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A MICROMETHOD FOR THE ESTIMATION OF OLIGOSACCHARIDES CONTAINING GLYCOSIDICALLY LINKED SIALIC ACID OR HEXOSES, OR BOTH, IN GLYCOPROTEINS

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ABSTRACT

The peeling reaction, the process by which oligosaccharides are degraded in alkali, was used as the basis for an assay to provide structural information about glycosidically linked oligosaccharides in glycoproteins. Glycoproteins were treated with 0.05M NaOH at 50° to induce release, and subsequent degradation ("peeling"), of glycosidically linked, but not of *N*-glycosydically linked, oligosaccharides. Among the degradation products generated from *O*-linked chains were three 3-deoxy sugar acids whose formation was correlated with certain structural features of the oligosaccharides. *N*-Acetylneuraminic acid was released from terminal positions in the oligosaccharides, and iso- and meta-saccharinic acids were derived from the degradation of 4-*O*- and 3-*O*-substituted hexoses, respectively. All of these sugar acids were detected colorimetrically by periodate oxidation and reaction of the product with 2-thiobarbituric acid. The ability of the method to generate 3-deoxy sugar acids was tested in 8 alkali-treated glycoproteins. 3-Deoxy sugar acids were detected only in those glycoproteins whose glycosidically linked carbohydrates contained *N*-acetylneuraminic acid, or 3-*O*- or 4-*O*-substituted hexoses, or both. As little as 0.12 μg of 3-deoxy sugar acid produced from 5 μg of human chorionic gonadotropin was sufficient for detection. This method is novel in its ability to distinguish sialylation of glycosidically linked carbohydrates. Furthermore, it combines the specificity of β-elimination with the sensitivity of the 2-thiobarbituric acid assay in targeting degradation products of the peeling reaction as candidates for an assay method.

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INTRODUCTION

Structural studies of many glycoproteins have shown that carbohydrate–peptide linkages are mainly of two types[1,2]: (*a*) glycosyloxy (*O*) linkages between L-serine or L-threonine and, principally, GalNAc, and (*b*) glycosylamine (*N*) linkages between L-asparagine and GlcNAc. A distinguishing feature of the GalNAc-*O*-Ser/Thr bond is its lability in alkali relative to that of GlcNAc-*N*-Asn. Treatment in mild alkali[3] selectively cleaves the *O*-linked chains from a peptide *via* a β-elimination reaction[1,4]. The oligosaccharides released are not, however, stable.

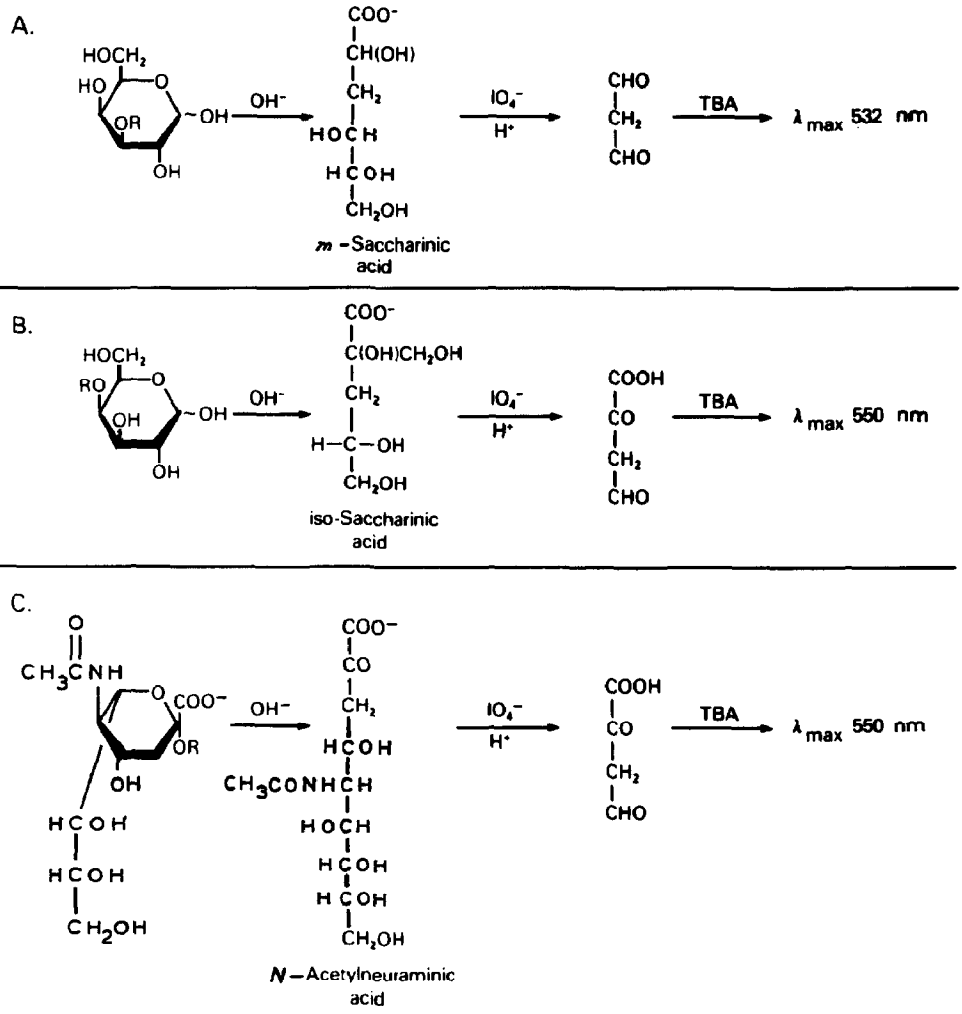

Fig. 1. Alkaline degradation of saccharide units. Oligosaccharides containing (A) 3-*O*- or (B) 4-*O*-substituted hexose units, or (C) *N*-acetylneuraminic acid units subject to "peeling" generate 3-deoxy sugar acids which, after periodate oxidation, can be detected colorimetrically by reaction with 2-thiobarbituric acid (TBA).

They undergo alkaline degradation in a stepwise process known[5–8] as "peeling", the products of which reflect certain structural characteristics of the oligosaccharide chain[7,9].

Peeling proceeds from the reducing end, generating degradation products in a stepwise fashion[8]. The extent of this peeling reaction, and the products which result, are a function of saccharide chain-structure and composition. D-Hexoses substituted at O-3 produce metasaccharinic acids as degradation products, whereas those substituted at O-4 afford isosaccharinic acids. Hexoses substituted at O-2 (*i.e.*, branch points) cannot form a β-alkoxycarbonyl structure and, consequently, are not converted into saccharinic acids[10]. The sugar moiety linking the oligosaccharide to the peptide, namely GalNAc, is generally 3-*O*-substituted, and so it constitutes a 3-substituted amino sugar through which the peeling reaction must proceed. When heated in alkaline solution, it is degraded to 3-acetamido-5-(1,2-dihydroxyethyl)furan with concomitant cleavage[11] of the glycosidic bond at O-3. Substitution of GalNAc at O-3 (or O-4) and of hexoses at O-3 or O-4 allows the peeling process to continue along the chain. Hexoses substituted at O-6 also produce saccharinic acids, but at a significantly lower rate. As the oligosaccharide is degraded, sialic acid residues in nonreducing positions are released.

It was noted that the saccharinic acids and NeuAc produced in this peeling process are 3-deoxy sugar acids[9] which can be detected in an assay[12] originally described for measurement of sialic acids (see Fig. 1). The 3-deoxy sugar acids are first converted by periodate oxidation into malonic dialdehyde or 3-formylpyruvate, and then this is treated with 2-thiobarbituric acid to afford a colored product. Although the complexity of degradation reactions which occur during this process makes a quantitative interpretation difficult, production of 3-deoxy sugar acids is diagnostic of these chain structures. The method should augment existing techniques to aid in purification and analysis of *O*-linked carbohydrate chains, especially those containing sialic acid.

EXPERIMENTAL

Chemical compounds. — Isosaccharinic [3-deoxy-2-*C*-(hydroxymethyl)-D-*erythro*-pentonic acid] and metasaccharinic acid (3-deoxy-D-*xylo*-hexonic acid), obtained as their crystalline lactones, were generous gifts from Drs. J. N. BeMiller, Purdue University, and G. G. Ashwell, NIH. Maltotriose, sialyllactose, *N*-acetyllactosamine, glycophorin, bovine submaxillary mucin, porcine dermatan sulfate B, ovalbumin, bovine transferrin, human chorionic gonadotropin, fetuin, and human α_1-acid glycoprotein were obtained from Sigma. All other chemical compounds were of the highest purity available.

Assay of NeuAc and SacA.* — The assay reported by Warren[12] was adapted to the micro scale by decreasing the sample volume from 200 μL to 50 μL with

**Abbreviations.* SacA, saccharinic acids; *i*-SacA, isosaccharinic acid; *m*-SacA, metasaccharinic acid; TCA, trichloroacetic acid; TBA, 2-thiobarbituric acid; hCG, human chorionic gonadotropin.

proportional diminution in reagents. The assay was performed in borosilicate glass tubes (12 × 75 mm) on 50-μL samples containing 0.2–5 μg of NeuAc/SacA in 20% TCA. To each sample was added 25 μL of 0.2M sodium metaperiodate in 9M (27N) phosphoric acid. After 20 min at room temperature, 10% sodium arsenite in a solution of 0.5M sodium sulfate–0.05M H_2SO_4 (250 μL) was added, followed by 500 μL of 0.6% 2-thiobarbituric acid in 0.5M sodium sulfate. The latter solution was most stable if the pH was adjusted[13] to 9.0 with M NaOH and it was stored in the dark at 4°. The assay mixture was heated in a boiling-water bath for 15 min, and then cooled briefly in an ice–water bath. The chromophore was extracted into an equal volume (1 mL) of cyclohexanone and the absorption measured at 550 nm (NeuAc, *i*-SacA) and 532 nm (*m*-SacA). Visible absorption spectra (450–600 nm) were measured with a Beckman DU-7 spectrophotometer (Beckman Instruments, Fullerton, CA).

Glycoproteins. — Glycoproteins were dissolved in water (1–5 mg/mL) and the resulting solutions were dialyzed before use. Concentrations were estimated by assay of protein[14] and of total NeuAc content after treatment[12] with 0.05M H_2SO_4 for 1 h at 80°.

Alkaline treatment. — Saccharides (36 nmol) or glycoproteins (5–100 μg) in 40 μL of 0.05M NaOH were incubated for 0–20 h at 50°. The reaction was stopped by neutralizing the solution with 2 μL of M HCl. Trichloroacetic acid [10 μL of 100% (w/v)] was added to precipitate protein, and, after centrifugation, the supernatant solution (50 μL) was assayed for 3-deoxy sugar acids generated by the reaction.

RESULTS

In initial experiments using the scaled-down assay, the fluorimetric detection of the TBA chromophore in 1-butanol[15] was explored, and no significant improvement in sensitivity over colorimetric detection in cyclohexanone was found.

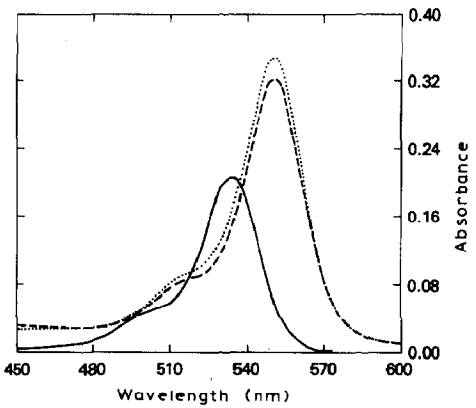

Fig. 2. Visible absorption spectra of 3-deoxy sugar acids (33 nmol) in the TBA assay. *N*-Acetylneuraminic acid (·····), isosaccharinic acid lactone (– – –), and metasaccharinic acid lactone (——).

As little as 0.32 nmol (100 ng) of NeuAc could be detected, and a linear response was obtained from 0.2–5 μg (data not shown). In addition to NeuAc, the assay detected two other 3-deoxy sugar acids, *i*-SacA and *m*-SacA. Isosaccharinic acids and NeuAc produced similar chromophores of approximately equal absorptivity[9] at λ_{max} 550 nm (see Fig. 2). The chromophore of *m*-SacA displayed λ_{max} 532 nm, with peak absorptivity ~60% that of the other two. Oxidation of *m*-SacA by periodate yields malonic dialdehyde in a process similar to oxidation of 2-deoxy-D-*erythro*-pentose. Both are detected[9] with a peak absorbance at 532 nm.

Detection of these 3-deoxy sugar acids as degradation products from "peeled" oligosaccharides was confirmed by using β-eliminated hCG (see Fig. 3). It was found that 0.12 μg of NeuAc–SacA generated from 5 μg of hCG could be detected reproducibly at 550 nm, consistent with the high *O*-linked sialic acid content of this glycoprotein[16,17], and that the 3-deoxy sugar acid response was linear from 5–100 μg of hCG.

Glycoproteins generate 3-deoxy sugar acids in a two-step process. The first step is release of the oligosaccharide unit from the protein by β-elimination, and the

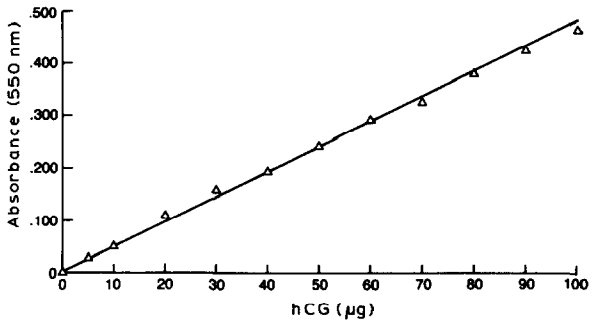

Fig. 3. Sensitivity of TBA assay for detection of 3-deoxy sugar acids from β-eliminated hCG. (Experimental conditions as described in the text.)

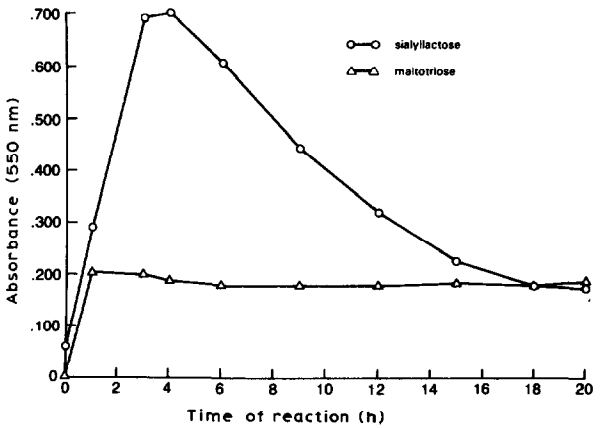

Fig. 4. Effect of β-elimination conditions on the trisaccharides α-NeuAc-(2→3)-β-Gal-(1→4)-Glc and α-Glc-(1→4)-α-Glc-(1→4)-Glc. (Experimental conditions as described in the text.)

second is formation of 3-deoxy sugar acids from degradation of released oligosaccharides. To determine whether the first step alone was sufficient for formation of 3-deoxy sugar acids, fetuin, another glycoprotein containing *O*-linked sialic acid[18], was β-eliminated without, and with, the addition of M $NaBH_4$ to prevent initiation of the peeling reaction (data not shown). In the absence of $NaBH_4$, alkali-treated fetuin produced a chromophore detected at 550 nm, indicating that the oligosaccharide–protein bond had been cleaved and that "peeling" had occurred. However, when $NaBH_4$ was present during alkaline treatment and "peeling" was prevented, no chromophore was detected. This result verified that formation of the 3-deoxy sugar acid chromophore requires both the cleavage step and subsequent "peeling" of the released oligosaccharide.

To isolate for investigation the second step in the process, β-elimination conditions were tested on two reducing trisaccharides (see Fig. 4). Maltotriose and sialyllactose (36 nmol) were incubated in 0.05M NaOH for 0–20 h at 50°, and assayed for 3-deoxy sugar acids as degradation products. Maltotriose was rapidly degraded to produce *i*-SacA, which reached its maximum level after 1 h of reaction and remained stable in alkali. In a similar fashion, alkaline treatment of sialyllactose resulted in the release of NeuAc, which was reflected in a rapid, initial increase in absorbance. Unlike maltotriose, however, absorbance peaked after 4 h

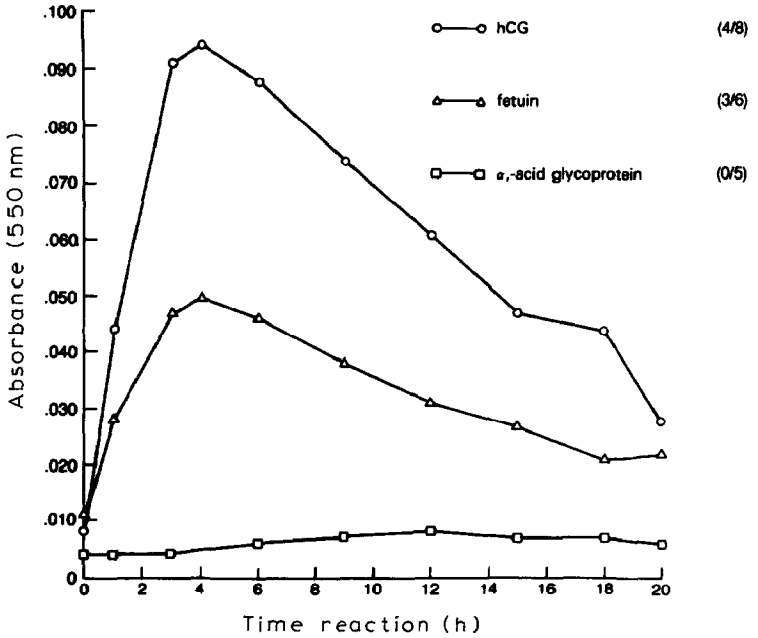

Fig. 5. Assay of glycoproteins after β-elimination. The major oligosaccharides released are: from hCG, α-NeuAc-(2→3)-β-Gal-(1→3)-GalNAc; from fetuin, α-NeuAc-(2→3)-β-Gal-(1→3)-[α-NeuAc-(2→6)]-GalNAc; and, from α_1-acid glycoprotein, none. The numbers in parentheses after the names of the proteins indicate the ratio of *O*-linked chains:total carbohydrate chains per molecule[16–18, 20–22].

of reaction and, following a linear decrease due to the instability of NeuAc in alkali[19], became stabilized at a minimum level after 20 h. This peak absorbance produced by the degradation of sialyllactose was 2.5 times that of maltotriose. Because the chromophores formed from the two 3-deoxy sugar acids produced in these reactions have similar absorptivities (see Fig. 2), and because equimolar amounts were compared, it appeared that the peeling reaction liberated NeuAc more efficiently than it produced *i*-SacA. In fact, only ~10% of degraded hexose proceeded to form *i*-SacA, while 70% of NeuAc was released from sialyllactose. The disaccharide *N*-acetyllactosamine produced no chromophore when assayed in this way, indicating that 4-*O*-substituted GlcNAc in a reducing position does not generate SacA.

The selectivity of this procedure was investigated for *O- vs. N*-linked oligosaccharides by using three sialylglycoproteins of known carbohydrate structure. Each had a unique number of *O*-linked oligosaccharides per protein molecule (indicated by the ratios of *O*-linked chains:total carbohydrate chains, shown in parentheses at the right in Fig. 5). Despite some microheterogeneity in these proteins, major oligosaccharides could be identified. Human chorionic gonadotropin[16,17], fetuin[18,20], and α_1-acid glycoprotein[21,22] (an amount of each normalized to 4 μg of total bound hexose + NeuAc, based on calculated weight percentages) were each dissolved in 40 μL of 0.05M NaOH and incubated for 0–20 h at 50°. After removal of the protein by TCA precipitation, supernatant liquors were assayed by the TBA method for NeuAc/SacA. Fig. 5 confirms that an A_{550} chromophore was detected from hCG and fetuin, the two glycoproteins that contain *O*-linked oligosaccharide chains. Moreover, the absorbance in each case roughly correlated with the relative abundance of *O*-linked carbohydrate in each glycoprotein. The steady decline in absorbance from a maximum at 4 h, similar to that seen after alkaline treatment of sialyllactose, indicated the presence of terminal sialic acid residues in the *O*-linked oligosaccharides of both glycoproteins. α_1-Acid glycoprotein produced no significant response in this assay during 0–20 h, a result which verified the failure of potentially reactive *N*-linked oligosaccharides to be released under these conditions.

Further support for the specificity of this method was obtained for five additional glycoproteins, all of which contain potentially reactive carbohydrate structures[1,2]. Two glycoproteins (glycophorin and bovine submaxillary mucin) and a glycosaminoglycan (dermatan sulfate B), each containing *O*-linked oligosaccharides, responded positively in the assay; two other glycoproteins containing only *N*-linked chains (ovalbumin and transferrin) did not (data not shown).

DISCUSSION

Direct confirmation of the presence of *O*-linked carbohydrate chains in glycoproteins can be obtained by isolation and structural analysis of the released oligosaccharide chains[3]. Short of this definitive and rigorous approach are a number of

rapid, qualitative methods, including chromatic silver staining of *O*-linked sialylglycoproteins[23], spectrophotometric detection of dehydroamino acids[24], enzymic removal of *O*-linked oligosaccharides followed by electrophoretic analysis of the deglycosylated protein[25], and the method described here. In our studies of a glycosylated lectin isolated from cortical granules of *Xenopus laevis* eggs[26], we attempted to use two of these rapid methods to obtain preliminary structural information on this lectin and to monitor oligosaccharide purification. Detection at 241 nm of dehydroamino acids was relatively insensitive, requiring 0.1–1 mg of glycoprotein. Enzymic removal using *O*-glycanase required pretreatment of the glycoprotein to remove sialic acid, and then was limited to hydrolysis only of unsubstituted disaccharides having a core structure[25] of β-Gal-(1→3)-GalNAc. By comparison, the procedure described here was more sensitive, and it was used without pretreatment, to detect a variety of sialyl-containing, *O*-linked oligosaccharides from different glycoproteins. Sensitivity of the TBA assay can be further enhanced by a recent h.p.l.c. adaptation[27]. An alternative method[28] was recently reported for assay of *O*-linked oligosaccharides which does not require the presence of NeuAc or certain other structural features.

Although *O*-linked chains were released, and 3-deoxy sugar acids were formed, the present β-elimination condition resulted in degradation of the polypeptide backbone (data not shown), compromising electrophoretic analysis of the deglycosylated protein. In some cases for which this method might be applied, recovery of the intact polypeptide may be desirable. A recent report[29] suggests that the integrity of the polypeptide chain can, in fact, be retained by using a lower NaOH concentration (5mM), thus permitting analysis both of the *O*-linked oligosaccharides released and of the intact, deglycosylated polypeptide.

Despite the nonstoichiometric production of 3-deoxy sugar acids, both conversion of substituted hexoses into SacA and release of NeuAc were reproducibly and linearly detected. As a qualitative, analytical tool, this procedure augments existing methods in its ability to distinguish *O*-linked oligosaccharides which contain NeuAc, or 3-*O*- or 4-*O*-substituted hexoses, or both, in glycoproteins. It is rapid and sensitive, and is particularly well suited to monitoring the purification of *O*-linked glycopeptides from proteolytic digests.

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