H.p.l.c. of oligo(sialic acids)

Application to the determination of the minimal chain length serving as exogenous acceptor in the enzymic synthesis of colominic acid

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A rapid, sensitive and easy h.p.l.c. method was developed for the quantitative analysis of oligosialic acids. This procedure, which permits the complete separation (in 23 min) of several sialyloligomers with a degree of polymerization of between 1 and 16, has been employed to establish the minimal chain length of oligomer accepted, as an exogenous acceptor, by *Escherichia coli* K-235 sialyltransferase complex (ST) leading to the synthesis *in vitro* of colominic acid. We showed that this membrane-bound enzyme catalyses the direct transfer of Neu5Ac residues (one by one) from CMP-Neu5Ac to an exogenous acceptor molecule which contains at least three Neu5Ac residues. Free Neu5Ac or (Neu5Ac)₂ were not recognized as substrates, whereas the maximal rate of polymer elongation was achieved when (Neu5Ac)₅ was used as substrate.

INTRODUCTION

Sialic acids are substances widely distributed in Nature. They have been found in some viruses, bacteria, protozoa and echinoderms and in all species of vertebrates (Corfield & Schauer, 1982a). In bacteria, the presence of sialic acid has been identified as a pathogenic determinant in capsular antigens of *Neisseria meningitidis*, *Escherichia coli* and *Pasteurella haemolytica*, which cause important diseases in humans and animals (Silver & Vimr, 1990). These microbes produce a homopolymer of *N*-acetylneuraminic acid with α -2,8 linkages (colominic acid), α -2,9 linkages or with mixture of α -2,8 and α -2,9 links (Corfield & Schauer, 1982a). Their biosynthesis requires the formation of 5-*N*-acetylneuraminate (Neu5Ac), its activation to CMP-Neu5Ac, and the transference of the sialyl moiety from the latter to a polymeric acceptor (Corfield & Schauer, 1982b). The incorporation of Neu5Ac from CMP-Neu5Ac into polymeric products is catalysed by a membrane-associated sialyltransferase complex (ST), as indicated in Scheme 1. Although the biosynthesis of colominic acid has been studied for many years (Troy *et al.*, 1982; Ortiz *et al.*, 1989), little information has been reported about the nature of the molecules that can act either as endogenous or as exogenous acceptors. Rodríguez-Aparicio *et al.* (1988b) showed that a protein complex plays an important role in the elongation of the whole polymer. More recently, Weisgerber & Troy (1990) have identified this protein (20 kDa) as the true endogenous acceptor. It has also been reported that some poly(sialic acid) with an unknown degree of polymerization (DP) can act as exogenous acceptors (Troy *et al.*, 1982; Rodríguez-Aparicio *et al.*, 1988b; Ortiz *et al.*, 1989). However, until now there has not been any evidence to show whether short sialyl polymers can be directly elongated by receiving Neu5Ac residues from CMP-Neu5Ac or whether they must be elongated from the sialylated protein complex. Furthermore, the minimal length of sialyl polymer required to start the elongation has not yet been established.

Determination of the number of Neu5Ac residues requires a sound method to analyse Neu5Ac as well as oligosialic acids with different DPs. Previous methods using gel-filtration chromato-

graphy (Kwiatkowski *et al.*, 1983), DEAE-Sephadex (Jennings *et al.*, 1985), t.l.c. (Iwasaki *et al.*, 1990), electrophoresis (Pelkonen *et al.*, 1988) and h.p.l.c. (Hallenbeck *et al.*, 1987) are time-consuming and/or are limited in the size of the oligosialic acid that is resolved. In the present work we developed a very rapid and sensitive h.p.l.c. method that allows the separation of Neu5Ac and oligo(sialic acids) with a DP ranging between 2 and 16. We also demonstrate the applicability of the procedure to the identification of the minimal length of the sialyl oligomer that can be used as an exogenous acceptor by the sialyltransferase complex from *E. coli* K-235.

MATERIALS AND METHODS

Strain and growth conditions

E. coli K-235 (A.T.C.C. no. 13207) was obtained from the American Type Culture Collection. *E. coli* was maintained and cultured as reported elsewhere (Rodríguez-Aparicio *et al.* 1988a,b).

Purification of poly(sialic acid)

Cells grown as reported by Rodríguez-Aparicio *et al.* (1988a) were separated by centrifugation (10000 g; 10 min) and the clear supernatant was concentrated (15-fold) by ultrafiltration through a Millipore concentrator (Minitrom TM system model, exclusion size < 10000). The concentrate was dialysed (Medicell International LTD dialysis tubing; exclusion size < 10000 *M_r*) against 100 vol of distilled water (4 °C, 24 h) and then freeze-dried. This sample was resuspended in 10 ml of water and treated with 0.75 % (w/v) streptomycin sulphate. After the mixture had been stirred for 30 min, the precipitate was removed by centrifugation at 10000 g for 20 min and discarded.

Hexadecyltrimethylammonium bromide (2 %, w/v) was added to the above supernatant and the resulting precipitate, containing poly(sialic acid), was removed by centrifugation (10000 g) for 10 min and resuspended with 5 ml of 1 M-CaCl₂. Then poly(sialic acid) was precipitated with 2.5 vol. of ethanol at -20 °C (2 h) and separated by centrifugation as described above. This operation was repeated twice to ensure a high degree of purification.

Abbreviations used: DP, degree of polymerization; ST, sialyltransferase complex.

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Scheme 1. Biosynthesis of colominic acid

$m-y$ is the minimal size of sialyl polymer that always remained bound to ST.

Fig. 1. H.p.l.c. separation of Neu5Ac and sialyl oligomers with different DP values (2-16)

CMP-Neu5Ac has a retention time of 4.8 min (results not shown). AUFS, absorbance (220 nm) units full scale.

The final pellet, containing poly(sialic acids), was resuspended in water, dialysed and freeze-dried. The quantity of poly(sialic acid) was determined by the Svennerholm (1958) method. Purity was higher than 95%.

Preparation of oligo(sialic acids)

Oligo(sialic acids) from *E. coli* K-235 were obtained by partial hydrolysis of the polysaccharides using the procedure of Jennings *et al.* (1985).

Fig. 2. A plot of ln(sialyl oligomer DP) against retention time

H.p.l.c. of sialyl oligomers

H.p.l.c. was performed by using a Spectra-Physics model SP 8800 chromatograph equipped with a variable wavelength UV/Vis detector SP 8450, a computing integrator SP 4290 and an anion exchange column (Zorbax SAX; 250 mm $\times$ 4.6 mm internal diameter). The column effluent was monitored at 220 nm.

Samples (100 μ l) containing 35 μ g of poly(sialic acid) were applied to the column previously equilibrated with 10 mM-phosphate, pH 3.5, containing 0.2 M-NaCl. The column was eluted first with a linear NaCl gradient from 0.2 to 1 M in 10 mM-phosphate, pH 3.5, for 5 min, and then a 1 M-NaCl concentration was maintained until the chromatography was completed. Flow rates was 1.5 ml/min over 10 min and then 3 ml/min until the end of chromatographic run.

Fig. 3. H.p.l.c. analysis of the different reaction products obtained when ST was incubated with sialyl oligomers containing three to six Neu5Ac residues for 0, 30, 60 and 120 min

Numbers indicated DP values (Neu5Ac residues).

Enzyme assay procedures

Cell-free extracts and ST were obtained as previously described (Troy *et al.*, 1982; Ortiz *et al.*, 1989). Enzyme fractions were prepared fresh daily.

Sialyltransferase activity was determined by either measuring the incorporation of [14 C]Neu5Ac from CMP-[14 C]Neu5Ac into

polymeric products (Ortiz *et al.*, 1989), or by measuring the transferase of Neu5Ac to oligo(sialic acids) with different DP values (exogenous acceptor). With this latter procedure, non-radiolabelled CMP-Neu5Ac (33 mM) was required. Samples of 75 μ g of oligo(sialic acids) were routinely used. After incubating the reaction mixtures for a preset time, membrane proteins were precipitated with 100 %-satd. $(\text{NH}_4)_2\text{SO}_4$ and the elongated sialyl

Fig. 4. Elongation of sialyl oligomers [(Neu5Ac)₃₋₈] (a-f) when incubated with ST and CMP-Neu5Ac for different times

The numbers indicate the DP values (Neu5Ac residues).

oligomers (which remain in the supernatant) were analysed by h.p.l.c. as described above.

RESULTS AND DISCUSSION

Different methods for the separation and analysis of oligo(sialic acids) has been reported. Thus PAGE analysis of capsular polysaccharides from *E. coli* was performed by Pelkonen *et al.* (1988); ion-exchange chromatography on DEAE-Sephadex A25 of free oligosaccharides or alditol oligosaccharides was described by Jennings *et al.* (1985) and Finne & Makela (1985) respectively. Other methods, based on t.l.c. (Iwasaki *et al.*, 1990) and gel permeation (Kwiatkowski *et al.*, 1983) have been reported, whereas an h.p.l.c. procedure, using a Mono-Q HR column, was developed by Hallenbeck *et al.* (1987). Although these methods have been applied with success for the study of sialic acids, all of them are disadvantageous with regard to the time required to separate those compounds. Thus, DEAE-Sephadex and gel permeation are carried out from 1 to 9 days per column run, and t.l.c. lasts several hours. Furthermore, these systems do not provide a suitable separation of many sialic acids and their derivatives, since by gel filtration, for example, it is only possible to separate oligosialic acids with a DP of 7, whereas t.l.c. only efficiently resolves oligo(sialic acids) with up to nine residues. On the other hand, the h.p.l.c. procedure described by Hallenbeck *et al.* (1987), although a good preparative procedure, presents several drawbacks when used as an analytical method, since: (a) the total amount of poly(sialic acid) used/run is very high (2.5 mg) and (b) to increase detection sensitivity, sialyl oligomers must be labelled with KB³H₄. This involves a lengthy treatment which requires several hours to prepare the standards and the samples.

The procedure described in the present paper avoids most of

the above disadvantages. Thus it is rapid (23 min per run); it does not need any transformation of oligo(sialic acids); it is very sensitive (30 µg of oligosialic acids is usually employed), and samples can be estimated directly by measuring A_{220} . Fig. 1 shows the profile of the appearance of the Neu5Ac and sialyl oligomers (purified by ion-exchange chromatography) containing a different number of Neu5Ac residues (between 1 and 16), whereas in Fig. 2 is represented the lnDP of sialyl polymers plotted as a function of the retention time. It can be observed that resolution decreases as the molecular mass increases (DP between 1 and 16). However, a good resolution even for oligosialic acids with DP > 16 could be obtained by changing the gradient and the flow rate (results not shown).

The method reported here also allows the separation of Neu5Ac from CMP-Neu5Ac and (Neu5Ac)₂, whereas, with other previously reported procedures (Hallenbeck *et al.*, 1987), sialic acid (Neu5Ac) and (Neu5Ac)₂ are eluted together.

Since this method allows a good separation and evaluation of CMP-Neu5Ac, Neu5Ac and oligosialic acids with a DP ranging between 2 to 16, it has been used for studying *in vitro* the biosynthesis of colominic acid.

We applied the above procedure to identify the minimal size of sialyl oligomer that can be used as an exogenous acceptor by *E. coli* K-235 sialyltransferase. This enzyme efficiently transfers Neu5Ac residues from CMP-Neu5Ac to an exogenous acceptor containing three or more sialyl residues (Figs. 3 and 4), the incorporation being maximal when (Neu5Ac)₅ was used as substrate. However, neither Neu5Ac nor di(sialic acid) serve as acceptors in the elongation reaction, and sialyl oligomers were therefore not produced (results not shown).

Figs. 3 and 4 show the time course of the appearance of the newly synthesized molecules [elongated oligo(sialic acids)] when sialyltransferase was incubated with different acceptors containing three to eight sialyl residues. It can be observed that: (a)

Fig. 5. Sialylation of ST when incubated with CMP-[¹⁴C]Neu5Ac in the presence (●) or absence (△) of exogenous acceptor [(Neu5Ac)₅]

the disappearance of each sialyl oligomer implies the formation of a different one containing a new Neu5Ac residue attached to the original molecule; and (b) the DP increases very slowly. From these results we conclude that Neu5Ac residues are not transferred in blocks, but rather one by one, suggesting that the synthesis of native poly(sialic acid) (colominic acid) is carried out, at least *in vitro*, by direct transfer of the Neu5Ac moiety from the CMP-Neu5Ac to the exogenous acceptor. Furthermore, when ST was incubated with CMP-[¹⁴C]Neu5Ac in the presence of a short exogenous acceptor [(Neu5Ac)₅], no sialylation of the protein was detected (see Fig. 5), suggesting, again, the direct transfer of Neu5Ac units from the CMP-Neu5Ac to the exogenous acceptor. However, it has been reported that, in the absence of the exogenous acceptor, formation of an endogenous acceptor (sialylated protein) is required (Rodríguez-Aparicio *et al.*, 1988b; Ortiz *et al.*, 1989; Weisberger & Troy, 1990).

The mechanism of colominic acid biosynthesis would presumably involve the existence of an enzyme (or enzymic system) that catalyses the formation of an oligosialic acid (primer) which contains at least three residues of Neu5Ac. A similar model has been reported for the biosynthesis of other sialyl polymers (trimer in CHP-134 cells; Livingston *et al.*, 1988) as well as for a different oligosaccharide (glycogen). In the latter case two

different enzymes subsequently catalyse: (a) the synthesis of the primer (glycogenin) and (b) the elongation of the polymer (glycogen synthetase) (Pitcher *et al.*, 1987, 1988). Obviously this question remains an object of further research in this field.

The findings shown above complete the previous results about colominic acid biosynthesis (Troy *et al.*, 1982; Rodríguez-Aparicio *et al.*, 1988a,b; Ortiz *et al.*, 1989), and suggest that the h.p.l.c. method here reported could be useful for the study of other enzymic activities involved in the biosynthesis of different Neu5Ac (or derivatives)-containing polymers.

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REFERENCES

- Corfield, A. P. & Schauer, R. (1982a) in *Sialic Acids: Chemistry, Metabolism and Function* (Schauer, R., ed.), pp. 5–50, Springer-Verlag, New York
- Corfield, A. P. & Schauer, R. (1982b) in *Sialic Acids: Chemistry, Metabolism and Function* (Schauer, R., ed.), pp. 195–265, Springer-Verlag, New York
- Finne, J. & Makela, P. H. (1985) *J. Biol. Chem.* **260**, 1265–1270
- Hallenbeck, P. C., Yu, F. & Troy, F. A. (1987) *Anal. Biochem.* **161**, 181–186
- Iwasaki, M., Inoue, S. & Troy, F. A. (1990) *J. Biol. Chem.* **265**, 2596–2602
- Jennings, H. J., Roy, R. & Michon, F. (1985) *J. Immunol.* **134**, 2651–2657
- Kwiatkowski, B., Boschek, B., Thiele, H. & Stirm, S. (1983) *J. Virol.* **45**, 367–374
- Livingston, B. D., Jacobs, J. L., Glick, M. C. & Troy, F. A. (1988) *J. Biol. Chem.* **263**, 9443–9448
- Ortiz, A. I., Reglero, A., Rodríguez-Aparicio, L. B. & Luengo, J. M. (1989) *Eur. J. Biochem.* **178**, 741–749
- Pelkonen, S., Hayrinen, J. & Finne, J. (1988) *J. Bacteriol.* **170**, 2646–2653
- Pitcher, J., Smythe, C., Campbell, D. G. & Cohen, P. (1987) *Eur. J. Biochem.* **169**, 497–502
- Pitcher, J., Smythe, C. & Cohen, P. (1988) *Eur. J. Biochem.* **176**, 391–395
- Rodríguez-Aparicio, L. B., Reglero, A., Ortiz, A. I. & Luengo, J. M. (1988a) *Appl. Microbiol. Biotechnol.* **27**, 474–483
- Rodríguez-Aparicio, L. B., Reglero, A., Ortiz, A. I. & Luengo, J. M. (1988b) *Biochem. J.* **251**, 589–596
- Silver, R. P. & Vimr, E. R. (1990) in *Molecular Basis of Bacterial Pathogenesis* (Iglewski, B. H. & Clark, V. L., eds.), pp. 39–60, Academic Press, New York
- Svennerholm, L. (1958) *Acta Chem. Scand.* **12**, 547–554
- Troy, F. A., Vijay, I. K., McCloskey, M. A. & Rohr, T. E. (1982) *Methods Enzymol.* **83**, 540–548
- Weisberger, C. & Troy, F. A. (1990) *J. Biol. Chem.* **265**, 1578–1587

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