

Free-radical depolymerization with metallic catalysts of an exopolysaccharide produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid

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Received 22 July 2005

Available online 13 December 2005

Abstract

Free-radical depolymerization with metallic catalysts has been investigated to depolymerize an exopolysaccharide (EPS), with a high molecular weight ($>10^6$ g/mol), produced by the bacterium *Alteromonas macleodii* subsp. *fijiensis* biovar *deepsane*. Three metals (copper, zinc and manganese) were used either alone or mixed. These metals led to the depolymerization of the high molecular weight bacterial polysaccharide with different efficiency. This study specifies some conditions required to produce, with good reproducibility and yield, EPS derivatives of reduced molecular weight ranging from 100,000 to 20,000 g/mol. The more influential parameters are specified. This is a first study to find suitable industrial conditions in the presence of these metallic catalysts.

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Keywords: Metal; H₂O₂; Free-radical depolymerization; Bacterial exopolysaccharide

1. Introduction

In the past decades, marine polysaccharides have appeared as a new source of biodegradable water-soluble polymers. Most of them are obtained from seaweeds (agar, alginates and carrageenans). More recently, a great interest in producing polysaccharides (especially exopolysaccharides, EPS) by microorganisms has arisen, representing an innovative approach to the biotechnological use of under-exploited resources. EPS often show original structural features that can be modified to design bioactive compounds and improve their specificity (Crescenzi, 1995; Manzoni, Rollini, Piran, & Parini, 2004; Volpi, 2003).

In this context, new bacteria have been collected near deep-sea hydrothermal vents characterized by extreme pressure and temperature conditions (Deming, 1998; Guezennec, 2002). Some thermophilic (Schiraldi & De Rosa, 2002) and also

mesophilic EPS-producing strains have been isolated, such as *Alteromonas macleodii* subsp. *fijiensis* (Raguénès et al., 1996), *Vibrio diabolicus* (Raguénès, Christen, Guezennec, Pignet, & Barbier, 1997a,b) and *Alteromonas infernus* (Raguénès, Portenlänger and Heusinger, 1997a,b). These new EPS have innovative structures with great potential for various biomedical purposes. For instance, native EPS secreted by *V. diabolicus* has been shown to be a strong bone-healing material (Zanchetta, Lagarde, & Guezennec, 2003) while, after chemical modifications, EPS from *A. infernus* have shown anticoagulant properties (Collic Jouault et al., 2001) and appeared as new heparin-like candidates.

The EPS HYD 657 (Cambon-Bonavita, Raguènes, Jean, Vincent, & Guezennec, 2002) from *A. macleodii* subsp. *fijiensis* biovar *deepsane* collected during the French Hydronaut voyage (Guezennec et al., 1994) has been selected for its abilities to enhance the skin immune defense system (Vacher & Fritsch, 2002) and used in cosmetics as the active ingredient under the Abyssine® 657 operation. In order to extend its area of interest, we have carried out investigations to obtain low molecular weight fractions from this high molecular weight EPS.

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In the literature, several methods are described for depolymerizing polysaccharides, such as irradiation (Bertolini, Mestres, Colonna, & Raffi, 2001), ultrasonic degradation (Portenlanger & Heusinger, 1997), free-radical depolymerization with or without metallic catalysts (Nardella et al., 1996; Volpi, Mascellani, & Bianchini, 1992; Yang, Li, & Guan, 2004), acid hydrolysis (Allan & Peyron, 1995; Cheng et al., 1999) or enzymatic depolymerization (Michaud et al., 2003). It has been shown by Guezennec et al. (1998) that the extended acidic hydrolysis of an exopolysaccharide causes severe depolymerization without preserving the monosaccharide ratios while enzymatic depolymerization requires a good knowledge of the structure of the EPS, thus making each EPS a particular case.

From a free-radical depolymerization process, Volpi et al. (1992) showed that it was possible to obtain polysaccharide fractions of low molecular weight (LMW) and constant composition starting from heparin or dermatan sulfate.

This work deals with free-radical depolymerization using metallic catalysts. Here, the metallic catalysts were selected in the interests of cosmetic applications, because their presence can enhance the anti-microbial properties of the EPS formulation to be used in skin and hair care. For the degradation of polysaccharides, free-radical depolymerization is an interesting route because it enables, with reproducibility and constant composition, the extent of the depolymerization to be controlled. Moreover, the reagents are inexpensive and suitable for use on a large scale. In this report, the three catalysts used were copper, zinc and manganese, alone or mixed. The influence of different parameters was investigated for these metals.

2. Experimental

2.1. Production and characterization of native EPS

The isolation procedure and characteristics of the HYD 657 strain have previously been reported by Cambon-Bonavita et al. (2002). HYD 657 is composed of neutral sugars (glucose, galactose, rhamnose, fucose and mannose) along with acidic sugars (glucuronic and galacturonic acids) and an unusual sugar identified by GC and GC-mass spectrometry analyses as a 3-O-(1 carboxyethyl)-D-glucuronic acid. This polysaccharide, as often observed for EPS, presents a high molecular weight (> 106 g/mol).

2.2. Molecular weight determination

Each fraction (2 mg/mL) was analyzed several times by high-performance size-exclusion chromatography (HPSEC) in 0.1 M ammonium acetate at a flow rate of 0.5 mL/min using a Superdex 200 (Amersham Pharmacia Biotech) column. Calibration was performed with pullulan standards, which are neutral glucans (Interchim), whereas our EPS are charged polysaccharides, so that such calibration does not allow exact measurement of the polysaccharide molecular weight. Area measurements and calculations of M_w (weight-average molecular mass), M_n (number-average molecular mass) and I

(polydispersity) were carried out using ARAMIS software (JMBS Développements, Le Fontanil, France).

2.3. Composition of EPS

The molar ratio of polysaccharides was determined according to Kamerling, Gerwig, Vliegenhart, and Clamp (1975); Montreuil et al. (1986). The monosaccharide residues were analyzed after acidic methanolysis of the polymer and subsequent GLC analyses as trimethylsilyl derivatives.

2.4. Viscosity determination

Viscosity was determined by a rotational viscometer Visco Tester GL/R (Haake). The result is a direct reading of the viscosity value in milliPascal.

2.5. Conditions of depolymerization of EPS

Free-radical depolymerization was investigated while varying different parameters (H_2O_2 /EPS ratio, flow rate, with or without pH control) and for three metallic salts: $CuSO_4 \cdot 5H_2O$ (Janssen Chimica), $ZnSO_4 \cdot 7H_2O$ (Prolabo) and $MnSO_4 \cdot H_2O$ (Merck). The following protocol is representative of the different experiments:

Two hundred milligrams of EPS HYD 657 was dissolved in water (100 mL) in a reaction vessel and 2 mL of 1.8×10^{-3} M cupric sulfate pentahydrate (corresponding to 0.18 mM of copper for 200 mg of EPS) was added. The temperature was kept at 50 °C. A 0.14% (w/v) H_2O_2 aqueous solution was then added at a flow rate of 30 mL/h. The reaction was stopped after 2 h. The reaction pH was maintained at pH 7 by continuous addition of 0.1 N sodium hydroxide. At the end of the reaction, the EPS was reduced by $NaBH_4$. The solution was concentrated, desalinated by ultrafiltration with a 500 Da cutoff membrane (Millipore) and then freeze-dried. Depolymerized EPS fractions were obtained with 70–90% yields.

The same experiment was carried out five times with the same batch of production of the EPS to check the reproducibility of the manipulation.

3. Results

3.1. Depolymerization with copper

Recently, the depolymerization of an EPS produced by *A. infernus* has been conducted by a radical process, using the catalytic system metal/ H_2O_2 . This process allows homogeneous (with a low polydispersity) LMW EPS to be obtained in a single step, and in good yield (Collic Jouault et al., 2001). Here, using the same method, the depolymerization has been performed on the complex EPS HYD 657 from *A. macleodii* subsp. *fijiensis* biovar deepsane, whose repeating unit is thought to be an undecasaccharide with three small side chains. Even though the structure of this EPS is not yet fully known, the repeating unit has been reported to be composed of neutral sugars (glucose, galactose, rhamnose, fucose and mannose) along with acidic

sugars (glucuronic and galacturonic acids) and an unusual sugar shown to be a 3-O-(1 carboxyethyl)-D-glucuronic acid.

First, we considered the starting solution of native EPS. Polysaccharides are often used as viscosity enhancers for various industrial purposes because of numerous intra- and intermolecular interactions via hydrogen bonds and, of course, the length of the chains. Another consequence is that temperature is a very important parameter to drive the viscosity of the solution. A good balance must thus be found to avoid the degradation of polymers and to prevent problems of handling and homogeneity.

From these considerations, variations in viscosity vs temperature and concentration were studied (Fig. 1a and b). The concentration of the solution of EPS was first set to 2 g/L and a linear decrease in the viscosity was observed with increasing temperature, ranging from 160 mPa at 10 °C to 60 mPa at 60 °C (Fig. 1a). At 60 °C, an increase in the viscosity was observed with an increase in the EPS concentration but this viscosity was lower than that observed at 30 °C (Fig. 1b). So, in order to work with a solution presenting a weak viscosity, we decided to perform all experiments at 50 °C with a concentration of EPS set at 2 g/L. During the depolymerization process, we also observed a rapid decrease in viscosity with the decrease in M_w (Fig. 2).

In the literature, iron and copper are the two metals most used to depolymerize polysaccharides by free-radical depolymerization. In this work, copper was preferred in order to determine standard conditions before the introduction of other cations. Moreover, copper is of interest for its biological activities in view of further applications in cosmetics.

The influence of the amount of copper cation and the experimental procedure on the depolymerization process was observed. Carboxylic moieties from acids and hydroxyl groups can be involved as electron donors in the chelation of cations, as has been well described in the literature (Angyal, 1989). For instance, a recent study concerning another EPS (Loaëc, Olier, & Guezennec, 1997) showed that metal capture is a chemical, equilibrated and saturable mechanism. In the case of Zn^{2+} , which has an ionic radius very close to Cu^{2+} (74 vs 72 nm), the binding

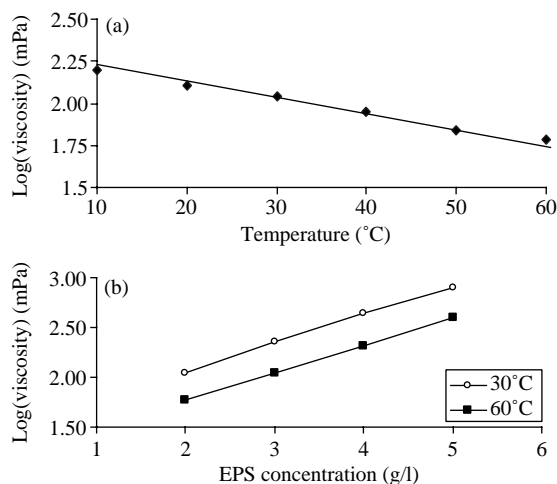


Fig. 1. Evolution of viscosity vs temperature (a) (EPS concentration = 2 g/L) and concentration (b) ($T = 30$ and 60 °C). A logarithmic dependence is showed in both cases.

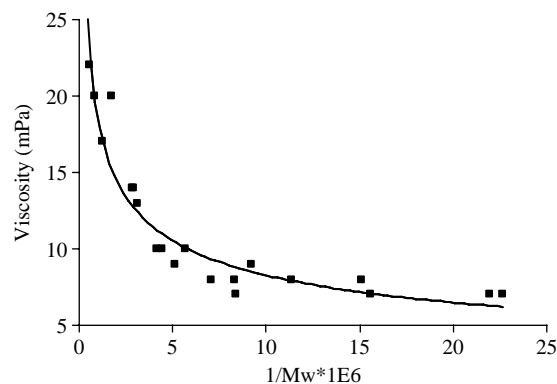


Fig. 2. Relation between viscosity vs $1/M_w$. Under M_w of about 200,000 g/mol, the viscosity of the solution remains constant at about 7 mPa.

capacity is about 80 mg of zinc per gram of polymer. Moreover, previous works in our laboratory showed that various cations from Re, Tc, Fe, Gd or Pb, were strongly chelated (unpublished works). Then, increasing amounts of copper sulfate were added to a solution of EPS (2 g/L), before introduction of H_2O_2 . For an H_2O_2 /EPS (w/w) ratio of 0.2, the system appeared saturated with metal for a concentration of about 2 g/L. So, high amounts of Cu^{2+} did not improve depolymerization (Fig. 3).

In order to limit the binding of copper ion, copper sulfate was added gradually by a peristaltic pump. The experiment was performed with a mass ratio of H_2O_2 /EPS of 0.2, hydrogen peroxide solution was added for 1 h at a flow rate of 1 mL/min, and 10 mL of $CuSO_4 \cdot 5H_2O$ (4.4 g/L) was added at a flow rate of 0.2 mL/min. The same experiments were performed with copper sulfate, copper chloride and copper acetate, using the same molar amount of metal. For these three counter-ions, the same results were obtained (i.e. M_w was about 20,000 g/mol for $R = 0.2$ and 10,000 g/mol for $R = 0.4$).

Another parameter that could influence the reaction course is pH. A neutral pH is required for an optimized depolymerization of the polysaccharide backbone by OH^\bullet and to avoid an acidic or basic hydrolysis of the polysaccharide. However, during the addition of metallic cation to polysaccharide solutions, a sudden decrease in pH immediately occurred, due to the chelation process. The pH of the solution then remained stable with values ranging from 4.0 to 5.2 at 50 °C depending on the initial concentration of the added cation. Without pH control, the reproducibility was not very good (Table 1) except for high

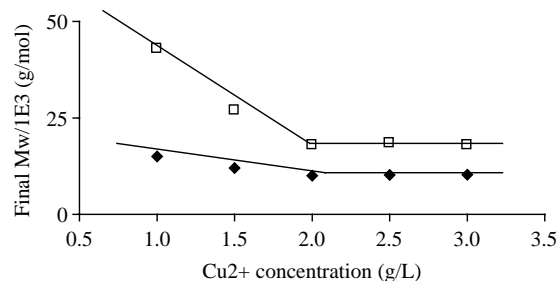


Fig. 3. Influence of Cu^{2+} on depolymerization. For an EPS concentration of 2 g/L and H_2O_2 /EPS ratio of 0.2, the limiting concentration of Cu^{2+} is about 2 g/L.

Table 1
Molecular weight, polydispersity and reproducibility according to different H₂O₂/EPS ratios and H₂O₂ flow rates

Entry	H ₂ O ₂ flow rate (mL/min)	R (H ₂ O ₂ /EPS)	pH control	M _w (×10 ³ g/mol)	I _p (M _w /M _n)	Reproducibility (n=4)
1	1.0	0.1	Yes	40–130	–	Bad
2	1.0	0.2	Yes	20–50	2.0	Bad
3a	1.0	0.4	Yes	10–20	2.5	Good
3b	1.0	0.4	No	15–25	2.5	Good
4a	0.5	0.1	Yes	40	2.0	Good
4b	0.5	0.1	No	60–100	–	Bad
5a	0.5	0.2	Yes	20	2.4	Good
5b	0.5	0.2	No	45–100	–	Bad
6a	0.5	0.4	Yes	10	2.5	Good
6b	0.5	0.4	No	20	2.5	Good

n, number of experiments.

Table 2
Monosaccharide contents of the low molecular weight EPS derivatives obtained after depolymerization for an H₂O₂/EPS ratio of 0.4 with or without pH control and in the presence of copper as catalyst, and compared to the native EPS

EPS	Rha (%)	Fuc (%)	Gal (%)	Glu (%)	Man (%)	GlcA (%)	GalA (%)	M _w (×10 ³ g/mol)	I _p
Native	3.4	1.8	21.7	12.3	1.8	6.1	7.3	3000	>5
pH control	2.3	1.4	10.5	7.6	1.3	2.3	6.1	10	2.5
No pH control	2.3	1	15.1	9.8	1.4	8.4	6.5	20	2.5

Rha, rhamnose; Fuc, fucose; Gal, galactose; Glu, glucose; Man, mannose; GlcA, glucuronic acid; GalA, galacturonic acid.

ratios of H₂O₂/EPS (Table 1, entries 3 and 6). For R (H₂O₂/EPS) equal to 0.4, in each case, final molecular weight was about 15,000 g/mol with a good reproducibility. Flow rate and pH control had no significant influence (Table 1, entries 3 and 6). The conditions of introduction of H₂O₂ were also considered. The radical depolymerization proceeds through the formation of free radicals from hydrogen peroxide and the catalytic action of copper. The active principle is OH• generated in situ with a Fenton reaction, for which Cu²⁺ is a very effective catalyst, only at trace level. The relative amount of H₂O₂, and so of radicals, is one of the keys to control depolymerization. In relation to this point, we showed that a continual addition of H₂O₂ was much better than a one-step initial addition to the starting mixture.

In Table 2, the average values corresponding to entry 6 of Table 1 are reported. The monosaccharide content analysis of depolymerized fractions showed some variations, which could imply non-selective degradation of the polymer. Only the contents of galactose, glucose and glucuronic acid are lower in the depolymerized EPS than in the native EPS. However, roughly the same osidic compositions are found with or without pH control in the LMW EPS and close to the native compound, except for the sugars cited above. In Table 3, the same remarks can be made about the osidic composition for two H₂O₂/EPS ratios (low and high).

Table 3
Monosaccharide contents of the low molecular weight EPS derivatives obtained for two H₂O₂/EPS ratios (R=0.2 and R=0.4) in the presence of copper as catalyst and compared to the native EPS

EPS	Rha (%)	Fuc (%)	Gal (%)	Glu (%)	Man (%)	GlcA (%)	GalA (%)	M _w (×10 ³ g/mol)	I _p
Native	3.4	1.8	21.7	12.3	1.8	6.1	7.3	3000	>5
R=0.2	2.7	1.9	15.5	9.5	2.1	2.6	7.1	20	2.4
R=0.4	3	1.7	11.2	7.6	1.3	2.8	7.2	10	2.5

Rha, rhamnose; Fuc, fucose; Gal, galactose; Glu, glucose; Man, mannose; GlcA, glucuronic acid; GalA, galacturonic acid.

3.2. Addition of other metals

After radical depolymerization in the presence of metal, a strong sorption of the cations by the EPS presenting well-known chelating properties is observed. So, when required, the elimination of them from the final product needs an additional step using a chelating resin or agent. In the literature, titanium, iron or copper are most often used to catalyze free-radical depolymerization coupling with hydrogen peroxide. In this work, we focus on metallic catalysts (or a mixture of cations) of interest in cosmetic applications. Metals such as iron, copper and zinc at low concentration are known as pro-oxidants in vitro but considered as antioxidants in vivo. So metals play an antioxidant role in vivo by protecting against free radical-induced oxidative damage; they protect against UV radiation, enhance wound healing, contribute to immune and neuropsychiatric functions and decrease the relative risk of cancer and cardiovascular disease (Rostan, DeBuys, Madey, & Pinnell, 2002; Stanner, 2003).

To the best of our knowledge, little is known about the use of zinc or manganese in such processes. First, the same molar amount of zinc or manganese as copper (0.18 mM for 200 mg of EPS) was introduced into the EPS solution. The temperature was kept constant at 50 °C and the pH was adjusted to 7.

Table 4

Molecular weight and polydispersity according to the metal used, H₂O₂/EPS ratio and pH control (flow=0.5 mL/min)

Entry	Metal(s)	R (H ₂ O ₂ /EPS)	PH control	$M_w (\times 10^3 \text{ g/mol})$	$I_p (M_w/M_n)$
1a	Cu	0.4	Yes	10	2.4
1b		0.4	No	20	2.5
2	Zn	0.4	Yes	10–100	–
		0.4	No	100	3
3	Mn	0.4	Yes	20–300	3.5
		0.4	No	12–430	–
4	Cu + Zn	0.2	Yes	25	2
		0.2	No	75–100	–
5		0.4	Yes	10	2.2
		0.4	No	5–45	–
6	Cu + Mn	0.2	Yes	65–120	–
		0.2	No	10	2.2
7		0.4	Yes	50–70	–
		0.4	No	<10	2.0

The results of molar mass determination show that it is possible to depolymerize with zinc sulfate or manganese sulfate alone but the efficiency of the depolymerization appears to be less good than with copper sulfate (Table 4, entries 2 and 3). The monosaccharide contents of the LMW EPS derivatives were very similar whatever the metal used and close to that of the native EPS (Table 5). However, when the depolymerization was performed in the presence of zinc or manganese, the HPSEC chromatograms showed different overlapping peaks, giving EPS derivatives very heterogeneous in size with a high polydispersity. According to the metal used, different trends were observed during depolymerization. Concerning manganese, reproducibility was very low with and without control of pH, under neutral and acidic conditions, respectively. These random results were probably the consequences of solid precipitations of manganese species during the depolymerization. Concerning zinc, results appear a little bit more surprising because, this metal is generally considered, in the literature, to not produce significant amount of oxidizing species. Blank experiments (without metal) did not show depolymerization of HYD 657, but preliminary experiments suggest that under these conditions, it seems that depolymerization is quite specific of this EPS. This point will be soon investigated to try to explain this origin of this process.

Next, synergism between copper and the other metals was studied in order to have two metals in the EPS derivatives at the end of the depolymerization process, with the possibility of obtaining some interesting biological properties. Equimolar mixtures of two metals (0.09 mM of each metal for 200 mg of EPS) were tested with and without pH control. Again, two different types of behavior were observed (Table 4, entries 4–7).

For the Cu/Zn system, the results are very close to those obtained when using copper alone. Copper and zinc are two di-cations with very similar sizes so similar chelation properties could be found. With the Cu/Mn system and without pH control, the molecular weight of the EPS derivatives is very low $M_w < 10,000 \text{ g/mol}$ (Table 4). Finally, we must say that the mechanism of the free-radical depolymerization with metallic catalysts is not well established. The radical degradation process occurs through the formation of free hydroxyl radical OH• from the hydrogen peroxide-metal redox system. Depending on the polysaccharide structures, these very reactive species mainly attack at the glycosidic bond but, in some cases, direct cleavage of the sugar ring can be observed. Presumably, the site of attack of the hydroxyl radical generated in the aqueous medium during the reaction is localized by the metal used. As has been shown, this EPS contains many carboxylic and hydroxyl groups which could be involved in binding metal ions as weak electron donors (Brown & Lester, 1979). Often a decrease in the content of glucuronic acid is observed, which is not the case for galacturonic acid. Now D-galacturonate complexes are stronger than D-glucuronate ones (Gould & Rankin, 1970), which can be explained by the formation of a complex at the axial position of O-4 in the former. Thus, glucuronic acid would be in more of a position to react with free radicals than galacturonic acid.

4. Conclusion

We have prepared, with high reproducibility, low molecular weight and size-defined EPS HYD 657 derivatives in the molecular weight (M_w) range from 100,000 to 20,000 g/mol, depending on the catalytic system and experimental conditions.

Table 5

Monosaccharide contents of the LMW EPS derivatives obtained after depolymerization with different metals and R (H₂O₂/EPS)=0.4

EPS	Metal	Rha (%)	Fuc (%)	Gal (%)	Glu (%)	Man (%)	GlcA (%)	GalA (%)	$M_w (\times 10^3 \text{ g/mol})$	I_p
Native	–	3.4	1.8	21.7	12.3	1.8	6.1	7.3	3000	–
LMW EPS	Cu	3.0	1.7	11.2	7.6	1.3	2.8	7.2	10	2.5
LMW EPS	Zn	2.8	1.5	17.7	9.8	1.3	4.8	7.3	21.5	4.8
LMW EPS	Mn	2.3	1.3	18.0	9.0	1.8	4.3	7.4	25	^a

^a Three peaks.

Copper was found to be a good catalyst whereas zinc and manganese used alone were much less efficient. However, synergies between the different metals were also studied and depolymerization was shown to be more efficient when a mixture of copper and zinc or manganese was used. To control the free-radical production, and thus, the efficiency and the reproducibility of the depolymerization, the most other influential parameters were pH control and the concentration of H_2O_2 . This latter parameter can be modulated both by the flow rate of H_2O_2 and the mass ratio $\text{H}_2\text{O}_2/\text{EPS}$. A low flow rate of H_2O_2 and a neutral pH (except for the mixture manganese sulfate–copper sulfate) are essential both to control the depolymerization (fractions above 20,000 g/mol) and to obtain good reproducibility. Now, studies are underway to explore the potential of depolymerized fractions, especially some of their biological properties.

Acknowledgements

The authors wish to thank the Lanatech subsidiary of Atrium Biotechnologies for financial support.

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