Determination of the number and distribution of oligosaccharide linkage positions in O-linked glycoproteins by capillary electrophoresis

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Abstract

O-Linked oligosaccharide chains are found in a wide variety of glycoproteins. Determination of the number of O-linked serine and threonine residues is an important step in glycoprotein characterization. Alkaline release of the oligosaccharide chains from the peptide chain followed by bisulfite addition to the unsaturated residues generates sulfonates. Hydrolysis of treated samples followed by derivatization with naphthalene 2,3-dicarboxyaldehyde and cyanide results in analytes which are separated and quantitated by capillary electrophoresis with UV detection. The utility of the method was demonstrated using bovine submaxillary mucin yielding results that agreed with previously published values.

1. Introduction

A variety of methods exist which can be used to determine oligosaccharide chain structure and peptide structure. However, there are relatively few procedures which can be used to determine the number and distribution of oligosaccharide linkage positions on the peptide chain. The current, most popular approach is to sequence a peptide with a commercial sequencer and note at which position sequencing is terminated. Subsequent comparison of these data to the sequence obtained after the carbohydrate chains have been enzymatically or chemically removed usually gives the desired information [1–3]. Ex-

amples of recent reports using this method are those of Schmid et al. [4] and Nakada et al. [5]. Alternative approaches include one which employs a biotinylated lectin/avidin-biotinylated peroxidase on-membrane staining technique [6].

In 1973, Pigman and Moschera [7] reviewed a reductive β-elimination procedure used to release oligosaccharide chains O-linked to serine and threonine residues and effect conversion to alanine and α-aminobutyric acid, respectively. The amino acid residues can then be released by acid hydrolysis and quantitated for compositional information [8]. Alternatively, the treated peptide can be subjected to sequence analysis [9]. One drawback to the reductive β-elimination is that the alanine which is generated in the procedure is also a commonly occurring amino acid in proteins. Therefore it is necessary to analyze

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an untreated sample in order to correct for endogenous alanine.

In a recent review on carbohydrate analysis, a relatively straightforward method similar to reductive β-elimination is given [10]. Originally described by Harbon et al. [11], it involves the treatment of the glycoprotein with a solution of alkaline sulfite in order to cause β-elimination of oligosaccharide chains O-linked to serine and threonine residues and conversion of the resulting unsaturated derivatives to their sulfonates (Fig. 1). The modified protein is then hydrolyzed to produce free amino acids which are then separated and quantitated. Using this procedure, any serine residue which was attached to an oligosaccharide will be converted to a cysteic acid (CA) residue while an O-linked threonine results in an α-amino-β-sulfonylbutyric acid residue (ASBA). Thus, the quantity of these two residues relative to the quantity of serine and threonine residues, respectively, gives the relative number of serines and threonines O-linked to oligosaccharide chains. Note that in this procedure two unique amino acids are formed which are not commonly found in peptide structures.

In order to increase sensitivity, this method has been modified by using radioactively labeled sulfite [12]. Though it gives useful results, it requires the use of radioactive substances. It also suffers from drawbacks common to other techniques which involve quantitation of the amino acid sulfonates in the presence of other amino acids. The sulfonates coelute at the void volume of an amino acid analyzer. The methods are quite tedious and time-consuming requiring desalting, ion-exchange chromatography for separation and then paper chromatography for identification.

With the advent of capillary electrophoresis (CE) it is possible to expedite the separation and quantitation of the amino acids involved in the procedure. Since CE in its simplest form, capillary zone electrophoresis (CZE), separates substances by size and charge, it seems well suited to analyze a reaction such as the alkaline-sulfite reaction in which the products have acquired an additional negative charge. CE also offers the advantages of highly efficient separations, low volume requirements and minimal sample preparation as compared to other separation techniques such as liquid chromatography. Specifically, we have developed a method whereby the amino acids, including the sulfonates, are derivatized and analyzed by CE. The amino acids are reacted with naphthalene-2,3-dicarboxyaldehyde (NDA) in the presence of cyanide to generate the cyanobenzylisoindole (CBI) deriva-
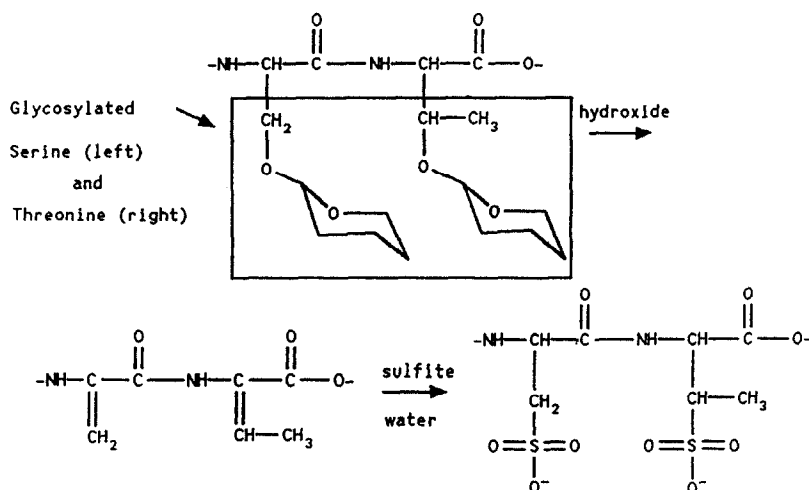

Fig. 1. Alkaline sulfite reaction causing β-elimination of O-linked serine and threonine residues and conversion of the unsaturated intermediates to sulfonates.
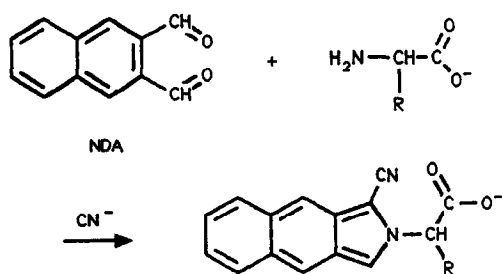
Fig. 2. NDA–cyanide derivatization reaction with primary amino acids.

tives (Fig. 2) and subsequently separated by CZE using UV detection. The separation of the CBI derivatives of selected amino acids by CE has been accomplished previously by us [13]. The CBI derivatives can also be detected with greater sensitivity by fluorescent, electrochemical and chemiluminescence detectors [14].

2. Experimental

2.1. Materials

Amino acids, glycoproteins and sodium dodecyl sulfate were obtained from Sigma (St. Louis, MO, USA). Sodium cyanide (99%) was acquired from Fluka (Ronkonkoma, NY, USA). Sodium borate (ACS), sodium sulfite (ACS), NaOH and concentrated HCl (ACS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and NDA was acquired from Oread Labs. (Lawrence, KS, USA). Solutions were prepared in Nanopure water (Sybron-Barnstead, MA, USA).

2.2. Alkaline sulfite reaction (β-elimination and bisulfite addition)

The conditions used in this reaction are adapted from Ref. [12]. Exactly 5.0 mg of glycoprotein were mixed with 100 μl of 5 mM α-aminoadipic acid (internal standard, IS). If the sample was a control sample, 300 μl of 6 M HCl was then added. Next, 1.5 ml of 0.5 M Na_2SO_3 in 0.1 M NaOH were added with thorough mixing. The solution is then incubated at 37°C in

a recirculating water bath for the indicated amount of time. The reaction is stopped by removing the solution, allowing it to reach room temperature, and combined with 400 μl of concentrated HCl. The mixture was sonicated and then evaporated at 50°C under a stream of dry air to remove HCl. To insure that the reaction was stopped, 400 μl of 6 M HCl was added, the mixture sonicated and evaporated.

2.3. Hydrolysis of treated glycoprotein and controls

Approximately 1.0 ml of 6 M HCl was mixed with the sample and the mixture sonicated. From the solution 190-μl aliquots were removed, evacuated and sealed to exclude oxygen for the solution. The vials were heated at 105°C for the indicated amount of time, 48 h in the standard protocol. Evaporation of the HCl solution from the cooled sample was followed by a 750-μl wash and evaporation to ensure removal of HCl. The residue was dissolved in 150 μl of water and filtered through a 0.22-μm filter.

2.4. Amino acid derivatization

The sample derivatization procedure used was a modification of that given by Lunte and Wong [14]. For derivatization of sample hydrolyzates, the indicated volume of sample, 10 μl in the standard protocol, was added to 5 μl of 10 mM NaOH. Next, 40 μl of 5 mM NaCN followed by 40 μl of 5 mM NDA in acetonitrile were added and mixed vigorously. The appearance of a fluorescent yellow-green color within a few minutes indicated that the reaction is proceeding. After the indicated amount of time, 1.25 h in the standard protocol, the sample was injected into the CE system.

Standard solutions of amino acids were derivatized in a similar manner. To 20 μl of 20 mM sodium borate (pH 9.0) were added 10 μl of a stock amino acid mixture. The mixture contained 0.56 mM of each amino acid. Addition of NaCN and NDA solutions were as above and the sample injected 1.25 h later.

2.5. Capillary electrophoresis

The instrument used in this work was an ISCO Model 3140 capillary electropherograph. Uncoated fused-silica columns of 100 cm (75 cm to window) × 50 μm I.D. were employed. The buffer composition in the columns and reservoirs was 20 mM sodium borate (pH 9.0). Operating voltage as 30 kV. These conditions are similar to those employed previously [13] for amino acid analysis. Although CBI derivatives exhibit an absorption maximum in the visible range at 420 nm, we found the 254 nm wavelength used for detection in these studies provided greater sensitivity. Samples were vacuum injected for 15 k Pa·s. The column was flushed after every run for 5 min with running buffer and after a maximum of six runs for 3 min each with 0.1 M NaOH, then water, then buffer.

Micellar electrokinetic capillary chromatography (MECC), used in the determination of the absolute amount of threonine, was run under identical conditions except that a 50 mM sodium dodecyl sulfate in 20 mM sodium borate (pH 9.0) buffer was used [15].

The electropherograph was interfaced with an IBM-compatible personal computer and output from the electropherograph was collected and analyzed using ICE software (ISCO).

3. Results and discussion

This method consists of four separate steps. These are: (1) treatment of the glycoprotein with alkaline sulfite to cause β-elimination and subsequent generation of sulfonates (Fig. 1), (2) hydrolysis of the treated glycoprotein, (3) derivatization of the released amino acids (Fig. 2) and (4) CE of the amino acid derivatives.

Optimization of the conditions used in steps 1, 2 and 3 were undertaken using mucin. Separation parameters for step 4 had been optimized previously by Weber et al. [13] and were used as described.

Finally, the method was applied to the analysis of bovine submaxillary mucin. This compound was chosen since it is a glycoprotein with a simple amino acid composition and a high percentage of serines and threonines O-linked to oligosaccharides.

3.1. Optimization of derivatization time (step 3)

Initial studies involved determination of the optimum reaction time for the derivatization procedure. A previous report [15] indicated that a minimum of 20 min is required. Observations in our laboratories indicated that amino acids with negatively charged side chains require additional time for derivatization especially if reagent is not present in large excess. Therefore, solutions obtained from the hydrolysis of an alkaline sulfite treated mucin sample were derivatized and allowed to react various lengths of time from 0.75 to 1.5 h before injection onto the CE system. Though the electropherograms displayed a broader mixture of amino acids, only serine, threonine, CA and ASBA were investigated, since these were the key amino acids for linkage analysis. Glycine in the mixture was also analyzed. It serves as a reference point to which the other four amino acids can be compared since it reacts quickly and the resulting derivative is quite stable. The height of each amino acid was measured and a plot peak height relative to glycine versus reaction time was made (Fig. 3). A reaction time of 1.25 h produces the greatest yield of CBI derivative and was thus chosen as the reaction time to be used for subsequent derivatizations. Note that the yield of the cysteic acid derivative is particularly sensitive to reaction time.

3.2. Hydrolysis time (step 2)

Since proteins vary dramatically in their resistance to acid-catalyzed hydrolysis and since the resultant amino acids also exhibit varying degrees of stability under the hydrolytic conditions, a detailed study was undertaken to see the effect of time of hydrolysis on the recovery of amino acids for both alkaline-sulfite treated (for 24 h) mucin and control mucin. The peak height of each amino acid relative to that of glycine was determined by CE following derivatization.
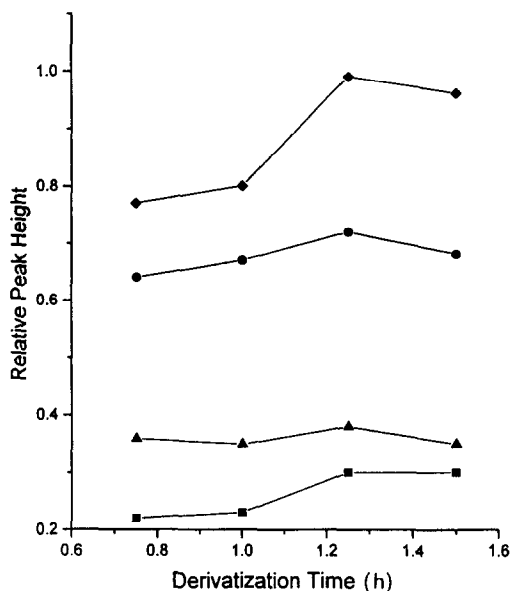
Fig. 3. Effect of derivatization time on the relative peak heights of amino acids in CE. Sample from mucin reacted with alkaline sulfite, hydrolyzed, derivatized and analyzed by CE as described in text. ● = Thr + Val; ▲ = Ser; ■ = ASBA; ◆ = CA.
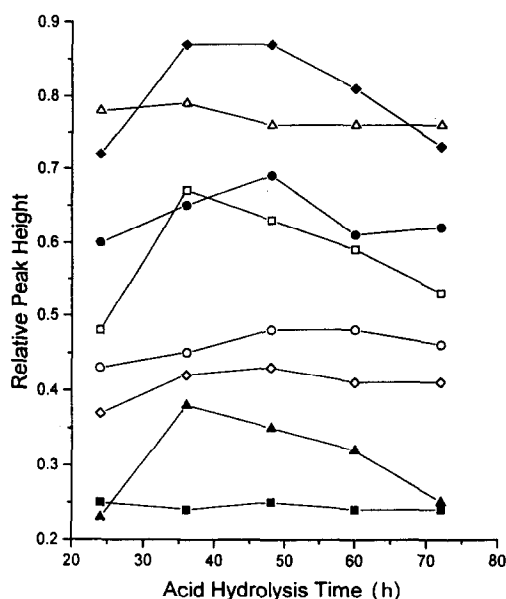

Fig. 4. Effect of acid-catalyzed hydrolysis time on the relative peak heights of amino acids in CE. Sample mucin reacted with alkaline sulfite, hydrolyzed, derivatized and analyzed by CE as described in text. □ = Thr + Val; ○ = Ser; ▲ = ASBA; ◆ = CA; △ = Ala; ■ = IS; ● = Glu; ◇ = Asp.

Glycine in the mixture was chosen as a reference since it is relatively stable to acid hydrolysis. Fig. 4 shows the effect of hydrolysis time on relative peak heights. Generally speaking, most amino acids exhibited an optimum recovery when treated for 48 h, although in the treated mucin samples, threonine, alanine and ASBA showed just slightly higher recoveries at 36 h. Thus, 48 h seems to be the optimum time for acid hydrolysis and this time was used for the standard protocol in all subsequent analysis.

3.3. Alkaline sulfite reaction time (step 1)/ glycosylation percentage

Earlier work involving the alkaline-sulfite reaction reported conflicting times for this reaction [12,16,17]. In order to ascertain the appropriate reaction time, the amino acid recovery as a function of reaction time was studied. Samples of treated mucin and controls were reacted for 24, 36, 48 or 60 h and then analyzed by the standard protocol. A plot of amino acid peak height relative to that of glycine versus reaction time is

shown in Fig. 5. In general, reaction time seems to have little affect on amino acid recovery. A notable exception appears to be the 24-h treatment which yields less CA and correspondingly more serine. This effect, which was reproducible, indicates that 24 h does not effectively convert all glycosylated serines to the sulfonate.

A more useful way of visualizing the data given in Fig. 5 is seen in Fig. 6. By using the appropriate response factors listed in Table 1 with the data from Fig. 5, one can determine the percentage of serine O-linked to oligosaccharide in samples at each reaction time. A different situation exists for threonine. In the borate buffer, threonine co-migrates with valine as a single peak. This is expected by CE theory since the amino acids are very close in size and shape [18]. However, in MECC, these amino acids are well separated [15] and MECC was performed on a sample by merely adding sodium dodecyl sulfate to the running buffer prior to analysis. Thus, by including the information provided by the MECC run, one can determine the per-
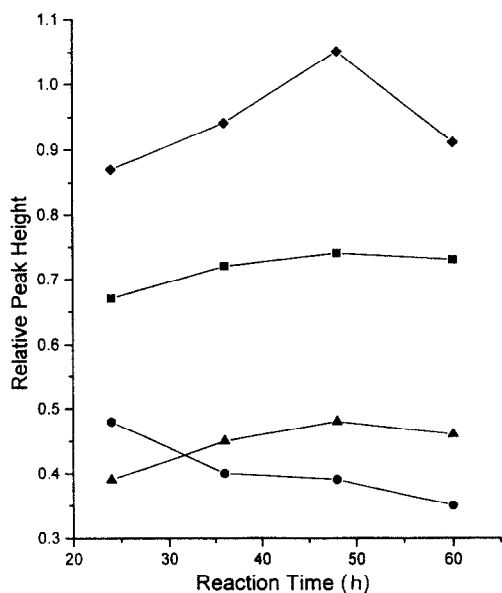
Fig. 5. Effect of alkaline sulfite reaction time on the relative peak heights of amino acids in CE. Sample mucin reacted with alkaline sulfite, hydrolyzed, derivatized and analyzed by CE as described in text. ■ = Thr + Val; ● = Ser; ▲ = ASBA; ◆ = CA.
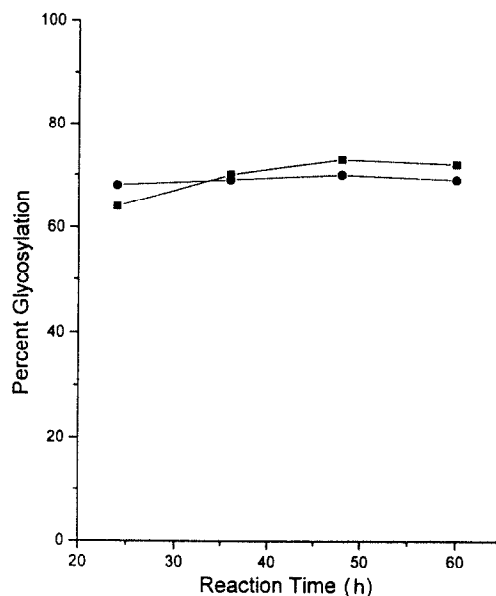

Fig. 6. Effect of alkaline sulfite reaction time on the percent glycosylation of serin and threonine in CE. Sample mucin reacted with alkaline sulfite, hydrolyzed, derivatized and analyzed by CE as described in text. ● = Thr; ■ = Ser. Percent glycosylation for Ser = [CA]/([CA] + [Ser]); Percent glycosylation for Thr = [ASBA]/([ASBA] + [Thr]).

centage of threonine O-linked to oligosaccharide in the same manner as done for serine. The 36-, 48- and 60-h reaction times give values close to each other, averaging 72% glycosylation for serine and 69% for threonine. This compares well to the values of 67% and 73% which we calculated from data reported for a core peptide of mucin [7].

3.4. Compositional analysis of mucin

A sample of bovine submaxillary mucin was subjected to the alkaline sulfite treatment, hydrolysis and derivatization. The mixture was separated by CE to give the electropherogram in Fig. 7B. Note the excellent resolution obtained in less than 20 min. When this electropherogram is compared to a control sample of mucin (Fig. 7A), the appearance of two new sulfonate peaks (migration times 15.7 and 16.8 min) and the corresponding reduction in the threonine and serine peaks (migration times 9.7 and 9.9 min) is noted.

Table 1
Response factors for selected amino acids and composition of bovine submaxillary mucin before and after alkaline sulfite treatment

Amino acid[a]	Response factors[b]	Untreated[c]	Treated[c]	Literature[d]
"Thr"	1.02	0.46	0.33	0.58
Ser	1.01	0.49	0.16	0.54
Ala	0.90	0.36	0.37	0.34
Gly	0.92	0.47	0.49	0.49
IS	1	–	–	–
Glu	1.06	0.27	0.27	0.18
ASBA	1.05	–	0.20	–
Asp	1.03	0.18	0.18	0.07
CA	1.01	–	0.41	–

[a] IS = Internal standard, α-aminoadipic acid; ASBA = α-amino-β-sulfobutyric acid; CA = cysteic acid, "Thr" = threonine and valine which coelute.
[b] Determined using relative peak heights.
[c] Micromole/milligram protein.
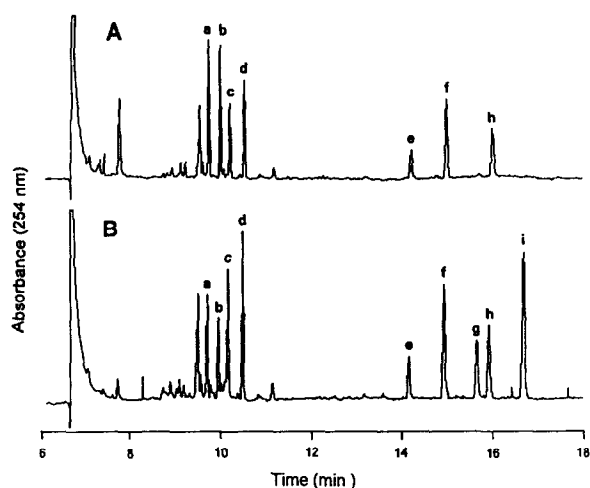[d] Relative values reported by Pigman and Moschera [7]. Conditions as in Fig. 7.

Fig. 7. Electropherogram of NDA-derivatized amino acids obtained from the hydrolysis of untreated (A) and alkaline sulfite treated (B) mucin. CE Conditions: 100 cm (effective length, 75 cm) × 50 μm I.D.; applied voltage, 300 V/cm; detection at 254 nm, 0.002 AUFS, 20 mM sodium borate buffer (pH 9.0). Peaks: a = Thr and Val; b = Ser; c = Ala; d = Gly; e = IS; f = Glu; g = ASBA; h = Asp; i = CA.

A solution containing equimolar amounts of eight amino acids found in alkaline sulfite-treated mucin samples and the internal standard was derivatized and separated by CE. This permitted determination of response factors used for the quantitation of amino acids in glycoprotein samples (Table 1). The mixture contains the sulfonated amino acids, CA and ASBA, that would be generated from serine and threonine respectively O-linked to oligosaccharides. Since ASBA is not commercially available, its constitutional isomer, homocysteic acid (HCA), was used instead. This substitution seems acceptable since the migration time of the two should be virtually identical according to CE theory and experimental results reported by Weber and Vaught [18]. Also, peak response factors should be nearly the same since the CBI moiety is the primary chromophore.

Also given in Table 1 is the amino acid composition of both treated and control mucin. Comparison of the amino acid composition for the mucin to reported values [7] shows good agreement except that the amounts of glutamate and aspartate are significantly higher. This could

be due to decarboxylation of these amino acids or incomplete deamidation of asparagine and glutamine during hydrolysis in the cited work.

Five consecutive injections of a treated mucin sample gave relative standard deviations from 2 to 5% for the amino acids listed in Table 1.

4. Conclusions

The alkaline sulfite treatment of a glycoprotein, followed by hydrolysis, derivatization and analysis by CE represents a useful alternative to the current methods employed for the determination of O-linked serine and threonine residues. Optimization of procedural conditions yield results comparable to literature values. CE offers a number of advantages over methods which utilize liquid chromatography such as low volume requirements and easy sample preparation. Since limits of detection for the derivatization method had previously been reported [13], this was not studied. However, the procedure could easily be scaled down yielding smaller volumes for CE analysis. Also, the use of a laser-induced fluorescence detector for detection of CBI derivatives [19] would further increase sensitivity.

Acknowledgements

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