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Biosynthesis of hetero-polysaccharides by *Acetobacter xylinum* - Synthesis and characterization of metal-ion adsorptive properties of partially carboxymethylated cellulose

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Abstract

Biological reconstruction of water-soluble carboxymethylated cellulose (CMC; D.S. = 0.47) has been achieved by culturing *Acetobacter xylinum* in medium containing CMC and D-glucose to give a novel hetero-polysaccharide having a carboxymethyl function. The novel extracellular polysaccharide, carboxymethylated-bacterial cellulose (CM-BC), had an ion exchange ability with enhanced specific adsorption for lead and uranyl ions compared to the original CMC and bacterial cellulose. The contribution of the hydroxy group at C-2 was confirmed by applying carboxymethylated chitin, which possesses acetamido group at C-2 of the glucose residue, as the carbon source of the incubation.

Keywords: Biosynthesis; Hetero-polysaccharides; *Acetobacter xylinum*; Carboxymethylated cellulose

1. Introduction

The biosynthesis of hetero-polysaccharide consisting of D-glucose (Glc) and *N*-acetyl-D-glucosamine (GlcNAc) has been studied using *Acetobacter xylinum* (Ogawa & Tokura, 1992; Shirai et al., 1994), which is well-known to produce bacterial cellulose (BC) as extracellular polysaccharide pellicles. It was found that the bacteria actively incorporated GlcNAc residues into pellicles following repeated subculture in medium containing GlcNAc. In a previous paper, we reported that GlcNAc residues were also incorporated at a relatively high ratio into the BC when either ammonium salts, 6-*O*-phosphorylated chitin (P-chitin), or 6-*O*-phosphorylated GlcNAc (6-P-GlcNAc) were added to the culture medium containing Glc (Shirai et al., 1994 and Shirai et al., 1996). Among P-chitins applied as co-substrates with Glc, only a water-soluble and lysozyme-susceptible derivative (P-chitin) enhanced the incorporation rate, although a much higher incorporation rate was observed with 6-P-GlcNAc as a carbon source. Recently, an incorporation mechanism was proposed in which the P-chitin added to the culture medium is depolymerized to a size capable of permeating the bacterial cell wall during the incubation, and then the oligomeric P-chitins are further depolymerized to an appropriate size inside the bacteria, which can then be followed by the formation of UDP-GlcNAc or UDP-chitin oligomer incorporated into a novel bacterial polysaccharide (Shirai et al., 1996).

Recently Takai and his co-workers reported that the production of BC was increased by addition of CMC and the product had stronger mechanical strength (Tajima et al., 1995). As we have found the depolymerization of the CMC added was occurred uduring *A. xylinum* incubation, the oligomeric CMC was assumed to be taken into the metabolic cycle of the bacteria and to be used as a carbon source of the extracellular polysaccharide. In this paper, we describe the results of incubating these bacteria in culture media containing carboxymethylated polysaccharides such as CM-chitin and carboxymethylated cellulose (CMC), purification of a novel polysaccharide, and their ion adsorption spectrum.

2. Materials and methods

2.1. Materials

Chitin was prepared from Queen Crab shells according to

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the literature (Hackman, 1954) and pulverized before use. The chitin powder was treated in 40 to 50% (w/v) aqueous sodium hydroxide to give an alkaline chitin, which reacted with monochloroacetic acid in 2-propanol to give CM-chitin after a freezing process (Tokura et al., 1983). The degree of carboxymethylation of the CM-chitin obtained was 1.0.

CM-cellulose with a 0.45 degree of carboxymethylation and other chemicals were purchased from Wako-pure Chemical, Co. Ltd. (Osaka, Japan) and used without further purification.

2.2. Subculture of Acetobacter xylinum

For incubation with CM-chitin, wild type *A. xylinum* ATCC strain 10245 was subcultured at 28 °C in Schramm-Hestrin (SH) medium (Hestrin & Schramm, 1954) containing GlcNAc as a carbon source, and repeatedly transfered to the new culture medium every 3 days. When CMC was employed as the carbon source, the bacteria was applied directly without subculture.

2.3. Fermentation and purification of polysaccharide

A 150 mL of SH medium in a 1 liter Erlenmyer flask was inoculated with 0.5 mL aliquot of the 3 d culture and incubated statically at 28 °C for 7 days. Pellicles produced at the surface of the culture medium were harvested and boiled successively in 2% (w/v) sodium dodecylsulfate for 3 h and in 4% (w/v) sodium hydroxide for 90 min. The resulting pellicles were rinsed extensively with deionized water until the washes became neutral, and after dried on stainless steel plates at 60 °C (sample for Na^+-form).

2.4. Determination of degree of carboxymethylation in BC

The pellicles were successively treated with 2 M hydrochloric acid-methanol (1:1, v/v) for 30 min, in methanol, and in acetone, and then dried to give a sample of H^+-form. The sample (60 mg) were stirred in 0.01 M aqueous sodium hydroxide (30 mL) for 20 min, then filtered and washed with deionized water. The filtrate and washings were combined and titrated with 0.01 M hydrochloric acid with use of bromothymol blue as the indicator to estimate the degree of carboxymethylation.

2.5. FT-IR analysis

IR spectra of both films of the Na^+- and H^+-forms were recorded with a Horiba FT-210 Fourier transform infrared spectrophotometer at a resolution of 4 cm^{-1}.

2.6. Estimation of carbohydrate consumption

The time-course of sugar consumption in the culture medium during the incubation was estimated by measuring of the reducibility of the medium using Schales' modified alkaline ferricyanide procedure (Imoto & Yagishita, 1971) briefly as follows. The culture medium was filtered through cellulose acetate membrane (0.45 nm). To 2.4 mL of aliquots of the filtrate were added a mixed solution of 10% aqueous tungsic acid (0.3 ml) and 0.67 M mixed acid (0.3 mL). The suspension was centrifuged to remove contaminating proteins (Merrill, 1924). The supernatant was diluted three times with deionized water for spectroscopic analysis. The absorbance of the sample was recorded at 420 nm with a spectrophotometer.

2.7. Determination of molecular weight

The molecular weight of the polysaccharide added in the culture medium was determined by a GPC method as follows. The culture medium was filtered through a cellulose acetate membrane filter (0.45 nm) and the filtrate was diluted to a sugar concentration of approximately 0.1%(w/v) by 0.01 M phosphate-citric acid buffer (pH 6.0). The resulting sample was analyzed in a Hitachi HPLC system equipped with a Shimadzu JC-6A refractive-index detector and an Asahi-pak GS-510 GPC column. The sample was eluted with 0.01 M phosphate-citric acid buffer (pH 6.0) at a rate of 0.5 mL/min.

2.8. X-ray scattering measurement

Wide-angle X-ray scattering (WAXS) of the carboxymethylated polysaccharide produced was measured with a Rigaku RAD-II SR X-ray diffractometer at 35 kV with a beam current of 30 mA using CuKα radiation (a wavelength of 0.1541 nm). A vacuum camera equipped with a 0.5 mm pin-hole collimeter was used.

2.9. Estimation of adsorption capacity for metal ions

A suspension of the carboxymethylated polysaccharide (2-13 g) in a mixed metal ion solution (20 mL) containing Mg, Ca, Ni, Mn, Co, Cu, Zn, Sr, Ba, and UO_2 ions (each 1.05×10^{-5} M) was stirred at 20 °C for 60 min. The supernatant solution was subjected to mass spectrometric analysis using a HITACHI P-7000 microwave induced plasma (MIP) mass spectrometry apparatus to measure the concentration of each metal ion. The amounts of adsorbed metal ion were plotted against the amount of the carboxymethyl groups in the polysaccharide, and the absorption capacity was estimated from the slope of the graph.

3. Results and discussion

3.1. Bacterial incubation in medium containing CMC

In order to examine the applicability of CMC as a substrate for *A. xylinum*, strain ATCC 10245 was incubated in a SH medium containing CMC as a sole carbon source. The

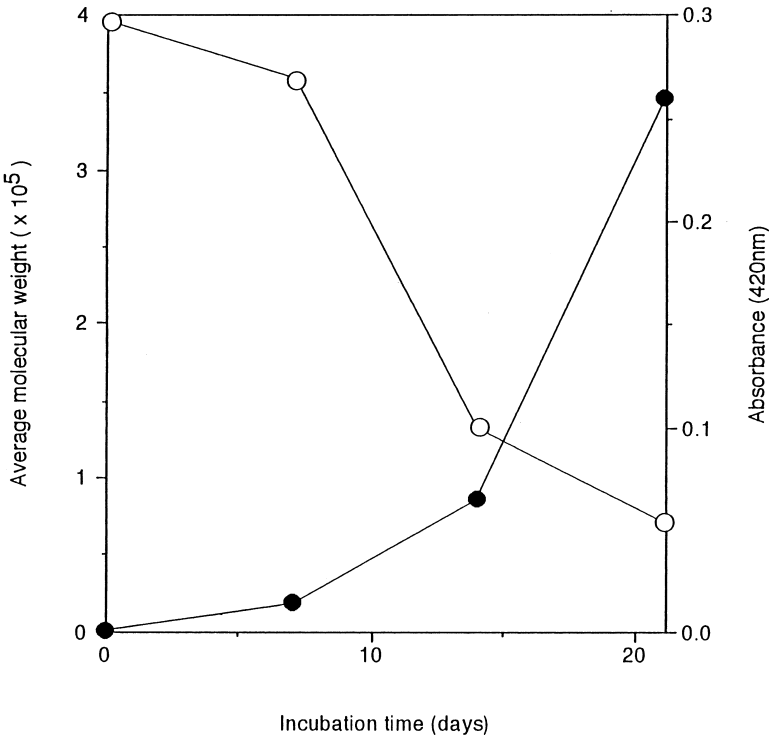

Fig. 1. Time-course carbohydrate consumption by *A. xylinum* in an SH medium containing CMC (1% w/v) as the sole carbon source; average molecular weight (●) and reducibilty (○). The culture was incubated statically at 28°C.

time-course of carbohydrate consumption in the culture medium was estimated by GPC analysis and measurement of reducibility (See Materials and Methods). The average molecular weight of CMC added gradually decreased from 400,000 to 60,000 within 21 days (Fig. 1). As reducibility of the medium increased during the period of incubation. Polysaccharide pellicles thus obtained from the surface of the culture medium were found to possess carboxymethylated residues. In the FT-IR spectrum of the pellicles shown in Fig. 2, the absorption of a carbonyl group was observed at around 1600 cm^{-1}. Furthermore, the degree of carboxymethylation was estimated to be 10% by acid-base titration. These results would be reasonable by consideration of the following assumptions.

1. (i) The bacteria has extracellular or cell-wall enzymes, which hydrolyze CM-cellulose added in the culture medium.
2. (ii) Carbohydrates so transported participate as carbon sources of the bacterial metabolic pathways. Considering the degrees of carboxymethylation of the original polysaccharides and those produced, either the carboxymethyl group must be removed by the bacteria or the carboxymethylated D-glucose residue would be re-formed into a D-glucose residue.

Beyond these considerations, we examined the influence of adding co-substrate into the culture medium, since Takai and his co-workers reported that the production of the pellicles was increased by incubation in Glc-CMC medium (Tajima et al., 1995). We also observed that the yield of the polysaccharide pellicles was significantly increased, when the incubation was carried out in a mixed HS medium containing both D-glucose (2 w/v%) and CMC. As shown in Fig. 3, the yield was increased 1.5-fold by addition of CMC (0.25 w/v%), and it became nearly constant where the concentration of CM-cellulose was 0.25-2% (w/v). We found that the degree of carboxymethylation reached a maximum (11.2%) at around 0.5% (w/v) CMC concentration in culture medium. Another experiment using CMC with lower molecular weight revealed slight increase in the production of pelicles and a decrease in degree of carboxymethylation. These results suggest that hydrolysis of CMC is a rate-determining factor in the biosynthesis of the polysaccharides, *i.e.*, reconstructed CMC (CM-BC).

3.2. Incubation in medium containing CM-chitin and glucose

Since CMC added to the culture medium of *A. xylinum* was reconstructed into a novel polysaccharide, our attention focused on another carboxymethylated polysaccharide, CM-chitin, in which the hydroxyl group at C-2 of the Glc residue is replaced by an acetamido group. Thus, an *A. xylinum* strain, repeatedly subcultured in GlcNAc, was incubated in a mixed SH medium containing 2% (w/v) of Glc and various ratios of CM-chitin (0-2% w/v) similarly as those for the incubation in the CMC-containing medium. The yields and the degrees of carboxymethylation of the

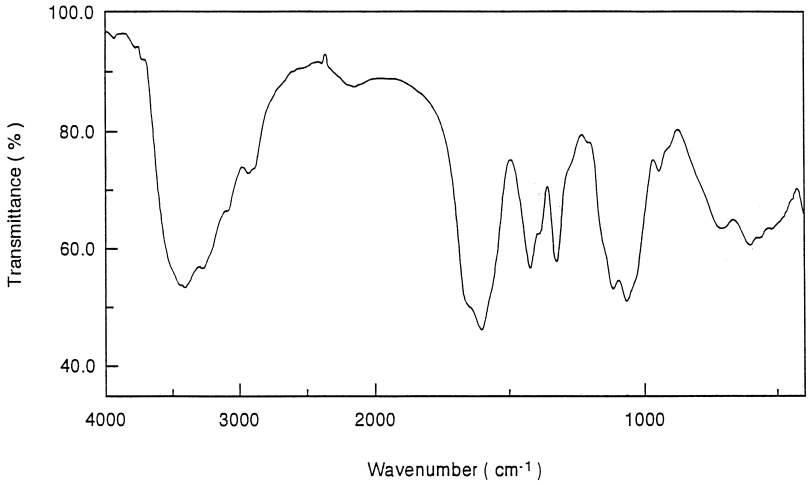

Fig. 2. IR spectrum of pellicles obtained by *A. xylinum* in SH medium containing CMC and D-glucose.

polysaccharide pellicles are shown in Fig. 4. The yield of the pellicles increased incrementally with increasing CM-chitin concentration (0.25 to 0.5%), and then decreased gradually at CM-chitin concentrations over 0.5%. A similar tendency was shown for the degree of carboxymethylation of the product (Fig. 4).

The novel bacterial polysaccharide from CM-chitin was characterized by FT-IR as shown in Fig. 5. A broad peak around 1550-1650 cm^{-1} was shown for the Na^+-form. Whereas sharp peaks assignable to C = O vibration of carboxylic acid, amido I, and amido II bands were observed in the spectrum of the H^+-form at 1730, 1640-1620, and 1530 cm^{-1}, respectively. These suggested clearly the presence of both carboxymethyl and acetamido groups in the polysaccharide. As the peak intensities of these three bands in the product are weaker than those of original CM-chitin, the incorporation of GlcNAc and CM-GlcNAc residues seemed to be extremely low. A low incorporation of the carboxymethyl group was also shown by acid-base titration of the product, in which the degree of carboxymethylation was 2-4% for total sugar residues. This was probably due to stereo-electronic effect of the substituent at C-2 position of the pyranosyl residue.

3.3. Ion-exchange profile of the product

The ion-exchange profile of the carboxymethylated bacterial cellulose (CM-BC) was characterized and compared with that of the original CMC. Fig. 6 shows the exchangeability of various metal ions plotted against the their ionic radii for two of the CM-BCs (degrees of carboxymethylation of 4.5% and 11.1%) in comparison to the original CMC

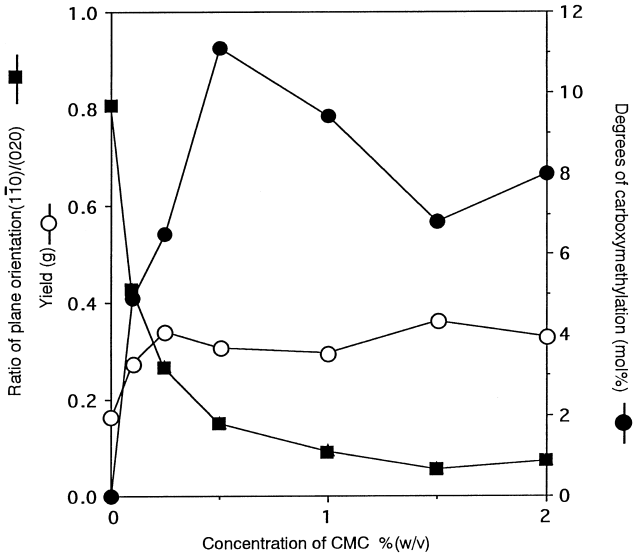

Fig. 3. Yield (○), degree of carboxymethylation (●), and ratio of plane orientation [(1$\bar{1}$0)/(020)] (■) of polysaccharide pellicles obtained by *A. xylinum* in a mixed SH medium containing D-glucose (2%) and CMC.

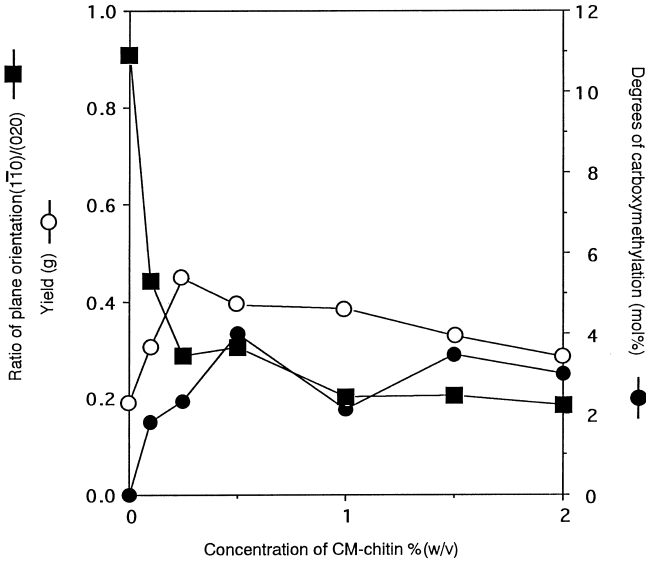

Fig. 4. Yield (○), degree of carboxymethylation (●), and ratio of plane orientation [(1$\bar{1}$0)/(020)] (■) of polysaccharide pellicles obtained by *A. xylinum* in a mixed SH medium containing D-glucose (2%) and CM-chitin.

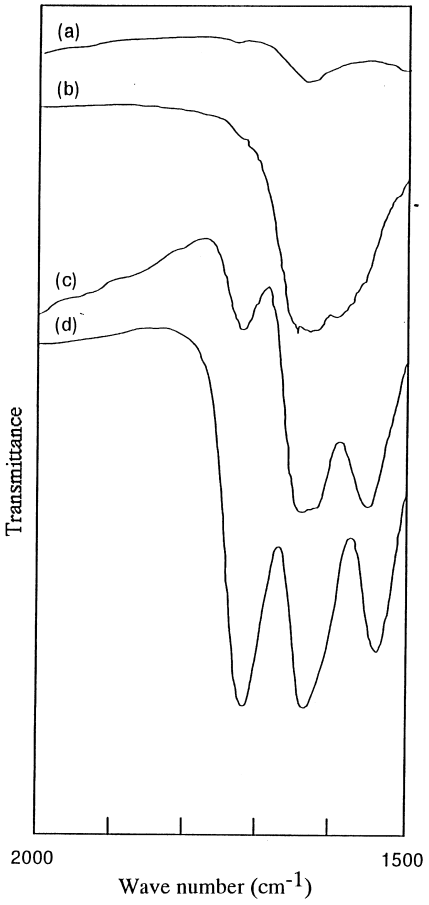

Fig. 5. Partial IR spectra of the pellicles produced by *A. xylinum* in mixed medium containing D-glucose and CM-chitin. (a) Bacterial cellulose; (b) Na^+-form of the pellicles; (c) H^+-form of the pellicles; (d) original CM-chitin. Base-line offset was done in recording each spectrum.

(D.S. = 45%). Most of the transition metal ions used were adsorbed to the extent of 1-4 mol% more towards the carboxymethyl groups introduced into the CM-BC. Ions with larger ionic radii such as lead and uranyl ions were found to be adsorbed strongly to the reconstructed CMC as compared to the original CMC and BC. As higher metal ion adsorption was observed for higher degrees of carboxymethylation, the main adsorption site was assumed to be the carboxymethyl group in addition to another coordination site geometrically arranged in the polysaccharide chain. Although all the metal ions evaluated here are divalent except for uranyl ion, they have quit different ionic radii. Certainly this variation, the so-called hard-soft acids and bases (HSAB) concept (Ho, 1977), has a dramatic effect on the numbers and types of metal ion bindings possible. Our novel polysaccharide possesses a number of chelating alkoxy functions and cation-exchanging carboxymethyl functions so that different metal-ion binding combinations are available. Closely allied with these factors, a multitude of three-dimensional structures may be available to this polysaccharide. Although the most preferred structure of the metal-ion complex is still uncertain, the high affinities observed for lead and uranyl ions are noteworthy and indicate utility of this novel polysaccharide as a synthetic adsorbent.

3.4. Wide-angle X-ray scattering analysis

As X-ray analyses have been extensively studied (Takai et al., 1975), examination of the wide-angle X-ray scattering (WAXS) pattern of the novel CM-BCs was carried out. The X-ray diffractogram revealed that ratio of the intensities of $(1\bar{1}0)/(020)$ plane decreased with increasing concentration

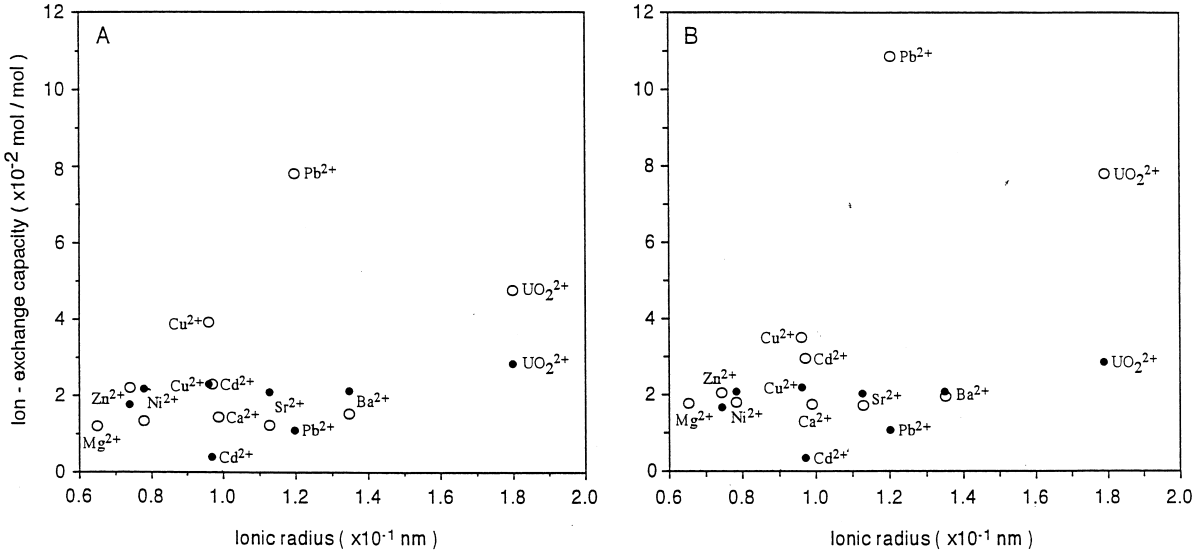

Fig. 6. Methyl ion adsorption profile of carboxymethylated BC (○) with degree of carboxymethylation of 4.9% (A) and 11.1% (B) produced by *Acetobacter xylinum*, and that of original CMC (●).

of the CM-chitin and CMC added to the medium. The decrease was almost independent on the degree of the substitution with carboxymethyl groups in the BCs (Fig. 3 and 4). This tendency may be attributable to a decrease in the regularity of the micro-fibril structure of the pellicle produced by the *A. xylinum* when incubated in the highly viscous culture medium containing polysaccharides. The degrees of the preferred orientation of the reconstructed CMC might influence the structure of the metal ion adsorption site.

4. Conclusion

Novel cellulose-like polysaccharides bearing carboxymethyl groups have been synthesized by incubation of *A. xylinum* in mixed culture medium containing D-glucose and CMC. A specific ion-exchange profile was shown by the novel polysaccharide that was distinct from that of original CMC. The carboxymethyl group at the C-6 position of Glc residue was assumed to be the driving force to attain the new specificity for ion-exchange. Although there are many problems to be solved in the production of CM-BC, the advantages of this specific ion-exchange profile would outweigh the problems associated with high cost of BC production.

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