Glycoconj J (2013) 30:791–800
DOI 10.1007/s10719-013-9483-9

A HPLC-based glycoanalytical protocol allows the use of natural O-glycans derived from glycoproteins as substrates for glycosidase discovery from microbial culture

Li Liu · Tharmala Tharmalingam · Eva Maischberger · Simone Albrecht · Mary E. Gallagher · Raúl Miranda-CasoLuengo · Wim G. Meijer · Pauline M. Rudd · Jane A. Irwin

Received: 5 February 2013 / Revised: 31 May 2013 / Accepted: 4 June 2013 / Published online: 25 June 2013

Abstract Many disorders are characterised by changes in O-glycosylation, but analysis of O-glycosylation has been limited by the availability of specific endo- and exo-glycosidases. As a result chemical methods are employed. However, these may give rise to glycan degradation, so therefore novel O-glycosidases are needed. Artificial substrates do not always identify every glycosidase activity present in an extract. To overcome this, an HPLC-based protocol for glycosidase identification from microbial culture was developed using natural O-glycans and O-glycosylated glycoproteins (porcine stomach mucin and fetuin) as substrates. O-glycans were released by ammonia-based β-elimination for use as substrates, and the bacterial culture supernatants were subjected to ultrafiltration to separate the proteins from glycans and low molecular size molecules. Two bacterial cultures, the psychrotroph *Arthrobacter* C1-1 and a *Corynebacterium* isolate, were examined as potential sources of novel glycosidases. Arthrobacter C1-1 culture contained a β-galactosidase and *N*-acetyl-β-glucosaminidase when assayed using 4-methylumbelliferyl substrates, but when defucosylated O-glycans from porcine stomach mucin were used as substrate, the extract did not cleave β-linked galactose or *N*-acetylglucosamine. Sialidase activity was identified in *Corynebacterium* culture supernatant, which hydrolysed sialic acid from fetuin glycans. When both culture supernatants were assayed using the glycoproteins as substrate, neither contained endoglycosidase activity. This method may be applied to investigate a microbial or other extract for glycosidase activity, and has potential for scale-up on high-throughput platforms.

Keywords Arthrobacter · Corynebacterium · Glycan analysis · Exoglycosidase · Endoglycosidase · O-glycan

Abbreviations

4-MU	4-methylumbelliferyl
PSM	Porcine stomach mucin
GU	Glucose unit
NP-HPLC	Normal phase HPLC
X-Neu	5-bromo-4-chloro-3-indolyl-α-D-*N*-acetylneuraminic acid sodium salt

Introduction

O-glycosylation, one of the most important post-translational modifications made to many proteins, is initiated by enzymatic addition of a single monosaccharide to serine/threonine *via* a hydroxyl group. Glycosylation initiated with the addition of α-*N*-acetylgalactosamine (GalNAc) is the most abundant form in nature, and is referred to as mucin-like O-glycosylation as this is found in mucin glycoproteins. Aberrant O-glycosylation

L. Liu · T. Tharmalingam · S. Albrecht · P. M. Rudd
Dublin-Oxford Glycobiology Group, National Institute for Bioprocessing Research & Training, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

T. Tharmalingam · E. Maischberger · M. E. Gallagher · J. A. Irwin (✉)
School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland
e-mail: jane.irwin@ucd.ie

R. Miranda-CasoLuengo · W. G. Meijer
School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

Present Address:
L. Liu
Glycomics and Glycan Bioengineering Research Center, Department of Food Science & Technology, Nanjing Agricultural University, Nanjing, China

occurs in many disorders, *e.g.* cancer [1] cystic fibrosis [2] and sialuria [3] Consequently, a detailed knowledge of O-linked glycan structure may be valuable in promoting an understanding of these disorders, or disorders characterised by mucus accumulation, *e.g.* asthma [4].

HPLC-based methods are among the most widely-used technologies for glycan analysis, due to their high sensitivity, capacity, reproducibility and their ability to provide detailed information about monosaccharide sequence and linkage [5]. Unlike N-linked glycans, which are removed by well-characterised peptide N-glycanases [6] the study of O-linked glycans using HPLC-based technology has been hampered by the lack of endo- and exo-glycosidases specific to O-linked glycans. The currently available endo-α-*N*-acetylgalactosaminases that hydrolyze O-linked glycans from proteins have a very narrow substrate spectrum, only hydrolysing core 1 glycan structures, or other core structures to a limited extent [7–11]. One company (New England Biolabs: www.neb.com) provides an O-glycosidase from *Enterococcus faecalis* [10] that hydrolyses core 1 and core 3 structures only. Comprehensive O-glycan release now relies on chemical methods, particularly hydrazinolysis [12] reductive β-elimination with sodium hydroxide and sodium borohydride [12], and non-reductive methods using ammonium hydroxide saturated with ammonium carbonate [13].

O-linked glycans that are labile to chemical reaction conditions normally experience further degradation after release (referred to as "peeling") [14] which complicates the final structural analysis. The removal of calcium, and possibly other cations, limits this process, but does not entirely eliminate it [15]. After glycan release, sequential digestion by a suite of exoglycosidases allows sequence mapping and linkage determination of the glycan structures. Despite their successful application to N-linked glycan studies, commercially available exoglycosidases may fail to completely digest certain O-linked glycans [16] as the monosaccharides differ in their sequence and linkage to those of N-linked glycans. The availability of a generic O-glycosidase that could non-specifically cleave O-linked glycans from glycoproteins, and exoglycosidases that are specific to unusual O-linked glycan structures, would thus facilitate the development of O-glycan study methodology.

One approach for identifying glycosidase activity is to use artificial substrates. These are labelled with a chromogenic/fluorogenic group, *e.g.* p-nitrophenyl, 4-methylumbelliferyl (4-MU) or 5-bromo-4-chloro-3-hydroxyindole (X-), which is released by enzymatic activity. However, not all the natural glycans containing the same terminal sugar can be digested by the enzymes that are active against these substrates. There are differences in structure and linkage between the artificial substrates and natural glycans, and some enzymes active against a particular terminal sugar may not hydrolyse all natural glycans bearing this sugar [17]. As a result, natural glycans and glycoproteins with known structures are essential for identifying all the glycosidase activities present in a biological extract.

In this study, the substrate specificities of enzymes were investigated by comparing their activities against 4-MU-labelled sugars with those against chemically released natural O-glycans containing the same terminal sugar. An HPLC-based protocol for glycosidase identification from microbial culture, without requirement for preliminary enzyme purification, was developed using natural glycans and glycoproteins as substrates. This was applied to culture supernatants from a psychrotrophic *Arthrobacter* sp. and a *Corynebacterium* isolate. The results show that this method is sensitive and reliable, and a sialic acid-degrading enzyme identified through this method from *Corynebacterium* was active against a natural target substrate.

Materials and methods

Materials

The O-linked glycoproteins foetal calf fetuin and porcine stomach mucin (PSM), 4-methylumbelliferone-linked (4-MU) monosaccharides, 5-bromo-4-chloro-3-indolyl-α-D-*N*-acetyl-neuraminic acid sodium salt (X-Neu), formic acid and 28 % ammonium hydroxide solution were purchased from Sigma-Aldrich (Tallaght, Dublin 24, Ireland). A fluorescent glycan labelling kit LudgerTag™ 2-AB (2-aminobenzamide) was purchased from Ludger Ltd (The Oxford BioBusiness Center, Oxford, UK). Amicon centrifugal filters (10 kDa molecular mass cutoff) were purchased from Millipore (Tullagreen, Carrigtwohill, Cork, Ireland).

Bacterial culture

Arthrobacter C1-1, a psychrotrophic bacterium [18] was obtained by courtesy of Dr. Radka Šaldová and was cultured in Brain Heart Infusion broth (BHI: Oxoid, Basingstoke, Hampshire, UK) at 15 °C for 2 days with shaking. The *Corynebacterium* sp. used in this study was obtained from a collection of bacterial isolates from the equine female reproductive tract, as part of an ongoing study into post-breeding endometritis [19] in mares. Sialidase activity was identified by adding 0.5 mM X-Neu into bacterial cultures (200 μl BHI). After overnight incubation at 37 °C, sialidase activity was qualitatively recorded by the appearance of a blue-green precipitate due to removal of the 5-bromo-4-chloro-3-indolyl moiety of the substrate.

Four hundred and sixty five bacterial colonies were screened for their ability to produce sialidase using X-Neu, and of these, 33 produced sialidase. The sialidase-producing bacterium was identified as *Corynebacterium* sp. by PCR amplification, sequencing and phylogenetic analysis of the

16S rNA gene. The primers used for amplification of bacterial 16S ribosomal RNA (16S rRNA) genes were 63F primer (5′-CAGGCCTAACACATGCAAGTC-3) and 1389R primer (5′-ACGGGCGGTGTGTACAAG-3). The phylogenetic affiliation of the isolate was determined by comparison with sequences in public databases using the Basic Local Alignment Search Tool (BLAST) algorithm [20] and the Ribosomal Database Project [21]. *Corynebacterium* sp. was stored in cryopreservative vials at −80 °C and grown on blood agar plates incubated at 37 °C for 2 days. Bacteria were inoculated into brain heart infusion (BHI) broth (200 μl) and incubated overnight at 37 °C in an orbital shaker. The bacterial culture was then transferred to a 20 ml sterile container containing fresh BHI broth and incubated for 12 h as described above, then transferred to 200 ml of fresh BHI broth in an Erlenmeyer flask and incubated overnight at 37 °C with shaking. Inoculation of 6 Erlenmeyer flasks yielded enough bacterial supernatant for further studies.

Normal phase (NP)-HPLC

A TSK-Gel Amide-80 4.6×250 mm column (Anachem, Luton, UK) mounted on a 2695 Alliance separation module equipped with a Waters temperature control module and a Waters fluorescent detector 2475 (Waters, Milford, MA, USA) were used for glycan analysis. A Waters Empower chromatography workstation software build 1154 was used for system control and data processing. The conditions for HPLC and profile analysis were the same as described previously [16].

Glycan release and enzyme assay

4-methylumbelliferyl (4-MU) substrates were used to screen for glycosidase activity: 4-MU β-D-galactoside, 4-MU α-D-galactoside, 4-MU *N*-acetyl-β -D-glucosaminide, 4-MU β-D-glucoside, 4-MU α-L-fucoside, 4-MU α-D-mannoside and 2′-(4-MU)-α-D-*N*-acetylneuraminic acid sodium dihydrate. The substrates were made up to 20 mM in dimethylsulphoxide and diluted to 0.25 mM in 0.1 M sodium citrate at pH 4.5, 6.5 and 8, to determine activity over this pH range. The assay was carried out as described by [22]. O-linked glycans from 50 μg fetuin were released by ammonia-based β-elimination as described by [13]. The dried released glycan sample was treated with 20 μl of 1 % (w/v) formic acid at room temperature for 40 min. The acid was subsequently evaporated to dryness in a speedvac, thereby removing the formic acid and preventing hydrolysis of sialic acid. 2-AB labelling of glycans was carried out as recommended by the manufacturer (Ludger Ltd). Samples were incubated at 65 °C for 2 h, and the excess 2-AB was removed by chromatography on Whatman 3MM chromatography paper. Glycans were eluted with water, concentrated to dryness using a speedvac, and re-suspended in 20 μl of water. The released glycans were subjected to normal phase-HPLC (NP-HPLC) for structural analysis [16].

Released and labelled glycans were analysed by NP-HPLC, and digestions using panels of exoglycosidases available for glycan analysis were performed to sequence the glycans. The enzymes employed in this study were as follows: *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18, ABS), 1–2 U/ml; bovine kidney α-fucosidase (EC 3.2.1.51, BKF), 1 U/ml; bovine testis β-galactosidase (EC 3.2.1.23, BTG), 2 U/ml; *Streptococcus pneumoniae* β-*N*-acetylglucosaminidase (recombinant in *Escherichia coli;* EC 3.2.1.30, GUH), 8 U/ml. These enzymes were purchased from Glyko (Novato, CA, USA), *Streptococcus pneumoniae* O-glycanase was purchased from Prozyme (available from Europa Bioproducts, Cambridge, U.K) and *Clostridium perfringens* sialidase was purchased from Sigma-Aldrich. The glycans were digested in 10 μl of 50 mM sodium acetate buffer, pH 5.5 for 18 h at 37 °C. The enzymes were removed by filtration using a protein-binding EZ filter (Millipore). Glycans were analyzed on NP-HPLC after digestion.

Bacterial cultures (*Arthrobacter* C1-1 or *Corynebacterium* sp.) were centrifuged at 4,600×*g* for 20 min and the supernatants were retained for enzyme assays using HPLC. Small molecules, including glycans and monosaccharides, were removed by ultrafiltration 3 times on a 10 kDa Amicon centrifugal filter, washing each filter with 2 ml of 50 mM sodium phosphate buffer, pH 7, on each occasion. The retentate, which contained enzyme activity, was re-suspended in 2 ml of the same buffer. In addition to the ultrafiltered culture supernatant (retentate), the same volume of sample was also taken from the BHI broth medium, untreated culture supernatant, and the filtrate containing low molecular weight compounds for glycan analysis on HPLC, for comparison. Water was used as a negative control and chemically released O-linked glycans were added to the retentate as a positive control. All glycan analyses using HPLC were carried out as described in the O-glycan release section, after labelling with 2-AB.

Exoglycosidase detection

Chemically released and 2AB-labelled O-linked glycans from the O-glycosylated proteins fetuin and PSM were added to the ultrafiltered bacterial culture supernatant and incubated overnight at 37 °C. The sample was then subjected to ultrafiltration to remove all the enzymes/proteins from which glycans had been released, and was analyzed by NP-HPLC. The HPLC profiles prior to and post digestion were compared. A shift of any peak after incubation indicated the presence of an exoglycosidase capable of digesting the corresponding terminal monosaccharide in the glycan structure. The culture to which glycans were added without incubation was used as the control.

Endoglycosidase digestion

To test bacterial cultures for endoglycosidase activity, 50 μg of fetuin was incubated with 100 μl *Corynebacterium* sp. culture supernatant (pH 7.0) or *Arthrobacter* C1-1 culture supernatant for 16 h at 37 °C. The protein was removed from the sample by ultrafiltration with Millipore centrifugal filters (10 kDa), to concentrate the sample. The glycans were present in the flow-through fraction. The filtrate was dried under vacuum and incubated with 20 μl of 1 % (w/v) formic acid. 2-AB (5 μl, made up as per manufacturer's instructions) was added to label the glycans and the mixture was incubated at 65 °C for 2 h. The excess 2-AB was removed by chromatography as described previously, and the glycans were eluted with water, dried by speedvac, and reconstituted with water as described for exoglycosidase digestion.

A control experiment was also performed in which exogenous O-glycosidase activity was added to a bacterial culture supernatant to confirm that the method could detect endoglycosidase activity present in the supernatant. Fetuin (50 μg) was incubated with ultrafiltered supernatant from *Corynebacterium* sp. (concentrated 10-fold and washed with 50 mM sodium acetate buffer, pH 5.5) to which 5 mU of *S. pneumoniae* O-glycanase was added. As the endogenous sialidase activity in the particular *Corynebacterium* sp. preparation used in this experiment was considerably lower than normal, 10 mU of *Clostridium perfringens* sialidase was added to 600 μl of *Corynebacterium* sp. culture supernatant to ensure desialylation of fetuin glycans. Parallel incubations of fetuin plus O-glycanase, and fetuin, O-glycanase plus 5 mU of ABS were also set up at pH 5.5. The glycans were labelled with 2-AB as described above and analysed using NP-HPLC.

Results and discussion

Assays with 4-MU substrates showed that the supernatant from the *Arthrobacter* C1-1 culture was active against 4-MU-β-D-

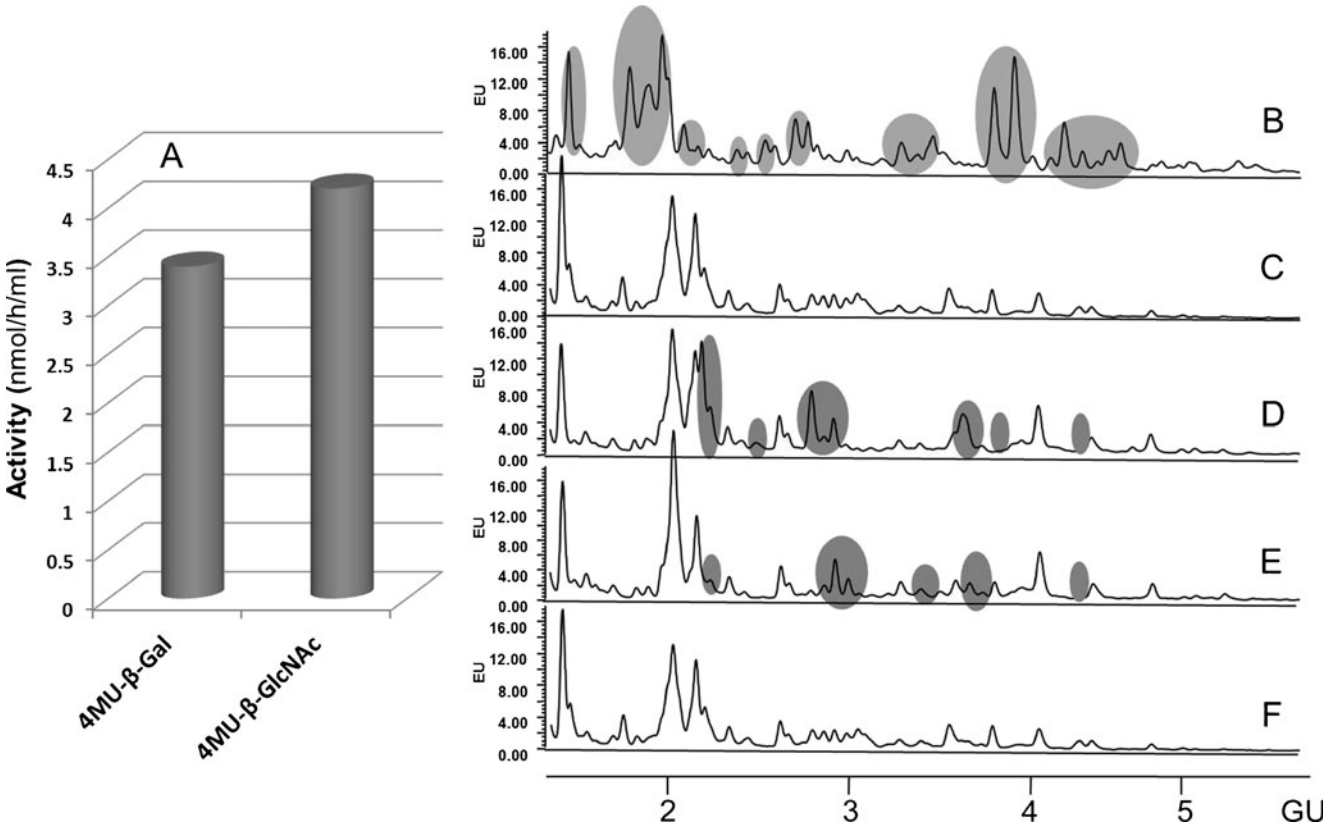


Fig. 1 A comparison of exoglycosidase activity of *Arthrobacter* C1-1 culture towards 4-MU substrates and porcine stomach mucin (PSM)-derived glycans. The structures of PSM-derived glycans were not determined due to the complexity. **a** Activity against 4-MU-β-D-galactoside and 4-MU-*N*-acetyl-β-D-glucosaminide, expressed as nmol/h/ml. **b** HPLC profile of native PSM-derived O-linked glycans. The peaks highlighted are the peaks shifted after experiencing bovine kidney fucosidase (BKF: acts on α 1-(6/2) linkages) digestion (**c**) HPLC profile of PSM-derived O-linked glycans after digestion with BKF. **d** Profile of BKF digested PSM-derived O-linked glycans after digestion by bovine testes β-galactosidase (BTG: acts on β 1-(3/4/6) linkages). The areas highlighted indicated the difference compared to C. **e** Profile of BKF-digested PSM-derived O-linked glycans after digestion by *S. pneumoniae* β-*N*-acetylglucosaminidase (GUH acts on ß1-(2/4/5) GlcNAc linkages). The areas highlighted indicated the difference compared to C. **f** Profile of BKF-digested PSM-derived O-linked glycans after digestion by *Arthrobacter* C1-1 ultrafiltered culture supernatant. No profile difference was observed compared to C. GU denotes glucose units

galactoside and 4-MU-*N*-acetyl-β-D-glucosaminide (Fig. 1a), but there was no activity against either 4-MU-α-L-fucoside or 4-MU-α-D-*N*-acetylneuraminic acid. The exoglycosidase activity of the bacterial supernatant was subsequently tested using natural O-linked glycans released from PSM as substrates, as shown in Fig. 1b. PSM was selected as a substrate, as it contains glycans containing galactose and *N*-acetylglucosamine shielded by a terminal fucose residue [23]. BKF was used to remove the terminal fucose to expose galactose and *N*-acetylglucosamine (Fig. 1c). The profile changes of native glycans after the BKF digestion are highlighted on Fig. 1c–e. Structures of the glycans were not shown in detail, as the peak shifts on HPLC show the differences between treatment of the glycans with different glycosidases. The presence of terminal galactose and *N*-acetylglucosamine in BKF-digested PSM-derived O-glycans was confirmed by the peak shift after digestions with BTG and GUH, respectively (Fig. 1d and e). After digestion with BTG, a split peak appears at approx 2.2 GU, with other peaks appearing at 2.8, 2.9, 3.6 and small peaks at 4.4 and 4.8 GU, and some of the peaks presented in Fig. 1c disappeared in Fig. 1d, in comparison with the profile in Fig. 1c. Digestion with GUH led to appearance of the peak at 2.9 GU, accompanied by a small peak at 3.0 GU and small peaks at 3.5, 3.7 and 4.4 GU, approximately. BKF-digested PSM-derived O-glycans were used as the substrates for digestion by the *Arthrobacter* C1-1 culture. However, when this was used, no peak shift was apparent on comparison of Fig. 1c and f, indicating that the two glycosidases active against the artificial fluorimetric substrates failed to digest the natural O-linked glycans containing the same terminal sugars. This suggests that the enzymes in this extract that degrade the artificial substrates may not be active against natural glycans. The use of artificial substrates is thus not reliable for identifying all glycosidases present in a microbial extract, and this process must be validated by testing culture media or bacterial extracts against natural glycans with known linkages. Care must also be taken that the substrate used for screening is sufficiently specific to detect only the enzymes of interest. One study used skimmed milk as a substrate for protease screening. However, as some milk proteins were glycosylated, the substrate was cleaved by glycosidases as well as proteases, as shown by hydrolysis of X-gal and other X-linked sugars by cultures containing proteases [24]. In

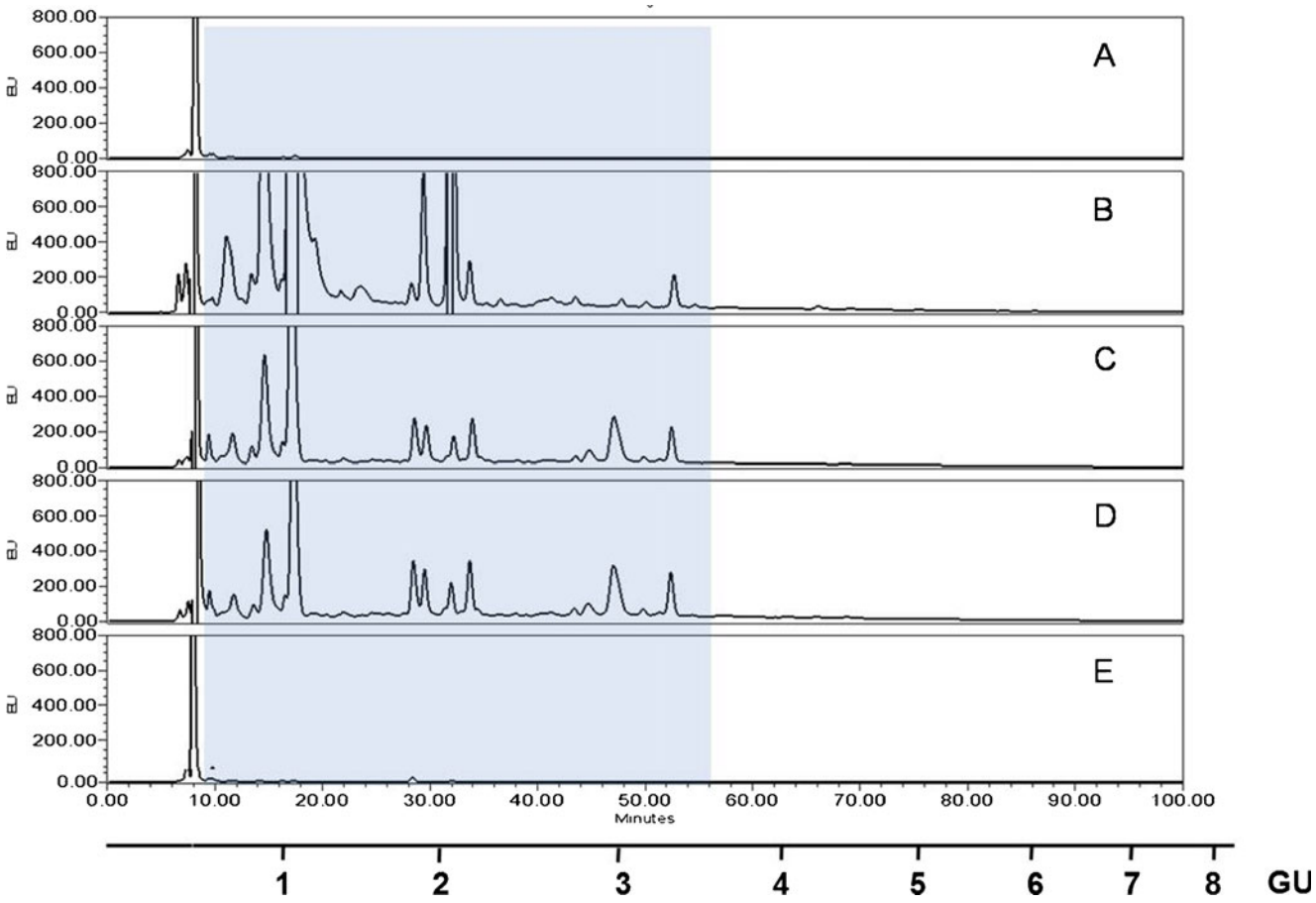


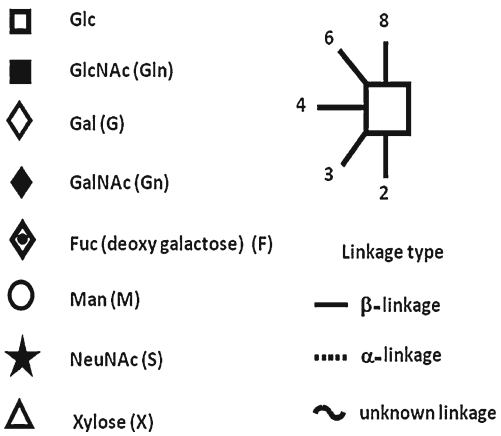
Fig. 2 A comparison of glycan profiles from BHI and the *Arthrobacter* C1-1 culture medium (grown in BHI) after different treatments, as illustrated using NP-HPLC. The peaks within the shadow (9–56 min elution time) represent the glycans present. **a** Profile of water blank with 2-AB label. **b** Profile of BHI broth glycans. **c** Profile of glycans from *Arthrobacter* C1-1 supernatant. **d** Profile of filtrate after ultrafiltration on Centricon filters (Mr cutoff 10 kDa). **e** Profile of retentate after ultrafiltration. GU denotes glucose units

another study, an α-fucosidase was found to be active against α1-3- and α1-4-linked fucose in natural glycans, but not against pNP-fucose [17]. The latter study showed that not all exoglycosidases are active towards an artificial substrate, confirming the need for a technology directly using natural glycans or glycoproteins as the substrates.

Two major requirements for the successful application of this protocol emerged during this study. Firstly, the method must be sensitive enough to detect any sugars present in microbial cultures. Secondly, any glycans or monosaccharides present in the culture under investigation must be removed efficiently to allow unambiguous detection of sugars released from glycan substrates. *Arthrobacter* C1-1 culture supernatant was used as an enzyme source to test the method (Fig. 2). The single major peak in the water blank (Fig. 2a) consisted only of the 2AB label. More peaks were present in the HPLC profile of the fresh BHI broth sample, comprising the glycans originally present in the BHI medium (Fig. 2b). The glycan profile of the culture supernatant was different to that of the fresh BHI broth, as shown by changes in peaks at 12 min and 28–35 min, likely to be due to the consumption of the glycans present in the medium and the production of new glycans and monosaccharides by the bacteria (Fig. 2c). The glycan profile of the filtrate was similar to that of the culture supernatant in Fig. 2c (Fig. 2d), while no major peak apart from 2-AB was observed in the profile of the retentate (Fig. 2e), since most of the sugars were in the filtrate due to their small size. Both the medium itself and the culture supernatant contained significant quantities of glycans, accounting for the dominant background of the HPLC profile, which could mask the target glycans during analysis. However, these were removed efficiently from the culture by ultrafiltration, thereby facilitating glycan analysis, while any proteins/enzymes in the culture remained in the retentate. An ultrafiltered culture supernatant to which chemically released O-linked glycans were added gave rise to an

Table 1 Structure of O-linked glycans released from fetuin

HPLC peak	Structure	Structure	GU value
a	Neu5Ac α 2-3 Gal---2AB		2.24
b	Neu5Ac α 2-3 Gal β1-3 GalNAc---2AB		2.94
c	Neu5Ac α 2-6 (Gal β1-3) GalNAc---2AB		3.25
d	Neu5Ac α 2-6 (Neu5Ac α 2-3 Gal β1-3) GalNAc---2AB		4.35
e	Neu5Ac α 2-3 Gal β1-4 GlcNAc β1-6 (Neu5Ac α 2-3 Gal β1-3) GalNAc---2AB		5.33



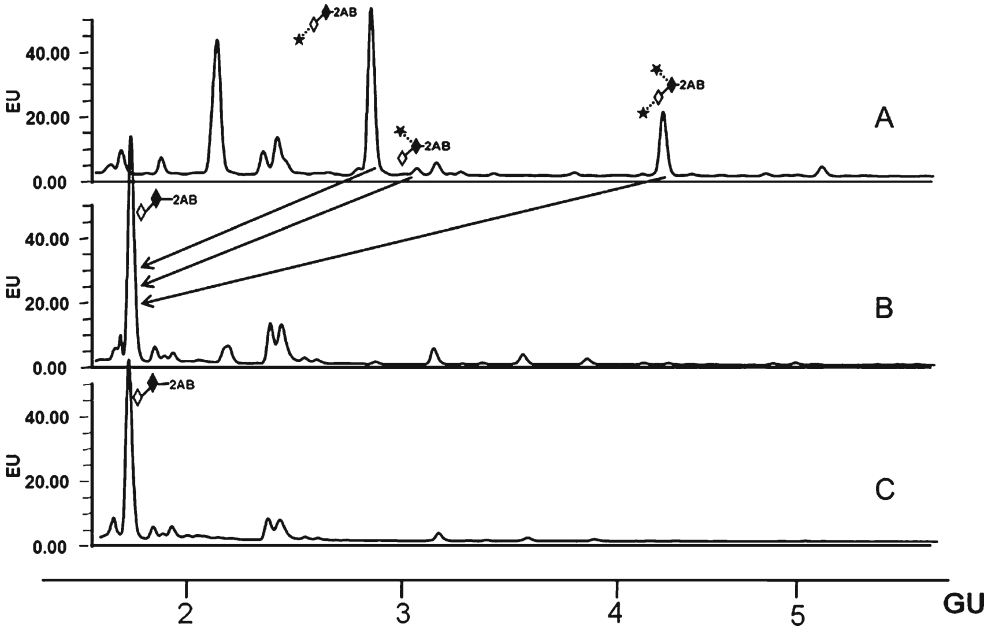


Fig. 3 A comparison of the digestion of fetuin O-glycans by *A. ureafaciens* sialidase (ABS: acts on α2-(3/6/8) linkages) and *Corynebacterium* sp. culture. **a** Profile of undigested fetuin O-glycans. These contain 3 sialic acid-containing core 1 structures. **b** Profile of fetuin O-linked glycans after ABS digestion. **c** Profile of fetuin O-linked glycans after digestion by the ultrafiltered *Corynebacterium sp.* culture. Symbols for glycan structures are shown beneath the Figure. GU denotes glucose units

HPLC profile identical to that of the O-linked glycans alone (data not shown). This indicated that the ultrafiltration method was efficient at removing the sugars present in the microbial culture, and the HPLC method is sensitive enough to monitor any change in the sugars and glycans in the sample.

The *Corynebacterium* culture was active against 2′- 4-MU α-D-*N*-acetylneuraminic acid, but as this displayed high backgrounds due to spontaneous 4-MU hydrolysis, p-nitrophenyl α-D-*N*-acetylneuraminic acid was used as substrate. The activity of the culture supernatant measured with this substrate was low, at 0.1 μmol/h/mg. *Corynebacterium* sp. supernatant after ultrafiltration was tested on 4-MU substrates and contained activity against α-fucosidase, β-*N*-glucosaminidase and β-galactosidase, which were optimal at pH 6.5. Fucosidase had no activity at pH 4.5 and 8, while β-*N*-acetylglucosaminidase activities at pH 4.5 and 8 were 23 and 34 %, respectively, of those at pH 6.5. β-galactosidase was not active at pH 4.5, and at pH 8 had only 4 % of the activity measured at pH 6.5. Activities of α-galactosidase and α-mannosidase were negligible.

Fetuin contains 5 sialylated O-linked glycans, listed in Table 1 (Fig. 3a) [16], which makes it a suitable substrate for sialidase discovery. ABS digestion confirmed the presence of sialic acid in all 5 glycans. The 3 sialylated core 1 structures were converted to core 1, and the Neu5Acα2-3Gal peak at 2.24 GU disappeared (Fig. 3b). Digestion by the *Corynebacterium* culture also resulted in a shift of all 5 peaks (Fig. 3c), giving rise to a profile similar to that in Fig. 3b. The peak shift indicated that the *Corynebacterium* culture contained one or more sialidases active against natural glycans with terminal sialic acid. The sialidase in the *Corynebacterium* sp. culture appeared to be capable of removing both α(2-3) and α(2-6)-linkages, as the profile after culture digestion was similar to

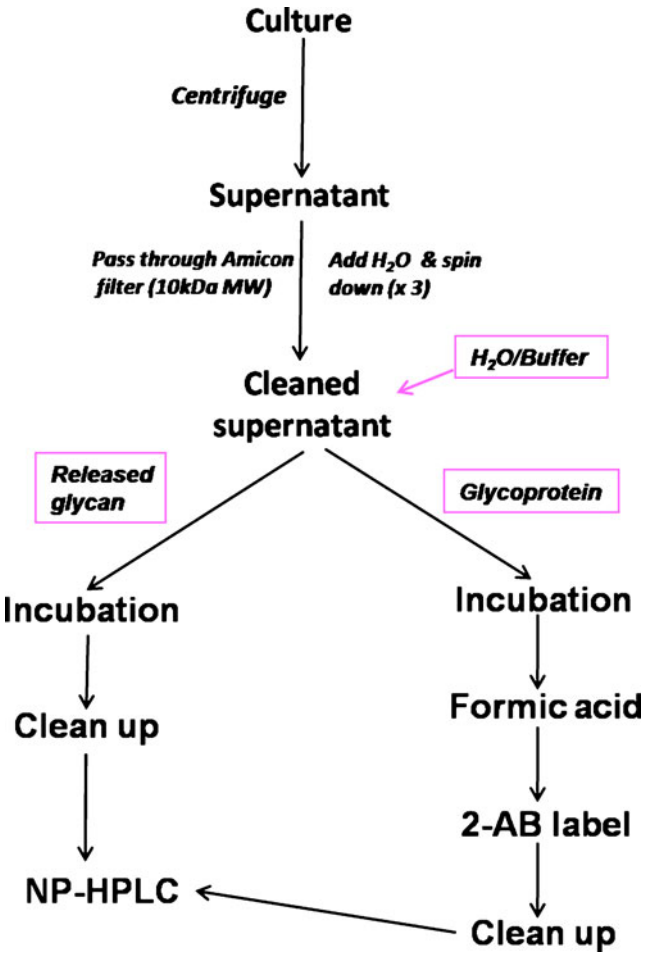


Fig. 4 A schematic diagram of the strategy for endo- and exo- glycosidase discovery, using HPLC with natural glycoproteins and released glycans, respectively, as substrates

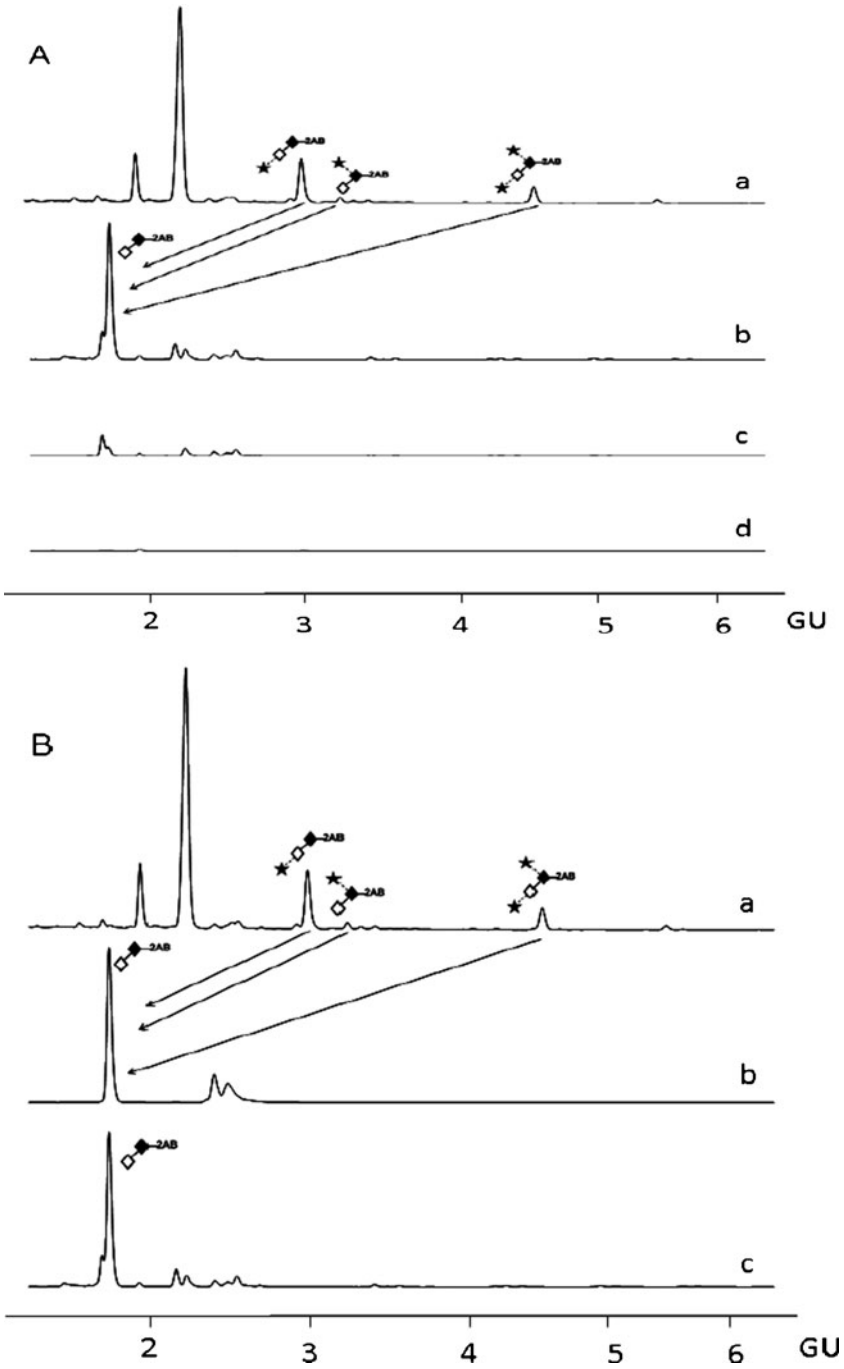


Fig. 5 Digestion of fetuin O-glycans by *Corynebacterium* sp. culture to which *S. pneumoniae* O-glycanase was added. Panel **A** displays profiles of the following: (*a*) Chemically released fetuin glycans. (*b*) Glycans from fetuin incubated with O-glycanase and *Corynebacterium* sp. culture. (*c*) Glycans from fetuin incubated with *Corynebacterium* sp. culture (*d*) Glycans from fetuin incubated with O-glycanase only. Panel **B** shows (*a*) Chemically released fetuin glycans. (*b*) Glycans from fetuin incubated with O-glycanase and *A. ureafaciens* sialidase (ABS) (*c*) Glycans from fetuin incubated with O-glycanase and *Corynebacterium* sp. culture. GU denotes glucose units

the profile after digestion with ABS, which removes both α-(2-3) and α(2-6)-linked sialic acid.

There are few, if any, suitable artificial substrates commercially available to probe cultures for endoglycosidase activity. We tested for endoglycosidase activity using fetuin and PSM as substrates, using the glycoprotein as substrate, rather than their released glycans (see Fig. 4 for schematic diagram of the method). We found no endoglycosidase active against any of the O-glycans attached to these two proteins in either the *Arthrobacter* C1-1 or *Corynebacterium* sp. cultures. However, to confirm that this method could detect an O-glycosidase present in a bacterial culture, we performed a control experiment in which fetuin was digested with ultrafiltered sialidase-containing *Corynebacterium* sp. culture supernatant to which commercially available *S. pneumoniae* O-glycanase had been added. The results of this experiment are depicted in Fig. 5. Figure 5a showed that when a mixture of culture supernatant and O-glycanase was added to fetuin and incubated overnight, the profile of the fetuin glycans shifted to give one predominant peak corresponding to core 1, which can only be released in its non-sialylated form from the protein backbone by the *S. pneumoniae* O-glycanase. When a similar experiment was

carried out using the *A. ureafaciens* sialidase ABS, instead of *Corynebacterium* sp. supernatant (Fig. 5b), a similar profile was obtained. Incubation of fetuin with *Corynebacterium* sp. supernatant or O-glycanase separately did not lead to the release of significant quantities of core 1 glycan.

In the extreme (albeit unlikely) case that cultures contain all the glycosidases capable of completely digesting the glycans, leaving nothing but monosaccharides, this method is not applicable, as the glycans attached to protein may also be sequentially digested by exoglycosidases without initial cleavage from the protein. This overall strategy could be applied to other microbial extracts. Synthetic O-linked glycans, and glycoproteins with structures identical to those of natural glycans, are preferable for identification of enzymes with particular substrate specificities. The synthesis of these structures has been studied using enzymatic, chemical or enzyme-chemical methods [25, 26] and their availability may facilitate future glycosidase discovery.

HPLC-based technology was initially developed for glycan structure analysis with the assistance of sequential digestion by selected exoglycosidases [5]. This methodology can also been applied to identify mucinase activities of bacterial cultures [27]. In addition to being robust, sensitive, reproducible and precise, HPLC-based technology has the following advantages. Firstly, use of the HPLC profile for monitoring the changes of glycan structures allows identification of potential enzyme activities and a direct comparison of the experimental result to that obtained using commercially available enzymes. Secondly, this method could be transferred to a high-throughput platform [28] to allow large-scale studies. In addition, the HPLC profile allows discrimination between the enzyme activity and lectins present in the culture. Enzyme digestion results in the shift of the target peak, while lectin binding results in the loss of the target peak without any shift as the glycan structure binding to the lectin is not detected by HPLC. This strategy shows promise for application for exo- and endo-glycosidase discovery, as it may be coupled with many technologies in other biological areas such as biosynthesis, metagenomics and robotics to facilitate fast, flexible and large-scale screening of many sources for glycosyl hydrolase activities.

Acknowledgments The authors thank The National Institute for Bioprocessing Research and Training (NIBRT) for funding for this project. Eva Maischberger and Raúl Miranda CasoLuengo were funded by a grant from the Irish Department of Agriculture, Fisheries and Food Stimulus Fund (RSF 06 408).

References

1. Storr, S.J., Royle, L., Chapman, C.J., Hamid, U.M., Robertson, J.F., Murray, A., Dwek, R.A., Rudd, P.M.: The O-linked glycosylation of secretory/shed MUC1 from an advanced breast cancer patient's serum. Glycobiology **18**, 456–462 (2008)
2. Xia, B., Royall, J.A., Damera, G., Sachdev, G.P., Cummings, R.D.: Altered O-glycosylation and sulfation of airway mucins associated with cystic fibrosis. Glycobiology **15**, 747–775 (2005)
3. Wopereis, S., Abd Hamid, U.M., Critchley, A., Royle, L., Dwek, R.A., Morava, E., Leroy, J.G., Wilcken, B., Lagerwerf, A.J., Huijben, K.M., Lefeber, D.J., Rudd, P.M.: Abnormal glycosylation with hypersialylated O-glycans in patients with Sialuria. Biochim. Biophys. Acta **1762**, 598–607 (2006)
4. Tarp, M.A., Clausen, H.: Mucin-type O-glycosylation and its potential use in drug and vaccine development. Biochim. Biophys. Acta **1780**, 546–563 (2008)
5. Liu, L., Telford, J.E., Knezevic, A., Rudd, P.M.: High-throughput glycoanalytical technology for systems glycobiology. Biochem. Soc. Trans. **38**, 1374–1377 (2010)
6. Royle, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M.: Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. Methods Mol. Biol. **347**, 125–144 (2006)
7. Brooks, M.M., Savage, A.W.: The substrate specificity of the enzyme endo-alpha-N-acetyl-D-galactosaminidase from *Diplococcus pneumonia*. Glycoconj. J. **14**, 183–190 (1997)
8. Fan, J.Q., Yamamoto, K., Matsumoto, Y., Hirabayashi, Y., Kumagai, H., Tochikura, T.: Action of endo-alpha-N-acetylgalactosaminidase from *Alcaligenes* sp. on amino acid O-glycans: comparison with the enzyme from *Diplococcus pneumoniae*. Biochem. Biophys. Res. Commun. **169**, 751–757 (1990)
9. Ashida, H., Yamamoto, K., Kumagai, H.: Trypsin inhibitory activity of bovine fetuin de-O-glycosylated by endo-alpha-N-acetylgalactosaminidase. Biosci. Biotechnol. Biochem. **64**, 2266–2268 (2000)
10. Koutsioulis, D., Landry, D., Guthrie, E.P.: Novel endo-alpha-N-acetylgalactosaminidases with broader substrate specificity. Glycobiology **18**, 799–805 (2008)
11. Goda, H.M., Ushiqusa, K., Ito, H., Okino, N., Narimatsu, H., Ito, M.: Molecular cloning, expression and characterization of a novel endo-alpha-N-acetylgalactosaminidase from *Enterococcus faecalis*. Biochem. Biophys. Res. Commun. **375**, 441–446 (2008)
12. Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., Parekh, R.: Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. Biochemistry **32**, 679–693 (1993)
13. Huang, Y., Mechref, Y., Novotny, M.V.: Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis. Anal. Chem. **73**, 6063–6069 (2001)
14. Kanamori, A., Inoue, S., Iwasaki, M., Kitajima, K., Kawai, G., Yokoyama, S., Inoue, Y.: Deaminated neuraminic acid-rich glycoprotein of rainbow trout egg vitelline envelope. Occurrence of a novel alpha-2,8-linked oligo (deaminated neuraminic acid) structure in O-linked glycan chains. J. Biol. Chem. **265**, 21811–21819 (1990)
15. Kozak, R.P., Royle, L., Gardner, R.A., Fernandes, D.L., Wuhrer, M.: Suppression of peeling during the release of O-glycans by hydrazinolysis. Anal. Biochem. **423**, 119–128 (2012)
16. Royle, L., Mattu, T.S., Hart, E., Langridge, J.I., Merry, A.H., Murphy, N., Harvey, D.J., Dwek, R.A., Rudd, P.M.: An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. Anal. Biochem. **304**, 70–90 (2002)
17. Ashida, H., Miyake, A., Kiyohara, M., Wada, J., Yoshida, E., Kumagai, H., Katayama, T., Yamamoto, K.: Two distinct alpha-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology **19**, 1010–1017 (2009)
18. Benešová, E., Marková, M., Králová, B.: α-Glucosidase and β-glucosidase from psychrotrophic strain *Arthrobacter* sp. C2-2. Czech J. Food Sci. **23**, 116–120 (2005)

19. Maischberger, E., Irwin, J.A., Carrington, S.D., Duggan, V.E.: Equine post-breeding endometritis: a review. Ir. Vet. J. **61**, 163–168 (2008)
20. Altschul, S., Gish, W., Miller, W., Myers, E.W., Lipman, D.J.: Basic local alignment search tool. J. Mol. Biol. **215**, 403–410 (1990)
21. Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M.: The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. **37**, D141–D145 (2009)
22. Irwin, J.A., Morrissey, P.E.W., Ryan, J.P., Walshe, A., O'Neill, S.M., Carrington, S.D., Matthews, E., Fitzpatrick, E., Mulcahy, G., Corfield, A.P., Dalton, J.P.: Glycosidase activity in the excretory-secretory products of the liver fluke, *Fasciola hepatica*. Parasitology **129**, 465–472 (2004)
23. Goso, Y., Tsubokawa, D., Ishihara, K.: Evaluation of conditions for release of mucin-type oligosaccharides from glycoprotein by hydrazine gas treatment. J. Biochem. **145**, 739–749 (2009)
24. Jones, B.V., Sun, F., Marchesi, J.R.: Using skimmed milk agar to functionally screen a gut metagenomic library for proteases may lead to false positives. Lett. Appl. Microbiol. **45**, 418–420 (2007)
25. Guo, Z., Shao, N.: Glycopeptide and glycoprotein synthesis involving unprotected carbohydrate building blocks. Med. Res. Rev. **25**, 655–678 (2005)
26. Matsushita, T., Sadamoto, R., Ohyabu, N., Nakata, H., Fumoto, M., Fujitani, N., Takegawa, Y., Sakamoto, T., Kurogochi, M., Hinou, H., Shimizu, H., Ito, T., Naruchi, K., Togame, H., Takemoto, H., Kondo, H., Nishimura, S.: Functional neoglycopeptides: synthesis and characterization of a new class of MUC1 glycoprotein models having core 2-based O-glycan and complex-type N-glycan chains. Biochemistry **48**, 11117–11133 (2009)
27. Carrington, S.D., Irwin, J.A., Liu, L., Rudd, P.M., Matthews, E., Corfield, A.P.: Analysing mucin degradation. Methods Mol. Biol. **842**, 191–214 (2012)
28. Royle, L., Campbell, M.P., Radcliffe, C.M., White, D.M., Harvey, D.J., Abrahams, J.L., Kim, Y.G., Henry, G.W., Shadick, N.A., Weinblatt, M.E., Lee, D.M., Rudd, P.M., Dwek, R.A.: HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. Anal. Biochem. **376**, 1–12 (2008)