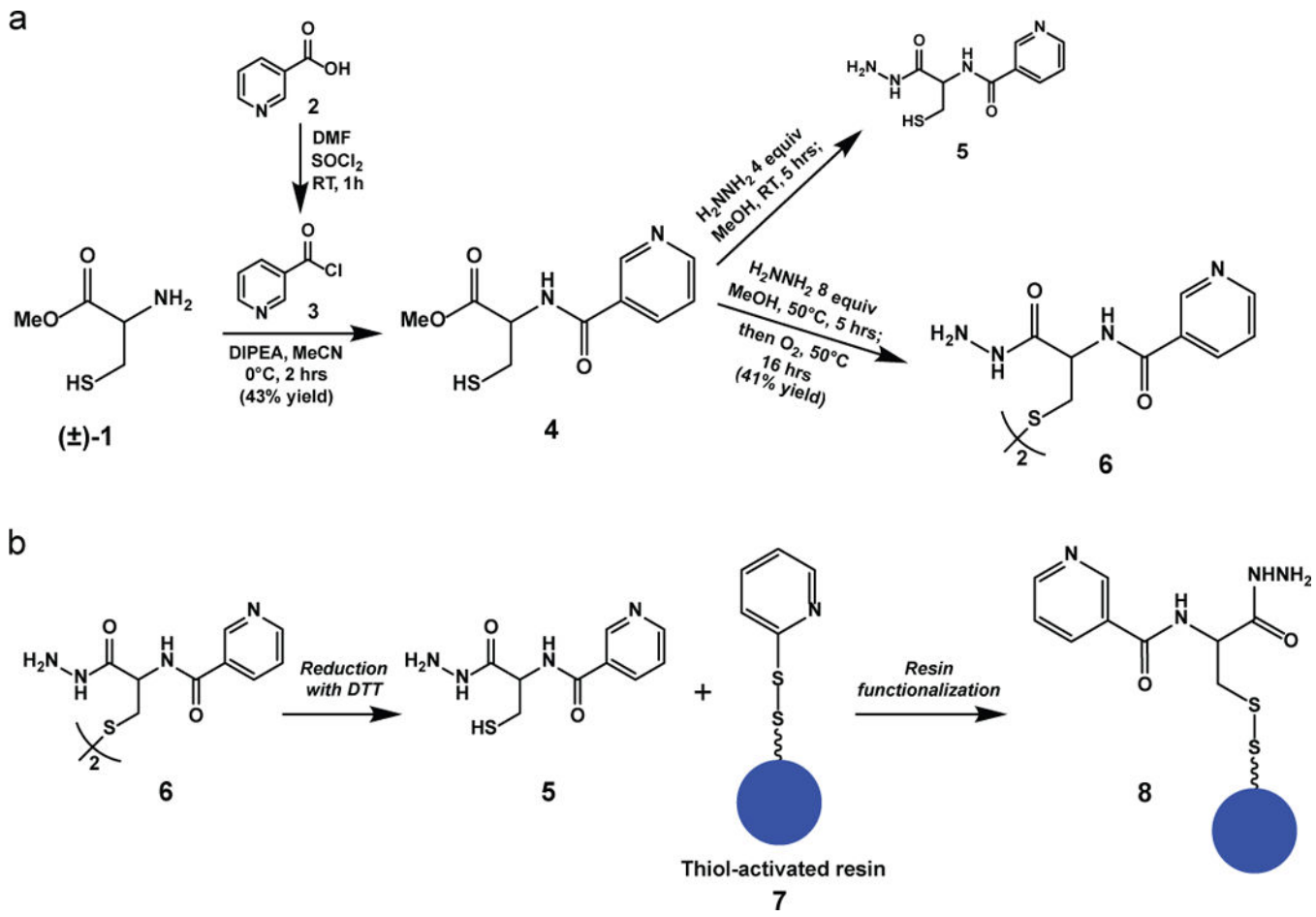

Figure 1.

Overall synthetic route of cationic hydrazide-functionalized resin. (a) Synthesis of cysteine hydrazide nicotinamide and (b) conjugation to thiol-activated resin.

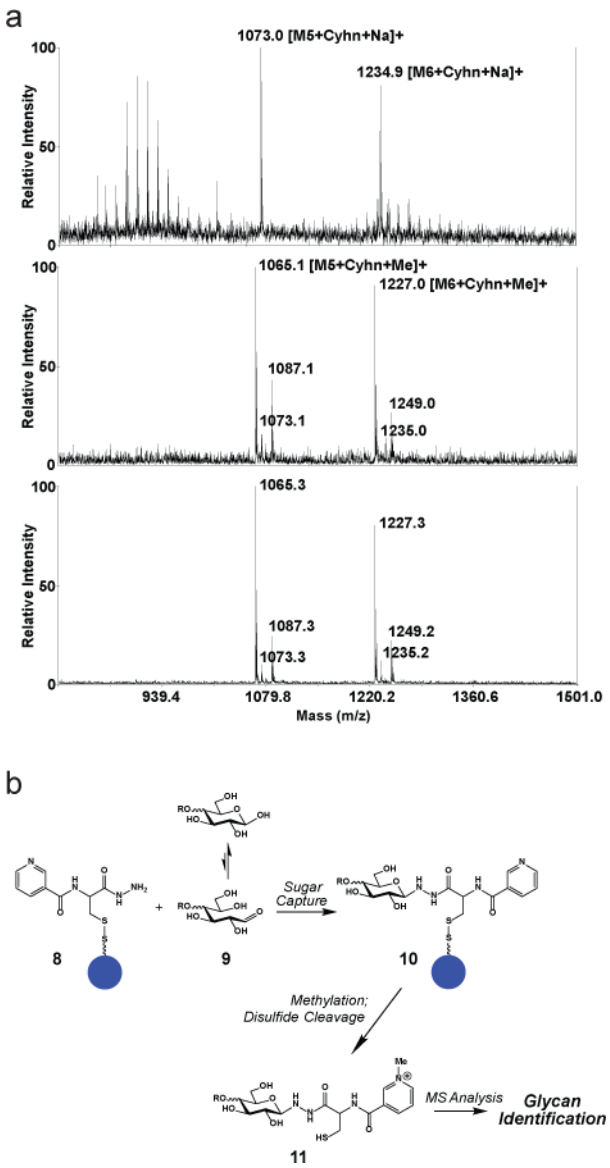

Figure 2.

Post-methylation of *Cyhn*-conjugates. (a) Optimization of the post-methylation condition; maltopentaose (M5) and maltohexaose (M6) enriched *Cyhn* resins were methylated. Upper panel, no methylation; middle panel, methylation with 20% iodomethane (MeI) in acetonitrile (ACN) at 50 °C for 2 h; lower panel, methylation with 20% MeI in ACN at 24°C for 10 h. The signal-to-noise ratio (SNR) of the *Cyhn*-conjugated M5 peaks in the upper, middle and lower panels was 6, 16, and 49, respectively. (b) Strategy for selective enrichment and identification of bacterial glycans.

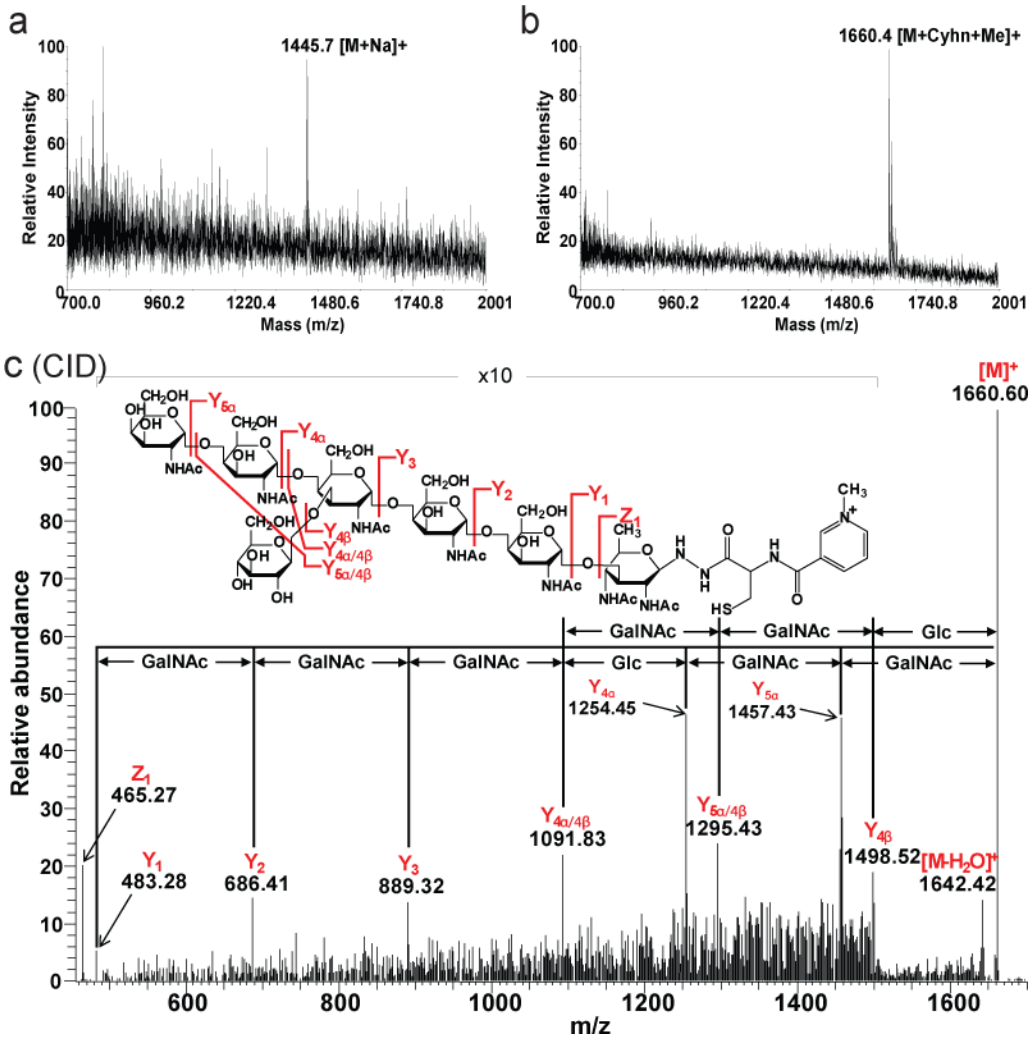

Figure 3.

Identification of bacterial free oligosaccharides (fOS) from periplasmic extracts of *C. jejuni*. MALDI-TOF MS spectra of enriched fOS_{Cj}. The enrichment was performed with periplasmic extracts from *C. jejuni* (a) by solid-phase extraction; (b) by the hydrazide-functionalized resins. The SNR was improved from 4 to 9 upon the methylation. (c) CID MS/MS spectrum of the fOS_{Cj}. These fragment ions were defined according to the nomenclature introduced by Domon and Costello [32]. Briefly, the glycosidic cleavage was designated as Y and Z (for the reducing end) and B and C (for the non-reducing end), and the cross-ring cleavage was designated as X (for reducing end) and A (for non-reducing end).

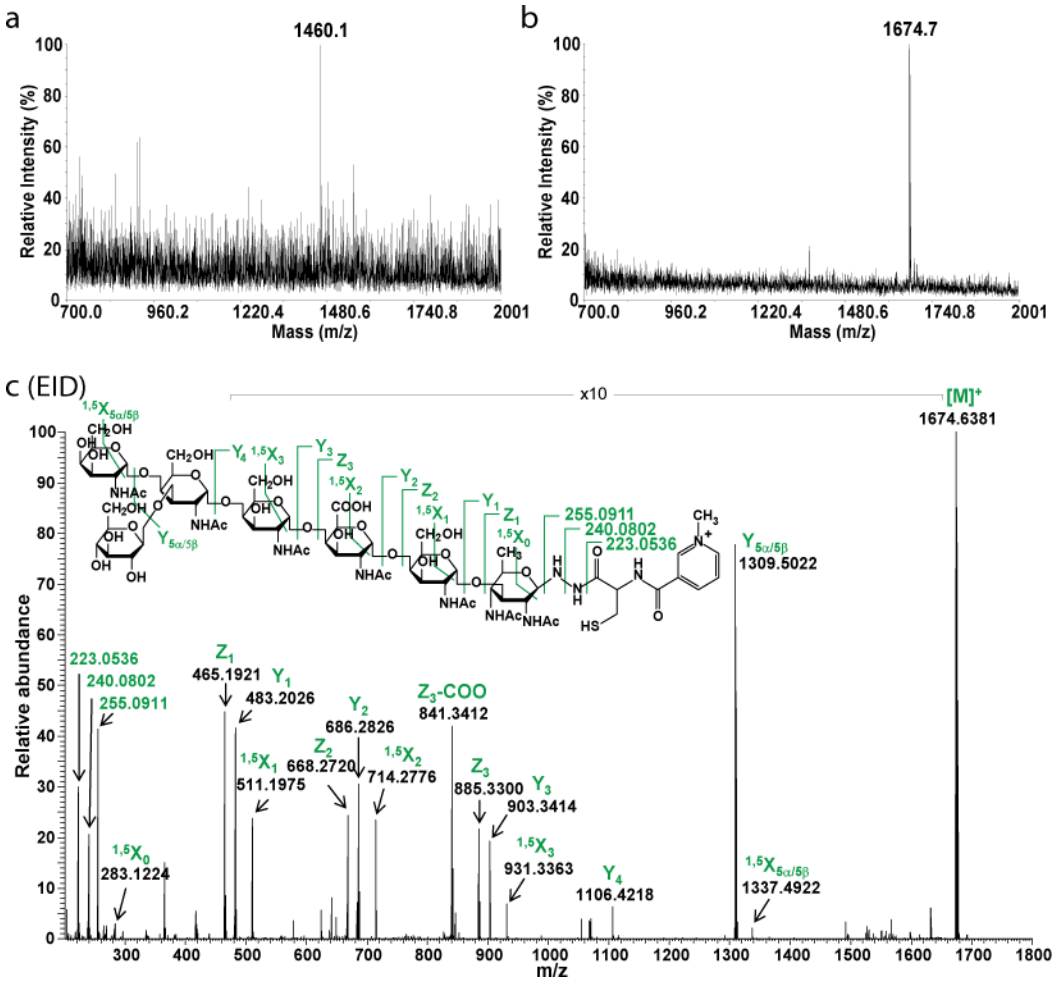

Figure 4.

Identification of fOS_{Cc} from *C. concisus*. MALDI-TOF MS spectra of enriched fOS_{Cc}. (a) The solid-phase extracted sample; (b) the hydrazide-enriched sample. Selected tandem MS spectrum of the fOS_{Cc}. (c) EID (30-ms with a voltage of −20 V) MS/MS spectrum.