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Structure characteristics of an acidic polysaccharide purified from banana (Musa *nana* Lour.) pulp and its enzymatic degradation

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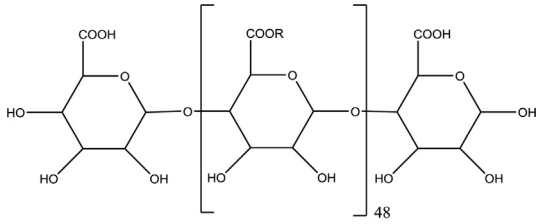
ABSTRACT

Banana is one of the most important fruits over the world. The chemical composition is critical for the organoleptic properties and health benefits. As one of the leading bioactive components in banana pulp, the polysaccharides may contribute to the beneficial health effects. However, their precise structure information remains unknown. A leading acidic polysaccharide (ABPP) of banana pulp was purified and identified by nuclear magnetic resonnance spectroscopy (NMR) and gas chromatography-mass spectrometry (GC–MS). →4-α-D-Gal*p*A-1→ and →4-α-D-Gal*p*AMe-1→ constituted the backbone. No branch chains were detected. The molecular weight was determined to be 8.9 kDa by gel permeation chromatography, which was smaller than previously reported fruit-derived polygalacturonic acids. The precise structure was identified as below. Digestion by enzyme would lead to production of oligogalacturonic acids and quick accumulation of 5000–7000 Da fraction.



R=H or methyl

1. Introduction

Fruits are important component of a healthy diet which offers diverse health benefits. Banana is one of the world's most important fruit crops as it is widely planted in tropical and subtropical regions [1]. It is known that banana can fight against intestinal disorder, and has antibacterial and antioxidant activities [2]. Banana has been reported to have abundant phytochemicals. Several bioactive steryl glucosides, such as campesteryl 3-β-D-glucopyranoside, stigmasteryl 3-β-D-glucopyranoside and sitosteryl 3-β-D-glucopyranoside, have been identified from *Musa acuminata* Colla cv. Cavendish [3]. Dopamine plays an important role in the physiological functions of human, such as sleep regulation, feeding, emotion, attention, cognitive function, olfaction, vision, hormonal regulation, sympathetic regulation and voluntary movement [4]. It has been found at a high level in the pulp of yellow banana (*Musa acuminata*), red banana (*Musa sapientum* var. baracoa) and peel of Cavendish banana [5,6].

Besides above bioactive phytochemicals, banana pulp is an abundant source of carbohydrates. Starch is the leading carbohydrate presented, and it has been well investigated for physicochemical properties [7]. The chemistry information of other bioactive polysaccharide fractions remains unknown. As far as we know, bioactive polysaccharides usually contribute much to the health benefits of fruits. Strawberry and mulberry polysaccharides have anti-inflammation potential via modulating cytokine

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secretion profiles [8]. Longan pulp polysaccharides, e.g. (1 → 6)-α-D-glucan, shows great immunomodulatory activity and anticancer cell proliferation activity [9]. It can induces lymphocyte proliferation and NO production. Therefore, it is necessary to investigate the chemical composition and structure information of polysaccharides in banana pulp.

In this work, the crude polysaccharides were extracted from banana pulp by hot water. Through purification by anion exchange chromatography and gel filtration chromatography, an acidic polysaccharide fraction was obtained. The chemical structure was identified by NMR. The molecular weight was determined by gel permeation chromatography. The molecular weight distribution after hydrolysis by pectinase was also measured.

2. Materials and methods

2.1. Materials

Fresh banana were collected from a local farm in Guangzhou. They were cleaned with tap water. The pulps were collected after removing the peels, and were immediately subjected to polysaccharides extraction.

2.2. Chemicals

Monosaccharide standards, hydroxylamine hydrochloride, acetic anhydride, methyl iodine and sodium borohydride were purchased from Aladdin Reagent Inc. (Shanghai, China). Galacturonic acid (Gal*p*A), glucuronic acid (Glc*p*A) and were purchased from Sigma Chemical Co. (St. Louis, MO). Dextran standards with different molecular weights were purchased from American Polymer Standards Corporation (Mentor, OH, USA). All the other chemicals used were of analytical grade.

2.3. Extraction of water-soluble polysaccharides from banana pulp

The water-soluble polysaccharides were extracted by hot-water extraction technique [10]. 500 g of banana pulp were added into 5 l of 80% (v/v) ethanol aqueous solution, and the ethanol-soluble chemicals were extracted. The pellets were collected by centrifugation at 5000*g*, and were subjected for further polysaccharides extraction. Excessive amount of water were added to the pellets. The extraction was incubated at 60 °C for 6 h. The supernatants were filtered and concentrated under a low pressure at 60 °C. Ethanol was added to the concentrated supernatants to a final concentration of 60%, and they were incubated in a refrigerator overnight at 4 °C. The crude polysaccharides were centrifuged at 8000 *g* for 20 min and lyophilized for 24 h.

2.4. Column purification of banana pulp polysaccharides

The polysaccharide was prepared as aqueous solution at 5 mg/ml. Equal volume of Sevag reagent (chloroform/butanol = 4:1, v/v) was added to remove the proteins. This step was repeated for 10 times. A DEAE Sepharose Fast Flow column (17 × 200 mm) was used for the initial column purification. The crude polysaccharides (100 mg) were dissolved in deionized water, filtered by 0.45-μm pore size membrane. A mid-pressure glass column (15 × 460 mm) was filled with DEAE Sepharose Fast Flow. Elution programme was conducted as below: phosphate buffer was eluted for 80 min, followed by gradient NaCl solutions (0.2, 0.4 and 0.8 M NaCl, each gradient for 80 min). The flow rate was maintained at 4 ml/min. All the eluates were collected by glass tubes. Through quantification by phenol-sulphuric acid method, the neutral and acidic fractions were collected respectively. As the yield of polysaccharides eluted by 0.2 M NaCl was higher than the other fractions, they were subjected to further purification by gel filtration chromatography. The polysaccharide sample was loaded onto a glass column (17 × 500 mm) filled with Sephadex G-75. Phosphate buffer was used as elution solvent at a flow rate of 0.5 ml/min. The main peak was collected, and the phoshpate was removed by ultrafiltration membrane. The purified polysaccharides were concentrated under a reduced pressure. Lyophilization was conducted to obtain the acidic banana pulp polysaccharide (ABPP).

2.5. Monosaccharide composition and absolute configuration

Reduction by l-cyclohexyl-3- (2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate was carried out to reduce uronic acid into corresponding alcohol. The polysaccharides were degraded into monosaccharides by hydrolysis with 2 M trifluoroacetic acid at 105 °C for 2 h. The hydrolysates were dried by rotary vacuum evaporator at 65 °C, and then aldononitrile acetate derivatization was conducted as below [11]. Hydroxylamine hydrochloride and pyridine were added to the hydrolysates. They were incubated at 100 °C for half an hour. Then 2 ml of acetic anhydride were added. The reaction was kept at 100 °C for half an hour. The acetylated derivatives were analysed by gas chromatography (Shimadzu, Kyoto, Japan). A Shimadzu RTX-5 capillary column was used for isolation and a flame ionisation detector was applied for monitoring. The following temperature programme was adopted: Column temperature was programmed from 140 °C/min. It increased to 180 °C at 1 °C/min, then increasing to 280 °C at 20 °C/min, holding for 10 min. Injection temperature: 230 °C; Detector temperature: 300 °C; Nitrogen was used as the carrier gas.

The absolute configuration of monosaccharide was determined as follows. The polysaccharides were reduced and acid hydrolysed. Two hundred and fifty microlitres of 0.625 M hydrochloric acid in (+)-2-butanol were added to the polysaccharide hydrolysates. The reactants were dried by vacuum rotary evaporator, and per-*O*-trimethylsilyl derivatives were prepared by adding *N*,*O*-bis(trimethylsilyl) trifluoroacetamide. The derivatives were analysed by gas chromatography, and compared with those prepared from d- and l-enantiomers of the external standards.

2.6. Methylation

The glycosidic linkages were analysed by methylation with methyl iodide and acetylation with acetic anhydride [12]. Reduction of uronic acids in ABPP by l-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate was conducted by using the protocol of Taylor and Conrad (Taylor °C, increased to 180 °C at 5 °C/min, then to 210 °C at 1.5 °C/min, holding for 5 min, increasing to 280 °C at 40 °C/min, holding for 15 min injection temperature: 230 °C. The ion source of mass spectrometer was set at 240 °C.

2.7. Nuclear magnetic resonnance (NMR) spectroscopy analysis

ABPP was dissolved in 0.5 ml of D_2O to a final concentration of 6% (w/v). ^1H and ^{13}C NMR spectra were recorded at 25 °C by a Bruker DRX-500 spectrometer (Bruker, Rheinstetten, Germany). The heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY), selective 1D total correlation spectroscopy (1D TOCSY) and H/H correlation spectroscopy (COSY) were measured. Chemical shift was expressed in ppm. Acetone (δ ^1H 2.22 ppm and ^{13}C 30.89 ppm) was used to calibrate the chemical shift.

2.8. Molecular weight determination

The molecular weight of ABPP were determined by using gel permeation chromatography. A Shimadzu LC-20A HPLC instrument equipped with a refractive index detector (Kyoto, Japan) was used. Three gel permeation columns were tandemly linked, including G6000PWXL, G5000PWXL and G3000PWXL (Tosoh Bioscience, Stuttgart, Germany). Dextran standards with molecular weight up to 2990 kDa were applied for standard curve preparation. The molecular weight was calculated with GPC software by retention time.

2.9. Digestion by pectinase

To understand the digestability of ABPP, it was subjected to hydrolysis by pectinase from *Aspergillus niger*. ABPP was dissolved in phosphate buffer (pH 5.0) and 1% (enzyme/ABPP, w/w) pectinase was added. The hydrolysis was incubated at 50 °C for different intervals. The reaction products were analysed by gel permeation chromatography. The molecular weight distribution was calculated by the dextran standards curve and the percentage of different molecular weight range was determined by the peak area integration.

2.10. Statistical analyses

All data were expressed as the means of three replicated determinations. One way of variance analysis was applied for determining differences between results. Duncan test was taken to compare the data. *P*-value <0.05 was regarded as significantly different.

3. Results and discussion

3.1. Purification of ABPP and determination of molecular weight

Banana pulp is an abundant source of carbohydrates. Even though starch accounts for the dominant carbohydrate fraction, the non-starch polysaccharides play an important role in the health benefits of banana as dietary fibre. Through hot water extraction, the crude banana pulp polysaccharides were precipitated by ethanol. DEAE Sepharose was used for isolation of banana pulp polysaccharides by electric charge. The anionic polysaccharides were isolated from neutral and cationic polysaccharides. As fruits usually don't synthesize cationic polysaccharides, this technique can well isolate the anionic and neutral polysaccharides in banana pulp. The neutral polysaccharides were eluted by phosphate buffer, while the anionic polysaccharides were eluted by NaCl in phosphate buffer. Due to the large molecular weight of neutral polysaccharide (larger than 2990 kDa, the largest dextran standard used in this work), it was not selected for further purification. The plant-derived anionic polysaccharides usually refer to the polysaccharides with uronic acid as one of the constitution units. Therefore, they are also called acidic polysaccharides. The acidic banana pulp polysaccharides were further purified by Sephadex G75 column to isolate the polysaccharide fractions due to difference in molecular weights. The leading acidic polysaccharide was collected and named ABPP. It was loaded into a gel permeation chromatography and the chromatogram is shown in Fig. 1. The retention time of ABPP was 57.3 min. A regression equation was computed by GPC software. It was as follows:

$$Y = 0.0011X^3 + 0.17X^2 - 9.15X + 166.74$$

where Y indicates molecular weight (Da), X refers to the retention time (min). The molecular weight of ABPP was calculated to be 8.9 kDa. It was located between 11,600 Da and 5220 Da dextrans in the chromatogram.

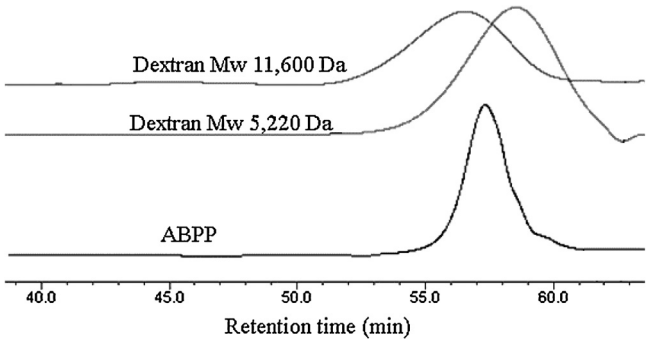

Fig 1. Molecular weight of ABPP determined by gel permeation chromatography. The standard regression equation is $Y = 0.0011X^3 + 0.17X^2 - 9.15X + 166.74$, where Y means molecular weight (Da), X means retention time (min).

Table 1
Monosaccharide compositions of ABPP.

Sugar[a]	Molar ratio	Glycosidic linkage	Molar ratio
Gal*p*A	1	→4)-D-Gal*p*A-(1→	1
Rha*p*	–		
Gal*p*	–		
Glc*p*	–		
Ara*f*	–		

[a] Ara*f*, arabinofuranose; Gal*p*, galactopyranose; Gal*p*A, galacturonic acid; Glc*p*, glucopyranose; Rha*p*, rhamnopyranose.

4.1. Monosaccharide composition and glycosidic linkages

As uronic acid will be degraded during acid hydrolysis, it is required to reduce the carboxyl into corresponding alcohol before acid hydrolysis. Through comparison of monosaccharide composition before and after reduction, only galacturonic acid was found to be the constitution unit of ABPP (Table 1). The absolute configuration was determined to be D-galacturonic acid. ABPP was subjected to reduction, acid hydrolysis, methylation and acetylation. The reaction products were analysed by GC–MS, which revealed the glycosidic linkage of D-galacturonic acid. It was presented as →4)-D-GalpA-(1→. The monosaccharide composition and glycosidic linkage indicated that ABPP was polygalacturonic acid.

In the view of plant polysaccharides, D-galacturonic acid is mainly occurred in two types of pectin structure. One is a homogalacturonic acid polymer, and another is constructed by repeating unit →4)-α-D-galacturonic acid-(1→2)-α-L-rhamnose-(1→ [13]. As no rhamnose was detected in ABPP, this acidic polysaccharide was a homopolymer made by →4)-D-Gal*p*A-(1→. Natural polygalacturonic acid is usually methyl esterified to different extents [14]. The common method to determine the methyl esterification degree is tritation. However, this method is not precise and the location of methyl ester in which galacturonic acid residues is not clear. We preferred to judge the methyl esterification information by NMR.

4.2. Structure identification by NMR

1D and 2D NMR spectra can well reflect the anomeric configuration, glycosidic linkage and side chain location of a polysaccharide [15,16]. The chemical shift was calibrated by acetone (^{1}H, 2.22 ppm; ^{13}C, 30.89 ppm). Table 2 lists the assignments of ^{1}H and ^{13}C chemical shift for all the glycosidic linkages. Only one glycosidic linkage was detected as →4)-α-D-GalpA-(1→. The reduced terminal and

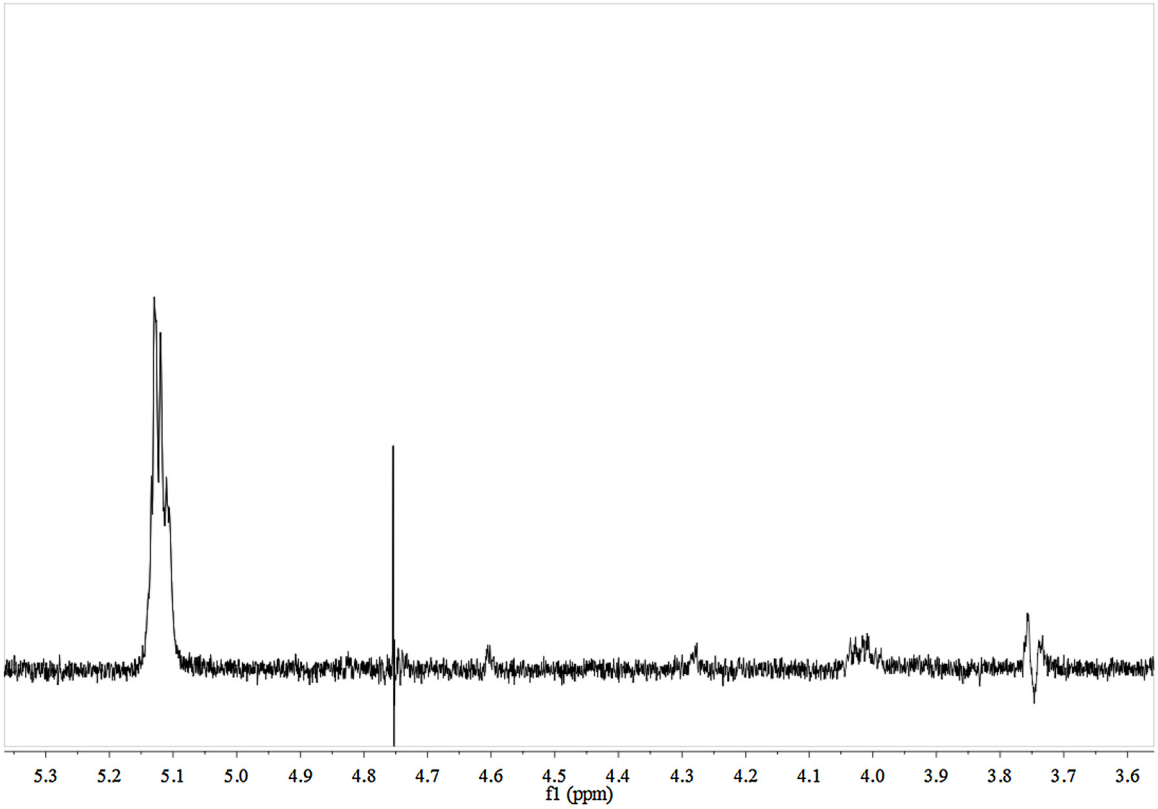

Fig. 2. 1D TOCSY spectra of ABPP when the anomeric H of →4)-α-D-GalpA-(1→ was selected.

Table 2
Chemical shift assignments for ABPP.

Glycosidic linkages		1	2	3	4	5	6	Me[a]
α-D-Gal*p*A –(1→	H	5.10	3.79	3.80	4.36	4.39		
	C	99.9	72.7	68.7	71.3	72.5	175.8	
→4)-α-D-Gal*p*A-(1→	H	5.12	3.77	4.01	4.28	4.60		
	C	99.5	72.7	69.5	77.8	72.2	176.8	
→4)-α-D-Gal*p*AMe-(1→	H	5.12	3.77	4.01	4.27	4.72		3.82
	C	99.5	72.7	69.5	77.6	71.9	171.8	53.5
→4)-α-D-Gal*p*A	H	5.32	3.84	4.01	4.28	4.60		
	C	92.9	68.7	69.5	77.8	72.2	176.8	

[a] Me refers to methyl ester.

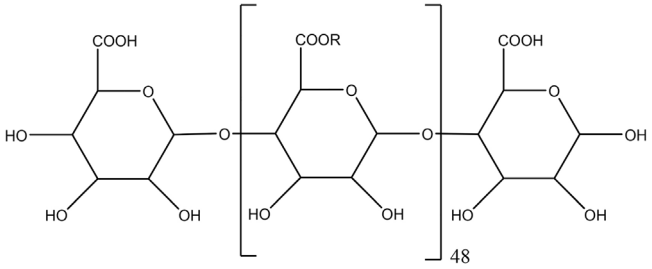

Fig. 3. The precise structure of ABPP. R = H or methyl.

non-reduced terminal were also detected. The results were in agreement with the glycosidic linkage analysis by GC–MS.

The anomeric signals of →4)-α-D-Gal*p*A-(1→ as 5.12/99.5 ppm (H-1/C-1). the chemical shift of H-1 indicated that the relative configuration of Gal*p*A was α-type. For glucose and galactose, when the chemical shift of H-1 is larger than 4.9 ppm, it suggests the monosaccharide is present as α-type configuration. Otherwise, the monosaccharide has a β-type configuration. In the Haworth conformation, α-type configuration means that the hydroxyl linked to C-1 is located at a different side to the hydroxyl linked to C-6. β-Type configuration indicates that both hydroxyls are located at the same side. As shown in Fig. 2, 1D-TOCSY spectra was scanned to reveal the H signals close to anomeric H of →4)-α-D-Gal*p*A-(1→. H-2 signal located at 3.77 ppm. The chemical shifts of H-3 (4.01 ppm), H-4 (4.28 ppm) and H-5 (4.60 ppm) could be judged by their decreased peak strengths. The peak at 4.76 ppm was the signal of water. A methyl ester signal was observed at 3.82/53.5 ppm. It indicated that some Gal*p*A was presented as Gal*p*A methyl ester. Through computing the areas of H in methyl ester and in anomeric H in Gal*p*A, the methyl ester degree was determined to be 12.5%. Methyl ester led to a shift to high field for the carboxyl C (171.8 ppm). As mentioned by literature [17], the chemical shift change of sugar residue induced by methyl esterification was also observed in this work.

As the molecular weight of ABPP is only 8.9 kDa, it was smaller than polygalacturonic acids commonly found in fruits. The commercial available polygalacturonic acid, such as from apple and citrus sources, have a molecular weight larger than 25 kDa [18]. Through calculating the molecular weight of each unit, ABPP was consisted of 50 galacturonic acids. The non-reduced terminal (α-D-Gal*p*A -(1→) was judged by the un-substituted hydroxyl linked to C-4, which had a downfield movement for H-4 and an upfield shift for C-4. The reduced terminal →4)-α-D-Gal*p*A was detected at 5.32/92.9 ppm for H-1/C-1.

Considering the glycosidic linkages and molecular weight, the structure of ABPP was drawn as Fig. 3.

4.3. Digestion by pectinase

As the bioactivity and physical properties of polygalacturonic acid are highly influenced by molecular weight, it is worthy to degrade it by enzyme and to investigate the moleclar weight

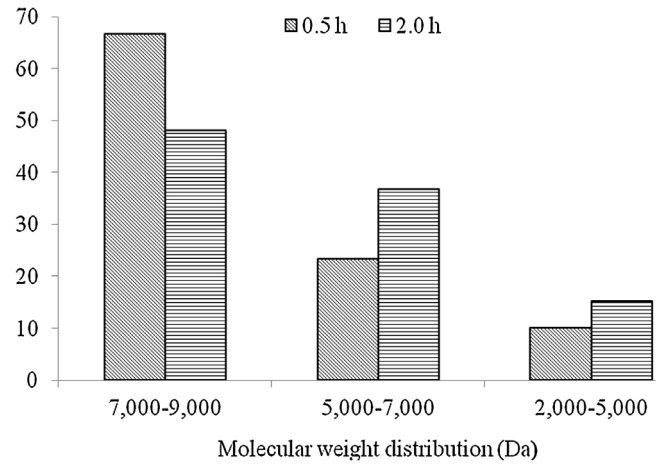

Fig. 4. The molecular weight distribution of ABPP hydrolysates digested by pectinase.

change. In this work, ABPP was digested by pectinase and the hydrolysates were analysed by gel permeation chromatography. The percentages of three fractions with molecular weights of 2000–5000, 5000–7000 and 7000–9000 Da were measured (Fig. 4). When 0.5 h of hydrolysis time was used, 23.3% of 5000–7000 Da fraction was detected. The percentage of 2000–5000 Da reached 10.1%. As pectinase usually cleaves oligogalacturonic acids as products [19,20], the decrease of ABPP molecular weight was originated from continual lose of oligogalacturonic acid units. Pectinase showed effective degradation on ABPP when 2.0 h of hydrolysis was applied, the 5000–7000 Da fraction accounted for 36.8% and the 7000–9000 Da fraction decreased to 48.0% .

5. Conclusions

An acidic polysaccharide was purified from banana pulp, and the precise structure was identified. It was a polygalacturonic acid with molecular weight of 8.9 kDa. Pectinase hydrolysis could lead to a fast accumulation of 5000–7000 Da fraction. These findings are helpful to understand the polysaccharide composition and further utilization for functional foods and medicines.

Acknowledgements

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