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Carbohydrate Research 328 (2000) 77–83

CARBOHYDRATE RESEARCH

The use of the 2-aminobenzoic acid tag for oligosaccharide gel electrophoresis

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Received 18 October 1999; accepted 17 January 2000

Abstract

Gel electrophoresis of fluorophore labeled saccharides provides a rapid and reliable method to screen enzymatic and/or chemical treatments of polysaccharides and glycoconjugates, as well as a sensitive and efficient microscale method to separate and purify oligosaccharides for further analysis. A simple and inexpensive method of derivatization and analysis using 2-aminobenzoic acid (anthranilic acid, AA) is described and applied to the extracellular polysaccharide released by the desiccation tolerant cyanobacterium *Nostoc commune* DRH-1. The results of these analyses suggest a possible protective functionality of two pendent groups, as well as a potential relationship between these groups and the desiccation tolerance of the organism.

Keywords: Anthranilic acid (2-aminobenzoic acid); Gel electrophoresis; Oligosaccharide; *Nostoc*; Cyanobacteria; Driselase

1. Introduction

The reductive amination of the reducing end of carbohydrates with fluorescent tags has become a widely used technique for improving the sensitivity limitations associated with this class of compounds [1–15]. A wide variety of fluorophores have been reported, ranging from 2-aminobenzoic acid (anthranilic acid, AA) to 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) (for reviews, see [1–3]). Such derivatives can be separated and quantified by high-performance liquid chromatography (HPLC) [4–7], capillary electrophoresis [8–12] and gel electrophoresis [5,13–15], and further analyzed by MALDI-TOF mass spectrometry [5,16], or by a combination of these methods [17,18]. With the availability of fluorescent tags, it has now become possible to routinely separate and quantify carbohydrates at picogram levels.

The use of fluorescent tags does have some limitations. For example, long separation times (>60 min) are required for the HPLC separations of many labeled carbohydrates. For the highest sensitivity, capillary electrophoresis makes use of laser-induced fluorescence detection [9,11], which is not commonplace in most laboratories doing carbohydrate work. Gel electrophoresis is very useful for comparing different treatments and/or isolates [14], but does not provide the level of quantification needed in many investigations.

Our research efforts are currently involved in unraveling the role of the complex polysaccharides present in the extracellular matrix of mucilaginous extremophiles, most notably

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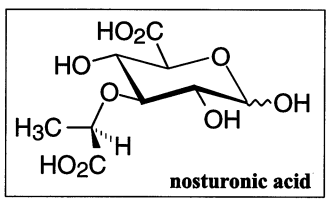
0008-6215/00/$ - see front matter
PII: S0008-6215(00)00045-8

cyanobacteria. In the course of this work, we have encountered a wide array of polysaccharide structures, each requiring a different set of protocols for preparation of the oligosaccharides necessary for structural determination. In these cases the amount of sample available is not limiting, the limiting factor is the total number of extremophiles and associated polysaccharides that need to be analyzed. During the course of these investigations, it became necessary to develop a relatively simple, rapid and inexpensive screening protocol for assessing oligosaccharide purity and the extent of degradation upon exposure of the polysaccharides to various depolymerization regimes (enzymatic, chemical, or both).

One of the extremophile organisms we have been investigating is the cosmopolitan cyanobacterium *Nostoc commune*, which produces large amounts of extracellular polysaccharide (EPS). This EPS has been strongly implicated in *Nostoc*'s desiccation tolerance [19,20], and may also have a role in physiological processes such as colony morphogenesis [21]. The polysaccharide released by *N. commune* DRH-1 was recently characterized [20], with the structure containing β-D-ribofuranosyl and β-3-*O*-[(*R*)-1-carboxyethyl]-D-glucuronosyl (nosturonic acid, NosA) pendant groups on a (1→4)-linked xylogalactoglucan backbone. During the structural elucidation of this EPS, we isolated and characterized a disaccharide (**1**), a trisaccharide (**2**), and a mixture of two closely related pentasaccharides (**3** and **4**), which, along with several other isolates, led to the EPS repeat unit shown as **5** [20].

Here we report a fluorescent derivatization method of carbohydrates using anthranilic acid (AA) in aqueous solution and its practical application in the polyacrylamide gel electrophoresis of oligosaccharides derived from *N. commune* DRH-1 EPS by partial acid hydrolysis and enzymatic degradation.

1 β-D-Glc*p*-(1→4)-D-Xyl*p*
β-D-NosA
1
↓
6
2 β-D-Glc*p*-(1→4)-D-Gal*p*
β-D-NosA
1
↓
6
3 β-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Xyl*p*
β-D-NosA
1
↓
6
4 β-D-Glc*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Gal*p*
β-D-NosA
1
↓
6
5 β-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Xyl*p*
3
↑
1
α-D-Rib*f*



nosturonic acid

2. Results and discussion

Derivatization of oligosaccharides with AA in aqueous solution.—Previously reported methods involved labeling with AA in organic solvents [4,5]. However, we found that higher molecular weight oligosaccharides were relatively insoluble in MeOH at the concentration used, and were difficult to recover from dimethyl sulfoxide (Me_2SO). Sato et al. [22] optimized AA-labeling of monosaccharides for capillary electrophoresis, including reaction solvent, pH, temperature and reaction time. They suggested aqueous 0.2 M AA and 1.0 M $NaBH_3CN$ as the optimum conditions for the reaction. We found this condition worked well for our oligosaccharides and small EPS fragments and no degradation occurred at the temperature used. Although AA is hard to dissolve in water, it does dissolve well when heated in the presence of $NaBH_3CN$ (Sato, personal communication). Therefore, the aqueous $NaBH_3CN$ is prepared first, and then the AA reagent is dissolved in this solution by heating at the reaction temperature (65 °C).

Recovery of AA-tagged saccharides.—Precipitation with acetonitrile (MeCN, final concentration $>85\%$ by volume) made it convenient to collect the AA tagged mono- and oligosaccharides, most of which were attached to the wall of the container after addition of MeCN and vortexing. This precipitate, after careful removal of MeCN solution, can be used directly for gel electrophoresis, although the remaining solids in the liquid can easily be obtained by microcentrifuge. Wash-

ing of the recovered precipitates with 95% MeCN (3 ×) eliminates most of the reaction by-products.

Chemical and enzymatic degradation.—AA-labeled oligosaccharides can be used in the reagent-array analysis method (RAAM) [23] for oligosaccharide structural elucidation as has been demonstrated with other fluorophore tags [14]. Electrophoretic analysis of AA-tagged saccharides could also be used in conjunction with chemical and/or enzymatic degradation in order to screen polysaccharides and glycoconjugates for their stability to various hydrolytic regimes. For instance, together with acid hydrolysis, AA-labeling and gel electrophoresis could be used to determine the possible differences in EPS structure from different extremophile organisms or the same organism in different habitats, or used as a routine procedure to monitor and/or modify recombinant glycosylated proteins (glycoengineering).

As an example, Fig. 1 presents the electrophoretic separation of AA-tagged oligosaccharides from *Nostoc* EPS. As can be seen in Lanes 2–4, AA-labeled **1**–**4** (obtained from *N. commune* DRH-1 EPS by partial acid hydrolysis and gel-filtration chromatography) were resolved based upon size and charge [20]. Due to the presence of two carboxyl groups in NosA, the penta- and trisaccharides moved much faster than the disaccharide. Based on this established 'library', we could assay the enzymatic degradation of the oligosaccharides as shown on Lanes 6 and 7. AA-tagged **1** (Lane 4) was cleaved by β-glucosidase (EC 3.2.1.210) to glucose and xylose-AA, as shown on Lane 6. In a TLC-based separation, glucose was also detected (data not shown). The two co-migrating pentasaccharides (**3** and **4**) after Driselase digestion gave a series of products (Lane 7), including unreacted pentasaccharide and trisaccharide **2**. Based on our structural work [20], we assume that the unreacted pentasaccharide was **3**, whereas **4** was degraded. Based upon this difference in enzymatic susceptibility, Driselase could be used to prepare **3** from the mixture, while **4** would be hydrolyzed to **2**.

Protective functionality of D-*ribofuranose and nosturonic acid in native* N. commune *EPS.*—Driselase is a fungal enzyme preparation containing exo- and endopolysaccharidases including cellulase, laminarinase and xylanase, and has been used in degradation and structural characterization of plant polysaccharides [24]. Driselase is ineffective at depolymerizing the *N. commune* DRH-1 EPS, which is not surprising considering the stability of extremophile polysaccharides in their native environment. In order to investigate whether or not partial acid hydrolysis would

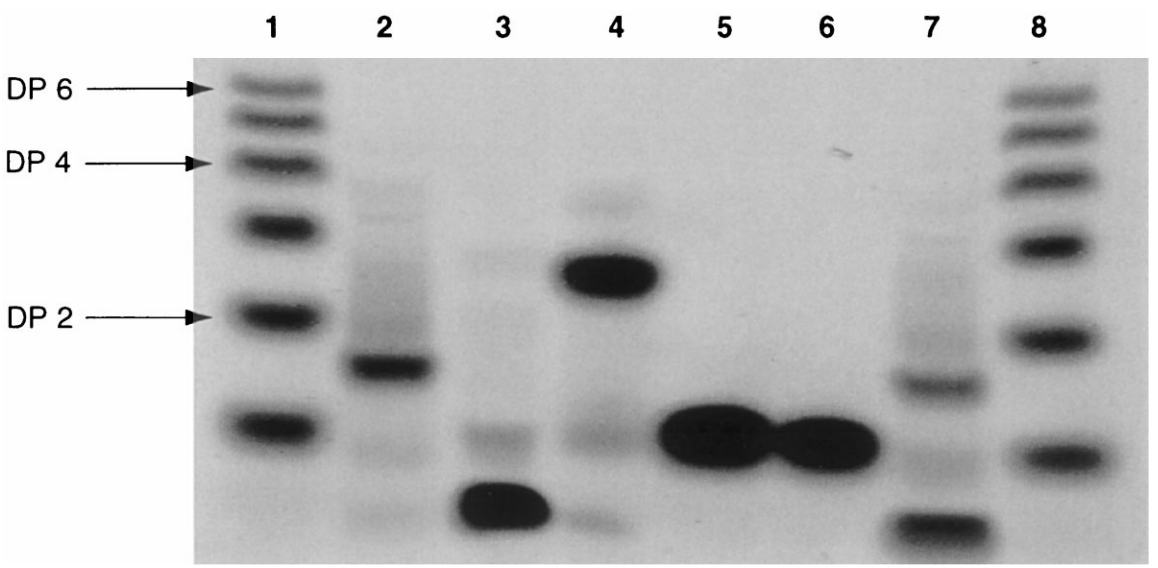


Fig. 1. Gel image of the electrophoretic separation of AA-labeled cello-oligosaccharides and *Nostoc* EPS oligosaccharides. Lanes 1 and 8: cello-oligosaccharide ladder, DP 1–6. Lane 2: AA-labeled **3** and **4**. Lane 3: AA-labeled **2**. Lane 4: AA-labeled **1**. Lane 5: AA-labeled xylose. Lane 6: AA-labeled **1** after Driselase digestion. Lane 7: AA-labeled **3** and **4** after Driselase digestion.

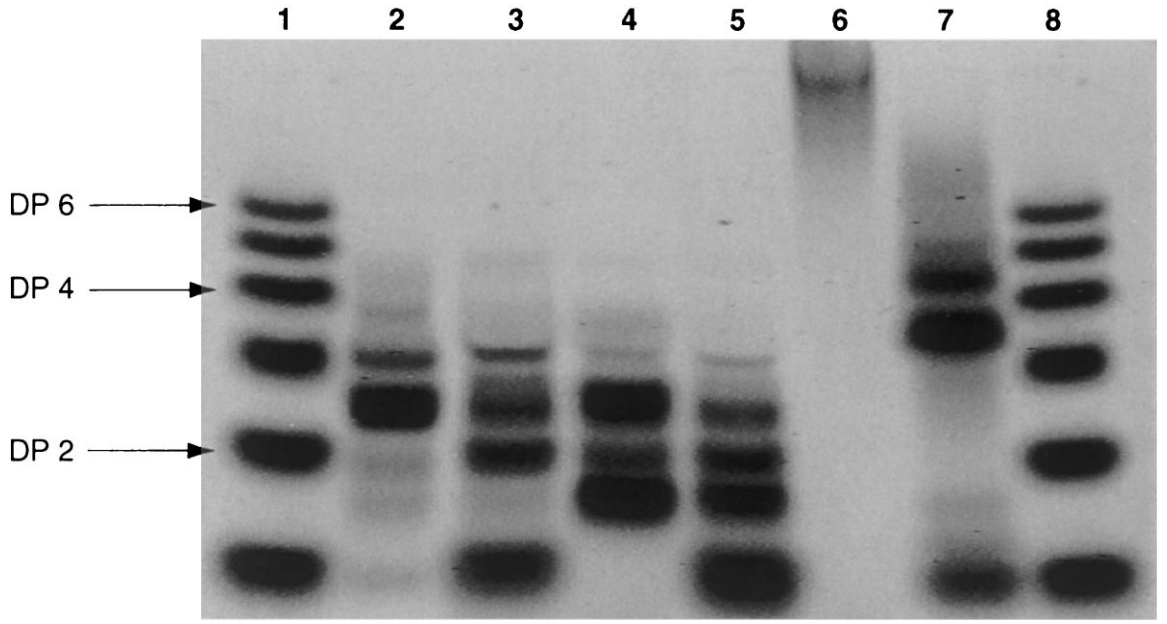


Fig. 2. Gel image of several AA-labeled and digested oligosaccharides. Lanes 1 and 8: cello-oligosaccharide ladder, DP 1–6. Lane 2: acid hydrolyzate *Nostoc* EPS, Bio-Gel P2 purified (intermediate molecular weight fraction) and AA-tagged. Lane 3: oligosaccharides of Lane 2 after Driselase treatment and AA-labeling. Lane 4: acid hydrolyzate *Nostoc* EPS, Bio-Gel P2 purified (low molecular weight fraction) and AA-tagged. Lane 5: oligosaccharides of Lane 4 after Driselase treatment and AA-labeling. Lane 6: lithium–ethylenediamine treated *Nostoc* EPS after BioGel P2 purification and AA-labeling. Lane 7: oligosaccharides after the Driselase digestion and AA-labeling of the Li–EDA-treated glycan of Lane 6.

provide a material more amenable to Driselase, the EPS was hydrolyzed and subsequently purified by size-exclusion chromatography. While the conditions chosen (80 °C, 1 M TFA) provided extensive depolymerization, the most significant effect was complete removal of all ribofuranosyl pendant groups. Several 'ribose-free' fractions were obtained, including one with a molecular weight slightly higher than that of **3** and **4** (Fig. 2, Lane 2). This fraction was digested with Driselase, AA-labeled and analyzed by gel electrophoresis (Fig. 2, Lane 3). In addition, a slightly lower molecular weight fraction from the acid hydrolysis and chromatograhic purification (Lane 4) was also treated in the same fashion (Lane 5). Clearly, the ribose-free oligosaccharide preparations were degraded by Driselase. In order to explain the stability of extremophile polysaccharides in the field, one could theorize that the ribofuranosyl moiety is a 'protecting group' on the xylogalactoglucan backbone. If this is the case, then one could potentially stabilize other polysaccharides by attaching ribofuranosyl pendant groups.

Driselase was also effective in hydrolyzing the EPS after removal of a majority of the uronosyl pendant groups via a lithium–ethylenediamine treatment [25]. The NosA-free glycan (Lane 6) was degraded to oligomers (Lane 7), again suggesting that hydrolase activity can occur after the 'protecting' moieties are chemically removed.

AA-tags for assessing fraction purity and performing structural work.—AA-labeling and PAGE analysis of oligosaccharides obtained by chemical and/or enzymatic treatment in combination with silica TLC (butanol–formic acid–water, naphthoresorcinol staining), are also useful in assessing the purity of gel-filtration fractions before submitting them to further analysis (NMR and/or MALDI). TLC alone in many cases is fully adequate for monitoring purposes, but does not have the resolution of the AA method. Furthermore, the oligosaccharide extracted from an excised gel band may be submitted to direct analysis by MALDI-TOF mass spectrometry [26]. The relatively small AA-tag is more amenable to mass spectroscopy than the larger tags such as ANTS and APTS. The utility of the AA-methodology for assessing fraction purity can be seen in Fig. 3. Bio-Gel P2 purification of a Driselase-treated sample provided tubes,

which were combined based on TLC analysis. Portions of the fractions were then AA labeled and analyzed. The resolution into different bands by gel electrophoresis provides a profile of the combined fractions, indicating which samples would be amenable to NMR analysis. Furthermore, each of the bands could be submitted directly to MALDI-TOF analysis, suggesting the method could be very useful for microscale purification and analysis.

Due to the relatively low cost and simplicity of the AA-derivatization procedure, in cases where sample limitation is not an issue, one can also perform the reaction on the 5–15 mg scale and submit the purified tagged oligosaccharides to NMR spectroscopic analysis. The reductive amination will have eliminated anomerization, providing for cleaner spectra. Furthermore, the ratio of aromatic protons to anomeric protons would provide a reasonable estimation of the degree of polymerization of the oligosaccharide.

Summary.—Gel electrophoresis of anthranilic acid tagged oligosaccharides provides a rapid and reliable method to screen enzymatic and/or chemical treatments of polysaccharides and glycoconjugates, and can also be used as a sensitive and efficient microscale method to separate and purify oligosaccharides. Anthranilic acid is an inexpensive reagent (e.g., compared with APTS, ANTS), and the resulting gels can be analyzed in most commercially available imaging systems.

3. Experimental

Materials.—Anthranilic acid, ethylenediamine, lithium wire, sodium cyanoborohydride and xylose were purchased from Aldrich Chemical Co. (Milwaukee, WI). β-Glucosidase (EC 3.2.1.21, from almonds), β-xylosidase (EC 3.2.1.37, from *Aspergillus niger*), α-galactosidase (EC 3.2.1.22; from green coffee beans), Driselase (from Basidiomycetes), glucose, cellobiose and xylobiose were purchased from Sigma Chemical Co. (St. Louis, MO). Cellotriose, cellotetraose, cellopentaose and cellohexaose were purchased from V-Labs Inc. (Covington, LA). All other reagents were of the highest purity available.

Oligosaccharide preparation.—Oligosaccharides from *Nostoc commune* DRH-1 EPS were primarily prepared by partial acid hydrolysis (1 M TFA, 80 °C), followed by gel-filtration chromatography on a Bio-Gel P2 column, as described previously [20]. Uronic acid-free glycan was prepared by selective cleavage with

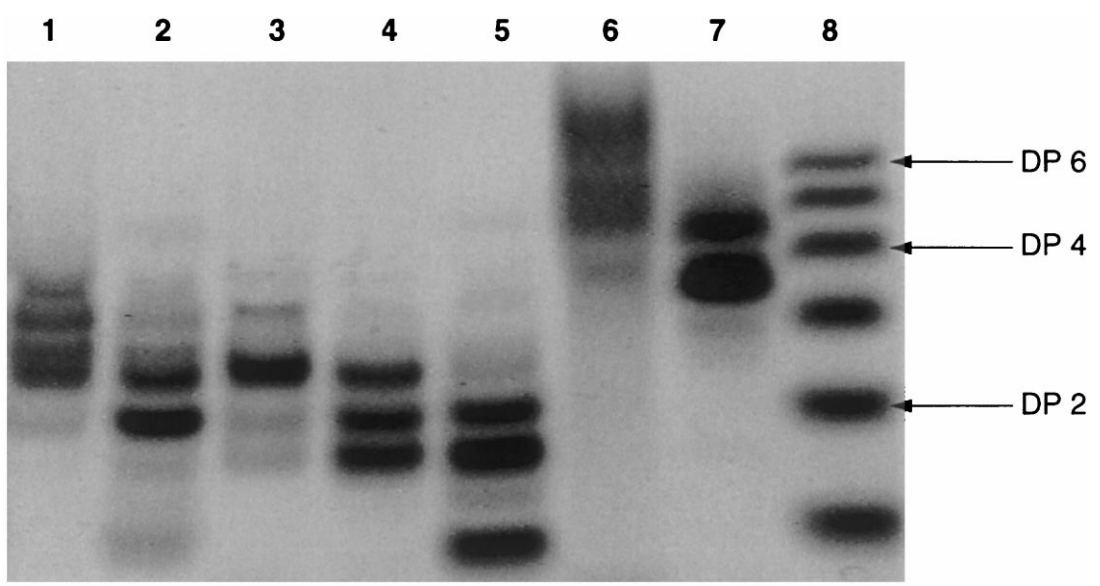


Fig. 3. Use of gel electrophoresis for analyzing column fractions. Lanes 1 and 2: fractions obtained from a Bio-Gel P2 purification and labeling of the Fig. 2. Lane 3 oligosaccharides. Lanes 3–5: fractions obtained from the sample shown in Fig. 2. Lane 5 after Bio-Gel P2 purification into three fractions. Lane 6 and 7: Li–EDA-treated glycan after Driselase digestion (Fig. 2, Lane 7), followed by Bio-Gel P2 purification into high (Lane 6) and low (Lane 7) molecular weight fractions. Lane 8: cello-oligosaccharide ladder.

lithium treatment of the EPS in ethylenediamine [25] and isolated on the Bio-Gel P2 column. The purified oligosaccharides or mixtures of oligosaccharides were then subjected to enzymatic digestion and/or labeling with AA as stated below.

AA labeling of saccharides.—Saccharide labeling with AA in aq solvent was performed according to Sato et al. [22], and sample cleanup was done following Anumula and Dhume [26] with minor modifications. The AA solution was prepared in aq 1.0 M $NaBH_3CN$ by heating at 65 °C with stirring. For a typical reaction (1 mg scale) 0.2 M AA (100–150 μL) was added to the sample in a small amber reaction vial containing a microstir bar. The reaction was performed in a Reacti-Therm (Pierce Chemical Co.) with stirring (65 °C, 2 h) and then cooled to room temperature. Upon the addition of MeCN (6 vol) and vortexing, most precipitates were attached to the bottom or on the wall of the vial. The liquid was then transferred with a Pasteur pipette to a microcentrifuge tube and centrifuged for 2 min. The supernatant was carefully removed; special attention was paid to the tiny jelly-like drops at the bottom, which should not be removed. The vial and the microcentrifuge tube were washed with 95% MeCN (3 × 1 mL) in the same way. The precipitates in the microcentrifuge tube, Pasteur pipette and reaction vial were then taken up with water (3 × 0.3 mL) into a small amber vial or a microcentrifuge tube, and freeze-dried. Freeze-dried samples were stored in freezer until use. The AA-labeled oligosaccharides were submitted to PAGE analysis, enzymatic digestion or gel-filtration chromatography on a Bio-Gel P2 column eluted with deionized water and monitored by UV (254 nm) and TLC [20].

Enzymatic digestion.—AA labeled or non-labeled oligosaccharides were digested with Driselase or combinations of exoglycosidases (β-glucosidase, β-xylosidase, α-galactosidase) at 37 °C using 25 mM NaOAc (pH 5.0) as the buffer. The reaction was monitored by TLC [20] and terminated at an appropriate time by boiling for 2 min. The products were freeze-dried, AA labeled when appropriate, and then analyzed by gel electrophoresis or separated on a Bio-Gel P2 column.

Polyacrylamide gel electrophoresis.—Gels of 20% polyacrylamide (1.5 mm thick, 70 mm high and 80 mm wide) were prepared in 1.5 M Tris (pH 8.0). Samples (2–10 μL, saccharide concentration less than 10μg/μL) were loaded with 0.005% bromophenol blue in 15% aq glycerol, and the gels were run in TBE/SDS buffer (pH 8.5) at 45 mA for 45 min. The gels were then imaged and documented with either a Glyko FACE® Imager (SE2000, 600 nm) or an Alpha Innotech ChemImager (Hoechst Blue filter).

Acknowledgements

The authors are grateful for the support of DARPA (Controlled Biological Systems), Grant # N00173-98-1-G005, and would also like to thank Dr K. Anumula (SmithKline Beecham Pharmaceuticals) for several helpful correspondences.

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