Horseradish Peroxidase Mediated Free Radical Polymerization of Methyl Methacrylate

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This paper reports the free radical polymerization of methyl methacrylate (MMA) catalyzed by horseradish peroxidase (HRP). A novel method was developed whereby MMA polymerization can be carried out at ambient temperatures in the presence of low concentrations of hydrogen peroxide and 2,4-pentanedione in a mixture of water and a water-miscible solvent. Polymers of MMA formed were highly stereoregular with predominantly syndiotactic sequences (syn-dyad fractions from 0.82 to 0.87). Analyses of the chloroform-soluble fraction of syndio-PMMA products by GPC showed that they have number-average molecular weights, M_n, that range from 7500 to 75 000. By using 25% v/v of the cosolvents dioxane, tetrahydrofuran, acetone, and dimethylformamide, 85, 45, 7 and 2% product yields, respectively, resulted after 24 h. Increasing the proportion of dioxane to water from 1:3 to 1:1 and 3:1 resulted in a decrease in polymer yield from 45 to 38 and 7%, respectively. Increase in the enzyme concentration from 70 to 80 and 90 mg/mL resulted in increased reaction kinetics. By adjustment of the molar ratio of 2,4-pentanedione to hydrogen peroxide between 1.30:1.0 and 1.45:1.0, the product yields and M_n values were increased. On the basis of the catalytic properties of HRP and studies herein, we believe that the keto−enoxy radicals from 2,4-pentanedione are the first radical species generated. Then, initiation may take place through this radical or by the radical transfer to another molecule.

Introduction

Enzymes have proven to be powerful catalysts for the polymerization of a wide variety of monomers and macromonomers.[1] Furthermore, enzymes represent a family of environmentally friendly catalysts. Though enzyme catalysis has been known for well over a century,[2] it has been largely restricted to aqueous systems until the relatively recent development of nonaqueous enzymology.[3−5] Nonaqueous enzymology has had a powerful impact on organic synthetic methods. In some cases, nonaqueous enzyme catalysis has provided synthetic routes to chemical transformations that are difficult, or even impossible, with conventional chemical catalysts. Important examples of reactions catalyzed by enzymes in organic media include the following: (i) the lipase-catalyzed synthesis of optically active polyesters,[6] (ii) lipase-catalyzed interesterification of triglycerides and fatty acids,[7] (iii) the regioselective oxidation of phenols by phenol oxidase,[8] (iv) peroxidase-mediated reactions that yield polymers with useful electrooptical properties such as polyphenols and polyaromatic amines,[9,10] (v) and the modification of polysaccharides to enhance their processability and to regulate their biodegradability.[11]

Horseradish peroxidase (HRP) is an oxido-reductase that acts on hydrogen peroxide and/or alkyl peroxide as an oxidant[12] and on several reducing substrates such as phenol, hydroquinone, pyrogallol, catechol, aniline, and *p*-aminobenzoate.[13] The oxidative coupling of a variety of substrates such as phenols and aromatic amines catalyzed by HRP in the presence of hydrogen peroxide have been reported in aqueous,[14] nonaqueous,[3−5,8−10,15−19] and interfacial systems.[20]

The potential of using HRP and other oxidases to catalyze the free radical polymerization of vinyl monomers was first reported by Derango et al.[21] The polymers were formed in the presence of a large excess of oxidant (monomer:oxidant, 1.66:1.0 v/v, for, e.g., 1.41:1.0 mol/mol for 2-hydroxyethyl methacrylate). Unfortunately, these workers gave only qualitative descriptors to describe whether a polymer was formed without further information on the polymer structure. Kobayashi and co-workers[22] reported the HRP-catalyzed polymerization of phenylethyl methacrylate. Similar to Derango et al.,[21] Kobayashi and co-workers[22] also published the formation of polymer using large quantities of oxidant (equimolar with respect to the monomer). More recently, HRP-mediated free radical polymerization of acrylamide in water was reported.[23,24] These polymerizations took place when *β*-diketones were used as initiators and the molar ratio of hydrogen peroxide to monomer was 1 to 66.[23,24] That *β*-diketones would react under such conditions is related to their weakly bonded α-hydrogens. Indeed, it had previously been shown that cyclic *β*-ketones, such as 5,5-dimethyl-1,3-cyclohexanedione, are substrates for chloroperoxidases which belong to the same subclass of enzymes as HRP (E.C. 1.11.a and 1.11.1.7, respectively).[25] By analogy to phenol, it was assumed that the enolic tautomeric form of 2,4-pentanedione is a key intermediate in the catalytic pathway.

One objective of this work was to explore the potential of enzymes to regulate the stereochemical configuration of

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methyl methacrylate (MMA) repeat units along chains. Stereoregular poly(methyl methacrylate)s, PMMAs, were first prepared by Fox et al.[27a] and Miller et al.[27b] The stereospecific polymerization of MMA relies on the use of a variety of lithium, magnesium and aluminum compounds. Preparation of highly syndiotactic PMMA is of great importance since it gives products that have a higher glass transition temperature, T_g. The high T_g of PMMA is exploited for industrial applications such as in optical fibers and disks. The stereoregulation of PMMA by ionic polymerizations requires the use of very low temperatures (around $-80\ °C$). Typical free radical polymerizations, carried out at $60-70$ °C, give PMMA with syndiotactic diad fractions of about 0.80.[28]

In this paper, the HRP-mediated polymerization of MMA in mixtures of water and water-miscible cosolvents was studied. The potential that the enzyme could interact in some way with the propagating chain to influence chain stereochemistry was investigated. Systematic variations of polymerization conditions, including the oxidase used, hydrogen peroxide and 2,4-pentanedione concentrations, water-miscible cosolvents, and the reaction temperature were carried out. The extent that variations in these reaction parameters influence the yield, molecular weight averages, and chain stereochemistry of the PMMAs formed was reported. Experiments were conducted to determine whether the mechanism of polymerization involved a direct electron transfer from the oxoiron(IV) π-radical cation to MMA, or whether chain initiation takes place through the radical species generated by 2,4-pentanedione.

Experimental Section

Materials. Horseradish peroxidase (type II, activity 235 purpullogallin units/mg), Horseradish peroxidase (type I, activity 100 purpullogallin units/mg), Soybean peroxidase (90 purpullogallin units/mg), *Arythomyces ramosus* peroxidase [75 purpullogallin units/mg, hydrogen peroxide (30% w/v)] were all obtained from the Sigma Chemical Co. Methanol, dioxane, acetone, tetrahydrofuran, and dimethylformamide were all of analytical grade and were used as received. Methyl methacrylate (MMA), obtained from the Aldrich Chemical Co., was fractionally distilled over calcium hydride under reduced pressure with a nitrogen atmosphere. 2,4-Pentanedione from Aldrich was distilled prior to use.

Instrumentation. The NMR data were recorded on a Bruker DPX300 (300 MHz) and Bruker AMX500 (500 MHz). The chemical shifts in parts-per-million (ppm) for proton (^1H) NMR spectra were referenced relative to tetramethylsilane (TMS, 0.00 ppm) as the internal reference. The stereochemistry of the polymer backbone was calculated by observing the NMR signals[29] due to the backbone methyl groups: syndiotactic triad, 0.81 ppm; atactic triad, 0.97 ppm; isotactic triad, 1.14 ppm. The standard deviation for the syn dyad fractions was calculated by taking the mean for three replicates of the NMR integration values.

The number-average molecular weights (M_n) of the polymer samples were determined by gel permeation chromatography (GPC) using a Waters HPLC system equipped with a model 510 pump, a Waters model 717 autosampler, a model 410 refractive index detector, and a model T-50/T-60 detector from Viscotek Corp. with 500, 10^3, 10^4 and 10^5 Å Ultrastyragel columns in series. Trisec GPC software Version 3 was used for calculations. Chloroform was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 0.2% w/v and injection volumes of 100 μL were used. Molecular weights were determined based on conventional calibration curve generated by polystyrene standards of low dispersity (Aldrich Chemical Co.). Since the PMMAs formed in this work were not completely soluble in chloroform, the molecular weight values reported are relevant to only part of the product.

Differential scanning calorimetry (DSC) was performed on a TA instrumental model 2920 DSC. The temperature program used was to increase the sample temperature from room temperature to 150 °C, to cool to 30 °C, and then to reheat to 150 °C. The heating rate was 20 °C min^{-1}, and the purge gas was helium. The cooling was performed using TA instruments refrigerated cooling systems using an equilibration step that gives rapid cooling. The glass transition temperature (T_g) was determined by the midpoint at half-height. The T_g results were taken from the second heating scan.

Enzyme-Catalyzed Polymerization of MMA in Binary Solvents. Methyl methacrylate (MMA) (5.6 mmol) was added to a solution of distilled water (0.7 mL) and organic solvent (0.3 mL) in a dual inlet ampule under nitrogen atmosphere. An example of a typical reaction is the successive addition under a nitrogen atmosphere of 0.2 mL of HRP (80 mg/mL, 16 mg of enzyme), hydrogen peroxide (0.092 mmol), and 2,4-pentanedione (0.136 mmol). The reaction mixture was maintained under nitrogen with stirring at room temperature for a predetermined time period. Then, the reaction mixture was poured into a large excess of methanol. The precipitate obtained was separated by filtration, washed with methanol, and dried (in vacuo, 50 °C, 30 mmHg, 24 h).

Results and Discussion

The reducing substrate 2,4-pentanedione and hydrogen peroxide were used in the ratio 1.5:1.0 mol/mol. The ratio of methyl methacrylate (MMA) to 2,4-pentanedione was fixed at between 42 and 44:1. MMA polymerizations were conducted in binary solvents that consisted of water and a cosolvent. The molecular weight values represent that for the soluble fraction since the PMMA products were partially soluble in chloroform. Attempts to totally dissolve the products using tetrahydrofuran, toluene, dichlorbenzene, and dimethylformamide as well as other solvents were unsuccessful. IR spectra of the insoluble products revealed IR bands and peaks that are similar to that of the chloroform-soluble stereoregular PMMA as well as PMMA obtained commercially. Thus, we conclude that the insolubility of the PMMA fraction is due to cross-linking reactions, the nature and frequency of which are not currently known.

Screening of Enzymes. Different commercial peroxidases were evaluated to compare their catalytic activities for MMA

Table 1. Different Oxidases Screened for MMA Polymerization Carried out at 25 °C for 24 Hours in Water:Dioxane (3:1,v/v)[a]

oxidase	% isolated yield	M_n ($\times 10^4$)	M_w/M_n	syn-diad fraction[b]	ca. % solubility in CHCl$_3$
HRP II	45	6.3	3.0	0.847	83
HRP I	5	3.3	1.6	0.848	76
SBP	48	9.3	6.8	0.850	95
ARP	5	N.D.	N.D.	N.D.	N.D.

[a] Oxidase = 80 mg/mL (16 mg), H_2O_2 = 0.092 mmol, 2,4-pentanedione = 0.136 mmol. N.D.: not detemined. [b] The standard deviation for the syn dyad fractions was determined by taking the mean for three replicate NMR integration intensities. The values of standard deviation ranged from 0.002 to 0.005.

Table 2. Effect of Water Miscible Cosolvents on MMA Polymerization Carried out at 25 °C for 24 h[a]

cosolvent	% isolated yield	M_n ($\times 10^4$)	M_w/M_n	T_g (°C)	syn-diad fraction[b]	ca. % solubility in CHCl$_3$
25% DMF	<2.0	N.D.	N.D.	N.D.	N.D.	N.D.
25% acetone	7.0	1.8	4.7	128	0.852	90
25% dioxane	45.0	6.3	3.0	131	0.847	90
50% dioxane	38.0	1.9	3.8	126	0.851	97
75% dioxane	7.0	2.8	4.3	N.D.	0.852	N.D.
25% THF	85.0	7.2	3.1	128	0.842	98
50% THF	58.0	6.4	2.6	N.D	0.841	89

[a] HRP II = 80 mg/mL (16 mg), H_2O_2 = 0.092 mmol, 2,4-pentanedione = 0.136 mmol. N.D.: not detemined. [b] See footnote, Table 1.

polymerization. These comparative studies were conducted at room temperature (25 °C), in a binary mixture of water−dioxane (3:1), for 24 h (Table 1). The oxidases evaluated were normalized based on weight, i.e., the same quantities by weight of the oxidases were taken to determine their activity for MMA polymerization. HRP II (type II) and soybean peroxidase appeared most promising. For example, the yields of PMMA when using soybean peroxidase (SBP), HRP II, HRP I and *A. ramosus* peroxidase were 48, 45, 5, and 5%, respectively. Furthermore, HRP II and SBP catalyzed polymerizations gave PMMA with high syn-diad fractions (0.85 and 0.85, respectively) and M_n values (63 000 and 93 000, respectively). However, the polydispersity index of the product was much lower using HRP II (3.0 instead of 6.8). On the basis of the above, HRP II was selected for more detailed studies of its ability to mediate MMA polymerizations.

Effect of Cosolvents. The room-temperature polymerization of MMA catalyzed by HRP II in the presence of hydrogen peroxide and 2,4-pentanedione was carried out in binary solvent mixtures consisting of water and different cosolvents. The water-miscible cosolvents dioxane, acetone, tetrahydrofuran, and dimethylformamide were evaluated (Table 2). Independent of the solvent mixture, the polymers formed, based on analyses by high field proton (^{1}H) NMR (see Supporting Information) were predominantly syndiotactic (syn-dyad fractions ranging from 0.84 to 0.85). When the binary mixture was water:DMF (3:1), the polymerization was very slow (<2% polymer yield in 24 h). The low yield in water/DMF is presumably due to unfavorable interactions between HRP and DMF resulting in enzyme deactivation. The yields of PMMA increased when less polar solvents (e.g., acetone, tetrahydrofuran, and dioxane) were used.

Specifically, for polymerizations in water/acetone, water/dioxane and water/THF (all 3:1 v/v, 24 h reactions), the PMMA yields were 7, 45, and 85%, respectively. Variation of the time of reactions in water:dioxane 3:1 v/v from 2 to 24 h resulted in an increase in both PMMA yield (10−45%) and M_n (15 000−63 000).

Ratios of water:dioxane (v/v) of 3:1, 1:1 and 1:3 were evaluated for HRP II mediated MMA polymerizations (Table 2). Increasing the ratio of dioxane to water as above resulted in decreases in polymer yield from 45 to 38 and 7%, respectively. HRP II normally loses activity as the proportion of the nonaqueous solvent increases.[30] This loss in enzyme activity is manifested here as lower product yield.[30] Analysis of the syndio-PMMA products by GPC established that they had M_n values between 18 000 and 72 000 (Table 2). Measurements by DSC, performed on PMMA products of high syndiotacticity (0.84 to 0.85 based on analysis of pentad sequences by ^{1}H NMR), showed they had T_g values of about 130 °C. For comparison, PMMA prepared by conventional free radical polymerization (bulk, AIBN, 60 °C) had a syn-diad fraction of 0.78 and a T_g of 123 °C.[31,32] The syn-diad fractions of PMMA prepared by convention free radical polymerization at 30 and 5 °C were 0.825 and 0.8475, respectively.[31]

Effect of Temperature. Previous work has shown that HRP II retains 100% of its activity at 5 °C and ≥70% of its activity when incubated at temperatures between 25 and 45 °C.[33] Therefore, HRP II catalyzed radical polymerization of MMA was performed at 5, 15, 20, 25, 34 and 40 °C for 24 h in water:dioxane (3:1). The isolated polymer yield was inversely proportional to the reaction temperature. Specifically, reaction temperatures of 5, 10, 15, 20, 25, 34 and 40 °C had product yields of 75, 56, 48, 45, 36 and 14%, respectively (product nos. 1−6, Table 3). Also, by decreasing the reaction temperature from 40 to 5 °C, the fraction of syn-diads increased from 0.82 to 0.87. Furthermore, decrease in the polymerization temperature from 40 to 5 °C resulted in a decrease in the induction time prior to monomer consumption from 70 min at 40 °C to 15 min at 5 °C. At present, we do not understand the factors that cause the induction period and, therefore, cannot offer an explanation for the observed temperature-induction period relationship.

Effect of HRP II, Hydrogen Peroxide, and 2,4-Pentadione Concentration. A study was conducted to determine how changes in the concentrations of HRP II, hydrogen peroxide, and 2,4-pentanedione alter the outcome of MMA polymerizations carried out for 24 h at 25 °C. Interestingly, changing the enzyme concentration from 14 mg (70 mg/mL) to 16 mg (80 mg/mL) resulted in an increase in the syn-diad fraction from 0.84 to 0.85 (product nos. 7 and 4, Table 3) and increase in polymer yield from 39 to 46%. Also, increase in the enzyme concentration from 16 mg (80 mg/mL) to 18 mg (90 mg/mL) leads to a further increase in polymer yield (46−56%). Thus, an increase in the enzyme concentration from 70 to 90 mg/mL resulted in an increase in reaction kinetics. The effect on HRP II catalyzed MMA polymerizations of varying the hydrogen peroxide concentration is also shown in Table 3. By increasing the concentration of H_2O_2 from 0.056 to 0.110 mmol, the percent yield

Table 3. HRP II Mediated Polymerization of MMA in Water:Dioxane (3:1) for 24 h: Effect of Temperature, Enzyme Concentration, Hydrogen Peroxide Concentration and 2,4-Pentanedione Concentration

no.	HRP II concn	H_2O_2 concn (mmol)	2,4-pentanedione concn (mmol)	temp (°C)	% isolated yield	syn-dyad fraction[b]	M_n ($\times 10^4$)	M_w/M_n	ca. % solubility ($CHCl_3$)
1	16	0.092	0.136	5	75	0.871	2.7	4.1	74
2	16	0.092	0.136	15	57	0.860	1.7	4.8	70
3	16	0.092	0.136	20	48	0.856	2.2	4.7	70
4	16	0.092	0.136	25	45	0.847	6.3	3.0	83
5	16	0.092	0.136	34	36	0.826	5.3	3.3	78
6	16	0.092	0.136	40	14	0.814	N.D.[a]	N.D	68
7	14	0.092	0.136	25	39	0.845	4.9	3.4	92
8	18	0.092	0.136	25	56	0.850	5.1	3.6	93
9	16	0.056	0.136	25	65	0.849	9.1	2.8	92
10	16	0.074	0.136	25	61	0.850	7.1	3.3	88
11	16	0.110	0.136	25	13	0.847	1.3	4.3	68
12	16	0.092	0.097	25	18	0.845	2.2	4.9	69
13	16	0.092	0.116	25	88	0.845	6.6	2.9	89
14	16	0.092	0.155	25	14	0.850	1.7	3.9	75

[a] N.D.: not detemined. [b] See footnote, Table 1.

decreased from 65 to 13% and the M_n decreased from 91 000 to 13 000 (product nos. 9–11, Table 3). It may be that the higher concentration of radical species generated at increased H_2O_2 concentration leads to an increase in termination reactions and, therefore, a decrease in product molecular weight.

Experiments conducted where the concentration of 2,4-pentanedione was varied gave extraordinary changes in the reaction kinetics. The polymer yield at 0.097 mmol 2,4-pentanedione (MMA 5.6 mmol; hydrogen peroxide 0.092 mmol) was 18%. The polymer yield increased from 18 to 88% when the concentration of 2,4-pentanedione was increased from 0.097 to 0.116 mmol (product nos. 12 and 13, Table 3). Further increase in the 2,4-pentanedione concentration to 0.136 and 0.155 mmol gave decreased product yields (45 and 14%, respectively; product nos. 4 and 14, Table 3). The optimal ratio of 2,4-pentanedione to hydrogen peroxide under the given reaction conditions studied was found to be between 1.3 and 1.45, whereby the yields are 88 and 45% and the M_n values are 66 000 and 63 600, respectively (product nos. 4 and 13, Table 3).

Mechanism of HRP-Mediated MMA Polymerization. Derango et al.[21] and Kobayashi and co-workers[22] used large quantities of hydrogen peroxide with respect to monomer and enzyme in their reports of HRP-catalyzed vinyl polymerization. In addition, Derango et al.[21] claimed to have initiated acrylamide and hydroxyethyl methacrylate polymerizations at room temperature in the absence of a proton donor. They believed that the reaction of HRP with hydrogen peroxide involves the formation of the oxoiron(IV) π-radical cation that may be contributing to the polymerization.[26] Similar to Derango, Kobayashi and co-workers[22] also did not use a proton donor to conduct phenylethyl methacrylate polymerization at room temperature. In contrast, Marechal and co-workers[23,24] conducted HRP-mediated acrylamide polymerizations at room temperature in the presence of a proton donor. They used a hydrogen peroxide-to-monomer ratio of 1/66 mol/mol.

In our studies of HRP–MMA polymerizations, in the absence of 2,4-pentanedione and using MMA to H_2O_2 (61:1 mol/mol), MMA polymerization did not take place. If the reaction occurred in the absence of 2,4-pentanedione, then we would expect that the oxoiron(IV) π-radical cation would be the intermediate responsible for initiation of MMA free-radical polymerization. Furthermore, in the absence of enzyme, and presence of 2,4-pentanedione and H_2O_2, no polymer was obtained. Thus, it does not appear that MMA polymerization takes place due to the homolytic cleavage of hydrogen peroxide. Therefore, we believe that the formation of primary radicals arises from the second and third steps of the enzymatic cycle. The keto–enoxy radicals are generated as the first radical species. Then, initiation may take place through this radical or by the radical transfer to any other molecule, i.e., enzyme impurity, amino acid residue, or hydrogen peroxide itself. Since the PMMA obtained is of very high molecular weight, we were unable to determine directly by NMR investigations whether the 2,4-pentanedione was at chain ends. Additional work will be necessary to confirm whether the initiator is indeed found at terminal chain positions.

It appears that in the absence of 2,4-pentanedione, an excess molar ratio of hydrogen peroxide with respect to the monomer is needed in order to generate free radicals that can initiate vinyl polymerizations. These conditions are similar to those generally used for HRP-catalyzed phenol and aromatic amine oxidations and free-radical polymerizations.[9,34] However, it may be desirable to use lower quantities of hydrogen peroxide relative to monomer in order to minimize enzyme deactivation and nondesired free-radical events such as cross-linking of chains or gel formation. In addition, a reduced concentration of a toxic reagent such as hydrogen peroxide is environmentally beneficial. Thus, when lower quantities of hydrogen peroxide relative to monomer are used, a mediator such as 2,4-pentanedione is needed to efficiently initiate MMA free radical polymerizations. This was similarly observed by Marechal and co-workers[23,24] in their studies of HRP-mediated acrylamide polymerization.

Conclusions

The HRP-mediated free radical polymerization of MMA was found to be a novel initiating system for the preparation

of highly syndiotactic PMMA. Normally, to attain high degrees of PMMA syndioregularity, it is necessary to use toxic metals or extreme temperatures. For example, ionic polymerizations and conventional free-radical polymerizations are often conducted at about −80 and ≥70 °C, respectively. HRP-mediated MMA polymerizations provide an "environmentally friendly" route to stereoregular PMMA. The polymerizations were conducted at ambient temperature in water/dioxane to form PMMA. The products were found to have syndiotactic diad fractions ranging from 0.82 to 0.87 and T_g values of about 130 °C. Detailed studies herein showed that the polymer yield, the extent of cross-linking, and molecular weight were strongly a function of reaction parameters such as the cosolvent, reaction temperature, and concentrations of HRP, hydrogen peroxide, and 2,4-pentanedione. The use of the low dielectric constant, and water-miscible cosolvents dioxane and tetrahydrofuran resulted in high polymer yields. Increases in enzyme concentration led to greater polymer yields. Also, by maintaining a stoichiometric ratio of 2,4-pentanedione to hydrogen peroxide of 1.30−1.45/1.0, higher polymerization rates and product M_n values resulted. An insoluble fraction of PMMA was found under all conditions studied herein for HRP-mediated MMA polymerization. That this insoluble fraction was not soluble in solvents that are known to dissolve high molecular weight syndiotactic PMMA allowed us to conclude that cross-linking accompanied propagation reactions. It will be interesting to determine the mechanism of cross-linking and ways to increase or circumvent such reactions. The generation of radical species was found to take place from hydrogen peroxide and 2,4-pentanedione through an oxidoreductive pathway**.** By analogy with the HRP-catalyzed oxidation of phenol, we believe that the enolic form of 2,4-pentanedione is directly involved in the generation of primary radicals.

Supporting Information Available. Figure 1, showing the ^{1}H NMR plot of syndioregular PMMA obtained by MMA polymerization mediated by horseradish peroxidase with hydrogen peroxide-2,4-pentanedione system. This material is available free of charge via the Internet at http://pubs.acs.org.

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