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Reductive amination of N-linked oligosaccharides using organic acid catalysts

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Abstract

The reductive amination of oligosaccharides with 8-aminopyrene-1,3,6-trisulfonate in several organic acids of varying strength was examined by capillary electrophoresis using laser-induced fluorescence detection. The relationship between the derivatization yield and the pK_a of the catalyst (organic acids) is in agreement with the general acid catalysis of the hemiacetal ring opening and the Shiff base formation, one of which is considered to be the rate-determining step of the reductive amination reaction. Derivatization in the presence of organic acids having higher acidity than acetic acid, the most commonly used catalyst, resulted in significantly higher derivatization yield and the highest yield was attained with the use of citric or malic acid as catalysts. This effect was even more prominent for oligosaccharides having N-acetylglucosamine at the reducing end compared to the similar size linear glucose oligomers. Sialylated oligosaccharides were derivatized at 37°C for 16 h with only less than 10% loss of the sialic acid residues. The derivatization procedures were tested on the N-linked oligosaccharides released enzymatically (peptide-N-glycanase F) from bovine fetuin and ribonuclease B.

Keywords: Catalysis; Derivatization, electrophoresis; Oligosaccharides; Aminopyrene trisulfonate; Organic acids

1. Introduction

Reductive oligosaccharides are usually labeled via reductive amination with charged or uncharged chromophores or fluorophores in the reaction mixtures containing 1–2 *M* acetic acid [1–13]. The specific function of acetic acid is not really understood yet but it was shown that derivatization yield is dependent on the concentration of the acetic acid [7,9–11]. The process of reductive amination of oligosaccharides starts with the aldehyde formation by opening the ring of the N-acetylglucosamine (GlcNAc) residue on the reducing end of the N-linked carbohydrate molecules. The second step is a nucleophilic addition: the labeling reagent's amino group forms a Schiff base with the sugar carbonyl. Since the equilibrium constant for this reaction is low, the amount of Shiff base does not accumulate with time. The third step, reduction with sodium cyanoborohydride produces the stable aromatic secondary amine. It is important to note here that the first two steps during this reductive amination reaction are known to be acid catalyzed. In a previous paper [12], the Bronsted equation was discussed as it expresses a linear free-energy relationship between labeling rates and acid dissociation equilibria and suggests that the efficiency of general acid catalysts increases with increasing acid strength. In that work,

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the role of various organic acid catalysts on the reductive amination of monosaccharides with 8-aminopyrene-1,3,6-trisulfonate (APTS) was also investigated. It was found that the use of organic acids with greater acidity than acetic acid ($pK_a < pK_a^{AcOH}$) significantly improved the labeling efficiency for most of the common monosaccharides including the N-acetylamino sugars. The use of a stronger acid catalyst, however, may increase the risk of desialylation, which has been known to occur in acidic medium at elevated temperatures [14,15]. This is presumably due to the internal acid catalysis by the protonated carboxyl group of sialic acid which facilitates the attack of water on the adjacent ketal group.

As a continuation of our previous work on derivatization of sugars [12,16], the goal of this study was to improve the labeling efficiency for N-linked oligosaccharides with APTS. Furthermore, since it is known that the rate of desialylation is expected to increase with elevating acid strength, we wanted to find an acid catalyst with a pK_a that allows more efficient APTS labeling than that obtained with acetic acid, while desialylation is still kept at a low level. This report describes the catalytic effect of seven organic acids, acetic-, succinic-, glycolic-, L-malic-, citric-, malonic- and maleic-acid with pK_a values ranging from 1.91 to 4.75, as potential catalysts during reductive amination studies with APTS derivatization of N-linked oligosaccharides released from glycoproteins.

2. Experimental

2.1. Chemicals

APTS was purchased from Lambda (Graz, Austria). Sodium cyanoborohydride (1.0 *M* solution in tetrahydrofuran (THF)) was obtained from Aldrich (Milwaukee, WI, USA). All oligosaccharides, organic acids, bovine fetuin, ribonuclease B and buffer components were purchased from Sigma (St. Louis, MO, USA). The maltodextrin ladder (M040) was obtained from Grain Processing (Muscatine, IA, USA). 6′-Sialyl-N-acetyllactosamine (6′-SLN) was purchased from V-Labs (Covington, LA, USA).

2.2. Derivatization of maltotetraose (G_4) and 6′-SLN

A mixture of dried 10 nmol G_4 and 10 nmol 6′-SLN was derivatized by addition of 2 μl 0.1 *M* APTS in water, 2 μl 1.8 *M* aqueous organic acid and 2 μl 1 *M* $NaBH_3CN$ in THF. The reductive amination was allowed to proceed at 75°C for 1 h or at 37°C for 16 h. The derivatization mixtures were diluted with water to 200 μl and then diluted further a 25-fold before capillary electrophoresis–laser-induced fluorescence detection (CE–LIF) analysis.

2.3. Oligosaccharides from ribonuclease B and fetuin

The N-linked oligosaccharides from bovine fetuin and ribonuclease B were cleaved by peptide-N-glycanase F (PNGase F) as follows. Ten μl of a 10 mg/ml solution of each glycoprotein was mixed with 10 μl of Enzyme Profiling Buffer (Glyko, Novato, CA, USA). Sodium dodecyl sulfate (0.4 μl of 5% aqueous solution) and 2-mercaptoethanol (0.7 μl of 1.4 *M* aqueous solution) were added and then the mixtures were placed in a boiling water bath for 5 min. After cooling, 2.5 μl of 7.5% Nonidet P-40 solution and 1 mU PNGase F were added. The enzymatic cleavage was allowed to proceed for 2 h at 37°C. Cold ethanol (75 μl) was then added to each tube to precipitate the proteins. After cooling for 1 h at 4°C, the tubes were centrifuged for 5 min at 10 000 *g*. The supernatants were dried in centrifugal vacuum evaporator, redissolved in 40 μl water, and then divided into four aliquots (10 μl each) for derivatization with four different acid catalysts (acetic, malic, citric and malonic acids). After drying in a centrifugal vacuum evaporator, the oligosaccharides were derivatized at 37°C for 16 h in the presence of 2 μl 0.1 *M* APTS in water, 2 μl 1.8 *M* aqueous organic acid and 2 μl 1 *M* $NaBH_3CN$ in THF, the same procedure used for derivatization of G_4 and 6′-SLN. Each of the derivatization mixtures were diluted to 200 μl and then diluted another 25-fold prior to sample injection to the CE–LIF system.

2.4. Capillary electrophoresis procedures

CE separations were performed on a P/ACE 2100

system equipped with a LIF detector (Beckman, Fullerton, CA, USA) as previously reported [13]. The separations were performed in a 19 μm×27 cm fused-silica capillary with a 20 s pressure injection (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa). Electrophoresis was performed at the voltage shown in each electropherogram. Running buffers were borate at pH 10.2 and phosphate at pH 2.5 at the concentrations indicated in the electropherograms. In the borate buffer based analysis, the capillary was sequentially washed between runs with 1 *M* sodium hydroxide and water (12 s of high pressure rinsing at 15 p.s.i.), followed by reconditioning with the running buffer for 1.2 min (high pressure rinsing at 15 p.s.i.). In the phosphate buffer condition, between runs the capillary was simply rinsed with buffer for 2 min.

3. Results and discussion

3.1. Derivatization of G_4 and 6′-SLN

6′-SLN was selected as a model linear oligosaccharide because it has GlcNAc at the reducing end and sialic acid at the non-reducing end, a usual combination found in most sialylated N-linked oligosaccharides released from glycoproteins. The preferred result for reductive amination is the high APTS labeling yield of 6′-SLN while the concomitant desialylation leading to the production of APTS-N-acetyllactosamine (APTS-LN) is minimized. To indicate the difference in reactivities during reductive amination for oligosaccharides having different sugars at the reducing and non-reducing ends

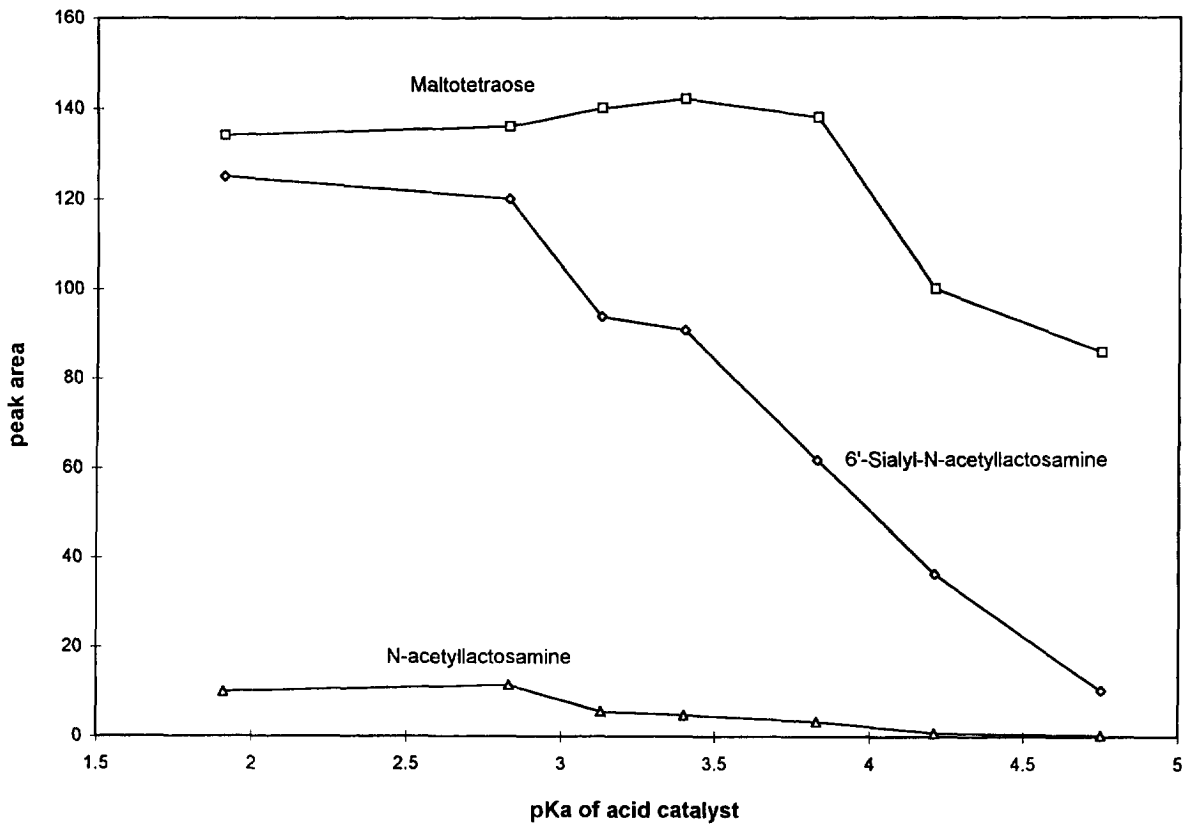

Fig. 1. Effect of organic acid pK_a on APTS labeling of 6′-SLN and G_4, as well as the formation of the desialylation product of N-acetyllactosamine at 37°C for 16 h. A mixture of 10 nmol G_4 and 10 nmol 6′-SLN was derivatized with 200 nmol APTS and 2 μmol $NaBH_3CN$ in a 6 μl reaction mixture containing 0.6 *M* organic acid. The reactions were terminated by the addition of 200 μl water and then diluted 25-fold by water for CE–LIF analysis. Separation buffer: 100 m*M* sodium borate pH 10.2; applied field strength 25 kV, current 17 μA; normal polarity.

(GlcNAc vs. Glc and Neu5Ac vs. Glc), similar derivatization conditions were used for G_4 in the same reaction mixture.

The results of the APTS labeling at 37°C for 16 h employing different organic acid catalysts are shown in Fig. 1. Significantly higher derivatization yields were obtained with all the lower pK_a organic acids compared to that produced in acetic acid. The increase in the 6′-SLN labeling efficiency relative to that in acetic acid ranged from three-fold in succinic acid (pK_a=4.21) to eight-fold in citric acid (pK_a= 3.13). The corresponding increase in yields of G_4-APTS, on the other hand, is only about 35% compared to that in the acetic acid reaction. The significantly greater increase in the labeling yield of GlcNAc compared to other sugars with increasing acid strength of the catalyst is in agreement with our earlier results obtained with a variety of monosaccharides [12]. The extent of desialylation based on the APTS-LN peak areas were all less than 10% in the 37°C, 16 h reaction (Fig. 2a), substantially lower than those observed with the 75°C, 1 h reaction (Fig. 2b). The peak due to the desialylated product APTS-LN was identified by monitoring the time-dependent

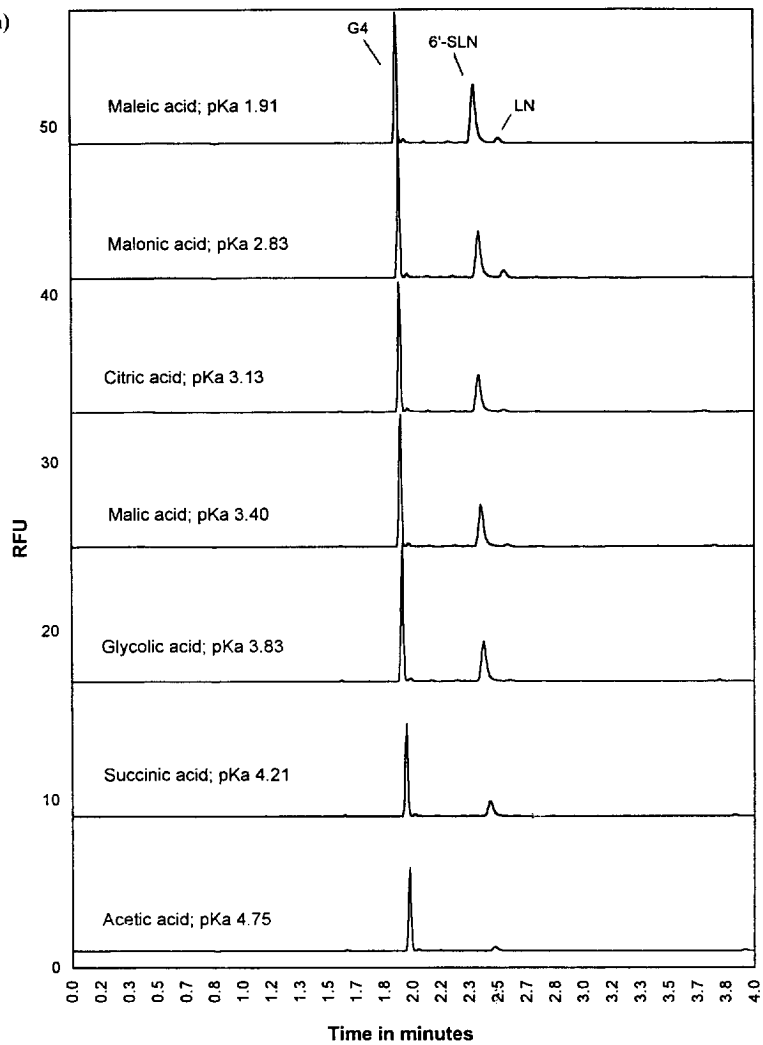

Fig. 2.

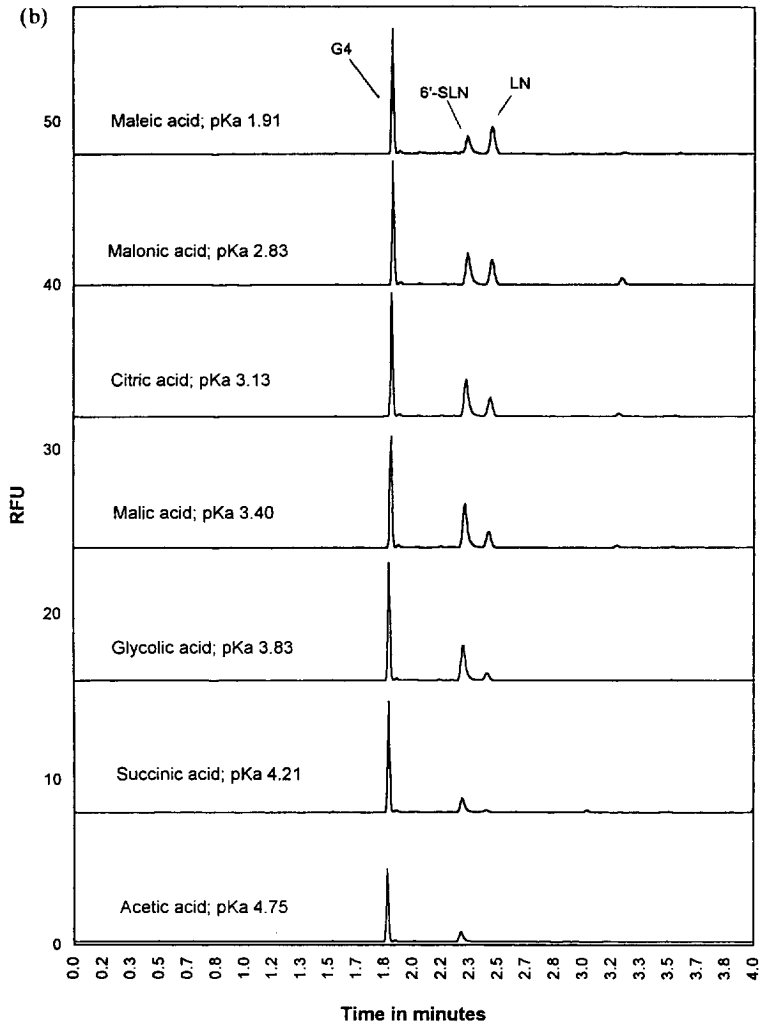

Fig. 2. (a) Effect of several organic acid catalysts on the APTS labeling of 6′-SLN and G_4 at 37°C for 16 h. Capillary electrophoresis separation conditions: as in Fig. 1. (b) Effect of several organic acid catalysts on the APTS labeling of 6′-SLN and G_4 at 75°C for 1 h. Capillary electrophoresis separation condition: as in Fig. 1.

changes in the electropherogram after addition of neuraminidase (EC 3.2.1.18) as reported for APTS-sialyllactose [17]. It can be observed that acid catalysts stronger than acetic acid yield more than 60% labeling of N-acetylamino sugars compared to G_4. Also note that the LN peak is slightly broader when compared to G_4, due to the stronger complexation between the borate and the galactose unit in this carbohydrate. With all the organic acids evaluated in this study, desialylation was found to be relatively low, ranging from 1.6% in acetic acid (pK_a=4.75) to 7.6% in maleic acid (pK_a=1.91) as the bottom curve depicts in Fig. 1. From the extent of labeling efficiency for 6′-SLN relative to G_4 and the degree of desialylation, our derivatization data suggests that derivatization at 37°C for 16 h in malic, citric and malonic acid appears to be a practical procedure for sialylated oligosaccharides. Alternatively, when a short labeling time is desired, the derivatization can be carried out at elevated temperatures if, for example, the carboxyl groups of the sialic acids are esterified prior to the reductive

amination. The resulting ester should be as able to withstand the acidic environment of the reductive amination as any neutral oligosaccharide.

3.2. Derivatization of branched N-linked glycans released from ribonuclease B and fetuin

The electropherograms of the APTS labeled oligosaccharides released by PNGase F from ribonuclease B and fetuin are shown in Figs. 3 and 4. Note that the signals for the oligosaccharides in both glycan pools having GlcNAc at the reducing ends, are about four to five times higher in malic, citric and malonic acids (acid catalysts with pK_a values of several units lower than acetic acid) compared to those obtained in acetic acid. Furthermore, it should also be noted, that the main peaks of the ribonuclease B and glycan pool have migration times in between those linear oligosaccharides containing seven to eleven glucose units when compared to the maltodextrin ladder. Fig.

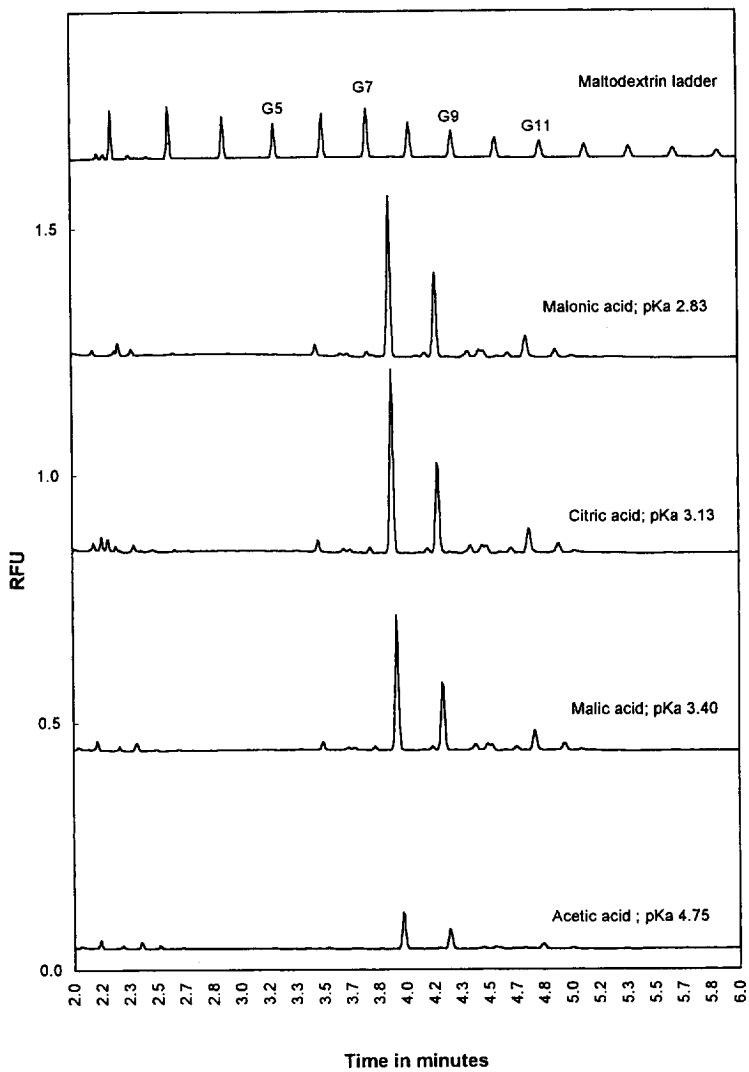

Fig. 3. Capillary electrophoresis separations of N-linked oligosaccharides released from ribonuclease B after APTS labeling in acetic, malic, citric and malonic acids. Separation buffer: 150 m*M* sodium phosphate pH 2.5; applied field strength 18 kV, current 27 μA; reversed polarity.

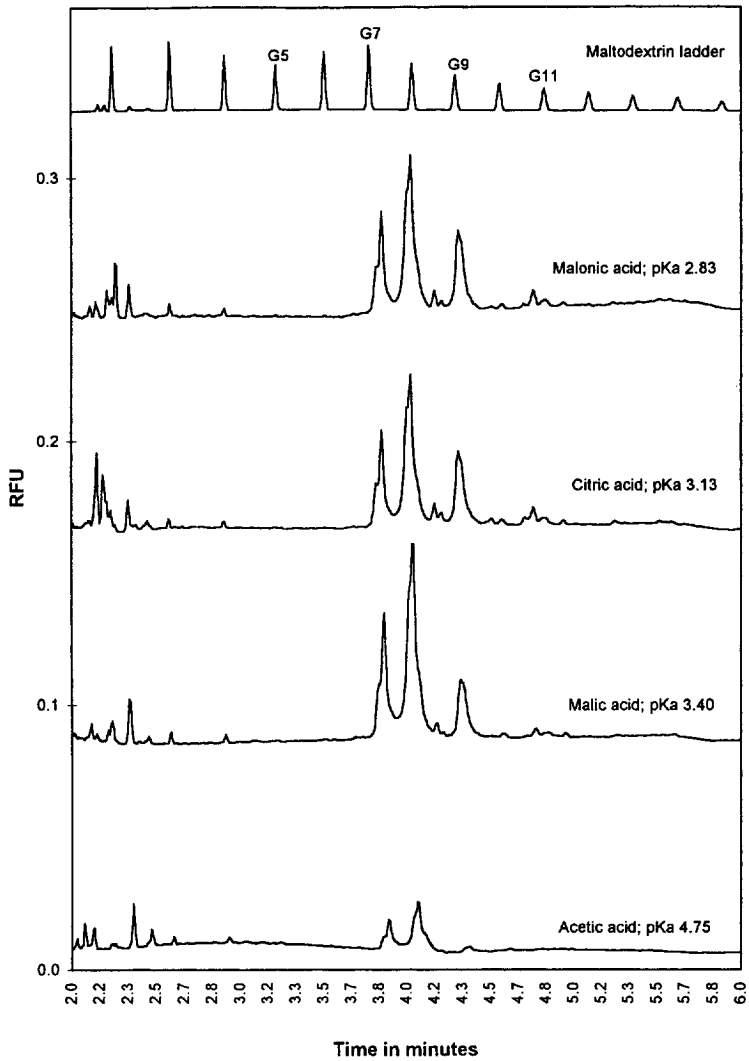

Fig. 4. Capillary electrophoresis separations of N-linked oligosaccharides released from fetuin after APTS labeling in acetic, malic, citric and malonic acids. Conditions: as in Fig. 3.

3 reveals that in the CE–LIF analysis of APTS labeled oligosaccharide, derived from ribonuclease B, the high mannose structures of Man-5 to Man-9 are very well resolved. The group of Man-7 peaks exhibits three distinctly different species corresponding to the three positional isomers suggested by Guttman et al. [18]. In the case of APTS labeled N-linked oligosaccharides released from fetuin, the degree of polymerization of the known major oligosaccharide components were found between seven to nine glucose units when compared to the maltodextrin ladder. Our results show that CE–LIF analysis using separation buffer around pH 2.0 produces a good correlation between migration time and the degree of polymerization for sialylated oligosaccharides, although at this low pH none of the positional isomers of the tri- and tetrasialylated triantennary structures were separated [19].

In summary, the above results show that the labeling efficiency is greatly improved for N-linked oligosaccharides with GlcNAc on the reductive end using catalysts more acidic than acetic acid, i.e.,

$pK_a < pK_a^{AcOH}$. Derivatization at 37°C with malic, citric and malonic acids produced high labeling yields with retention of more than 90% of sialic acid residues on the oligosaccharides. These results are consistent with the general acid catalysis step being the rate-limiting step in the reductive amination labeling procedures using APTS and the method may be applicable to the reductive amination of sugars with other derivatization agents.

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