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ORIGINAL CONTRIBUTION

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Horseradish peroxidase-catalyzed polymerization of *p*-hydroxycinnamic acid for synthesis of reactive microspheres

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Abstract In this article, we report the experimental synthesis of reactive polymer microspheres of poly(*p*-hydroxycinnamic acid). Enzyme-catalyzed polymerization of poly(*p*-hydroxycinnamic acid) using horseradish peroxidase as a catalyst and hydrogen peroxide as an oxidant took place in a mixture solution of methanol and phosphate buffer solution; it was found that the fraction of methanol in the mixture solution strongly affected the yield of powdery polymer materials. The chemical structure of the polymers was characterized by ^{1}H-NMR and FT-IR spectroscopies, and the molecular weight was measured by gel permeation chromatography. The ^{1}H-NMR chart of the obtained polymer was almost the same as that of the monomer; FT-IR spectra indicated the existence of carboxyl groups. The weight-average molecular weight of the soluble part in tetrahydrofuran was found to be 1,451. Dispersion polymerization of *p*-hydroxycinnamic acid was carried out in a mixture solution of methanol and phosphate buffer solution by adding a dispersion stabilizer. Of the several such polymers tested, poly(vinyl alcohol) was found to be the most effective in producing reactive poly(*p*-hydroxycinnamic acid) microspheres.

Keywords Reactive polymer microspheres · Enzyme-catalyzed polymerization · Dispersion polymerization · Polymer dispersant · *p*-Hydroxycinnamic acid

Introduction

Enzyme-catalyzed polymerization has been attractive in both fundamental and applied fields; in particular, much attention has been given to the discovery of environmentally benign polymerization in which the reaction proceeds under mild conditions [1]. Furthermore, this polymerization process, catalyzed by enzymes such as oxydo-reductase (lipase, horseradish peroxidase, etc.) has introduced polymers having properties that are quite different from those of polymers prepared by traditional polymerization processes, that is, radical polymerization, polycondensation, and so on. So far, enzyme-catalyzed polymerization has been investigated for substituted phenols [1, 2, 3, 4, 5, 6], lactone [7, 8], and aromatic amines [9].

p-Hydroxycinnamic acid is one of the substituted phenols; its molecule has a carboxyl group, a phenolic hydroxyl group and a double bond, as shown in Fig. 1. Radical polymerization may be retarded or terminated by the phenolic hydroxyl group of *p*-hydroxycinnamic acid, so that the polymer cannot be synthesized. Polycondensation between carboxyl and phenol groups would be a promising way to synthesize poly(*p*-hydroxycinnamic acid), except that functional groups are consumed by polycondensation, rendering the product unreactive. Enzyme-catalyzed polymerization is likely to provide a reactive poly(*p*-hydroxycinnamic

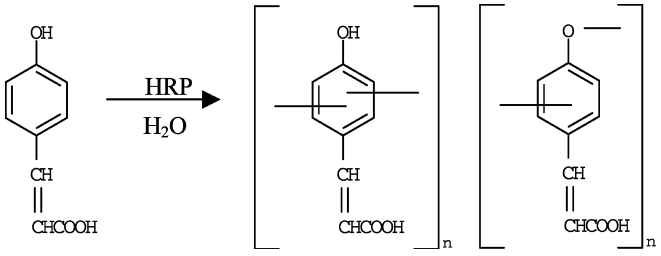

Fig. 1 Enzyme-catalyzed polymerization of *p*-hydroxycinnamic acid

acid) with carboxyl groups as reactive functional groups as shown in Fig. 1, because it has been found in previous studies on the polymerization of phenol and cresols that the obtained polymer is composed of a mixture of phenylene and oxyphenylene units.

This study focused on the preparation of reactive polymer microspheres of poly(*p*-hydroxycinnamic acid) by enzyme-catalyzed polymerization. It has been pointed out in previous papers that enzyme-catalyzed polymerization is significantly affected by solution properties such as the ratio of organic solvent to water. The first approach in this work was therefore the selection of a solution suitable for the polymerization; subsequently, we attempted in situ formation of reactive polymer microspheres of poly(*p*-hydroxycinnamic acid) using suitable dispersion stabilizers.

Materials and methods

Reagent Horseradish peroxidase was supplied by Wako. Reagent-grade *p*-hydroxycinnamic acid was used as a monomer without further purification. Aqueous hydrogen peroxide (30% wt) solution was used as an oxidant. All other chemicals of analytical grade were obtained from Wako and used as received.

Polymerization A prescribed amount of *p*-hydroxycinnamic acid was dissolved in a solvent to make a solution of the desired concentration. The solution of *p*-hydroxycinnamic acid was added dropwise into a pH 7.2 phosphate buffer solution containing horseradish peroxidase under vigorous stirring. Subsequently, the 30% hydrogen peroxide solution (0.56 ml) was added in 20 portions at intervals of 15 min. The reaction progressed for 6 h at room temperature after full addition of hydrogen peroxide solution. The obtained product was separated by suction filtration and was washed by Elix water and methanol. Solvents used in this work were acetonitrile, acetone, 1,4-dioxane, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), ethanol and methanol.

Functional microspheres prepared by dispersion polymerization A prescribed amount of *p*-hydroxycinnamic acid was dissolved in methanol. An aqueous solution of a water-soluble polymer and horseradish peroxidase was charged into the methanol solution of *p*-hydroxycinnamic acid. The water-soluble polymer was employed as a dispersion stabilizer; its concentration was kept at 25% wt of the monomer. After the addition of hydrogen peroxide, dispersion polymerization continued for 24 h with moderate stirring by a magnetic stirrer. The obtained precipitate, recovered by suction filtration with a glass filter, was washed with Elix water, and dried in vacuo.

Analysis The molecular weight of the obtained polymer was measured by gel permeation chromatography (GPC) (Tosoh, HLC 8120GPC) using a controlling unit (Tosoh GPC-8020). The measurement conditions were as follows: 1.2 ml/min flow rate, THF eluent and 313 K temperature. The following columns were connected in series: TSK guard column Super H-H, TSK Super HM-H (two columns) and TSK Super H2000. A calibration curve was made with standard polystyrene (Tosoh, PS oligomer kit, molecular weight range of 495–1,110,000).

The chemical structure of the prepared polymer was analyzed by FT-IR (Shimadzu, FTIR-8600PC) and NMR (Varian, Gemini-200) spectroscopies. FT-IR measurement was carried out by the KBr method. In NMR measurement, the solvent was dimethylsulfoxide (DMSO-d_6) and the standard was trimethylsilane. Scanning electron microscopy (SEM) was performed with JEOL JSM-6300 to investigate the morphology and particle diameter of the prepared microspheres.

Results and discussion

Polymerization of *p*-hydroxycinnamic acid in solvent

The solvents tested for enzyme-catalyzed polymerization of *p*-hydroxycinnamic acid using horseradish peroxidase included acetonitrile, acetone, 1,4-dioxane, DMF, THF, ethanol and methanol. Table 1 shows the yields of poly(*p*-hydroxycinnamic acid) in the mixture solution of each organic solvent with a phosphate buffer solution. The fraction of organic solvent was kept at 0.6. It was found that the methanol/phosphate buffer solution system provided the highest yield of polymer. The polymer yield was only 2.3% when acetone was used as a co-solvent. The monomer was not dissolved in acetonitrile. No precipitate was formed in the other mixtures of organic solvent/phosphate buffer.

The addition of aqueous hydrogen peroxide turned the transparent brown solution of horseradish peroxidase and *p*-hydroxycinnamic acid into a black turbid solution of the prepared polymer precipitate. This phenomenon was only observed when methanol and

Table 1 The effect of organic solvent on yield of poly(*p*-hydroxycinnamic acid) by enzyme-catalyzed polymerization. The fraction of organic solvent was kept at 0.6.*THF* Tetrahydrofuran, *DMF* *N*,*N*-dimethylformamide

Entry	Organic solvent	Yield (%)
1	Acetonitrile	-
2	Acetone	2.3
3	1,4-Dioxane	-
4	DMF	-
5	THF	-
6	Ethanol	-
7	Methanol	47.9

acetone were used as the reaction medium. No color change was observed without horseradish peroxidase. This indicates that color change is evidence that enzyme-catalyzed polymerization is proceeding.

Uyama et al. found that polymerization of phenol in 1,4-dioxane/buffer solutions produced polymeric material in higher yields [2]. Ichinohe et al. carried out the polymerization of phenylenediamines in aqueous 1,4-dioxane solutions and produced poly(phenylenediamine)s [9]. It was also reported that polymerization of syringic acid catalyzed by horseradish peroxidase had been successfully carried out in acetone/buffer solutions, acetonitrile/buffer solutions, 1,4-dioxane/buffer solutions and methanol/buffer solutions [1]. However, in this work, polymer materials could not be recovered in organic solvent/buffer solutions except when methanol and acetone were used as co-solvents. At the present stage, the effect of the solvent on the reactivity of *p*-hydroxycinnamic acid in enzyme-catalyzed polymerization cannot be specified in detail. Presumably, it is due to the low solubility of the monomer and/or the low activity (denaturation) of enzyme in the solution used.

Figure 2 illustrates the effect of solvent composition on polymerization of *p*-hydroxycinnamic acid in the case of methanol as a co-solvent, the highest performing co-solvent. It was found that the methanol fraction of the mixture solution strongly affected the yield of poly(*p*-hydroxycinnamic acid). The yield of polymer product increased with increasing methanol fraction until the methanol fraction reached 0.6. Further addition of methanol reduced the polymer yield, and no polymer product was synthesized in the solution of which the methanol fraction was over 0.8. This tendency is similar to that previously reported for polymerizations of phenol derivatives and phenylenediamine in 1,4-dioxane/buffer solution [5, 9]. Deterioration of polymer yield at the higher methanol fractions was presumed to be the result of lowered enzyme activity.

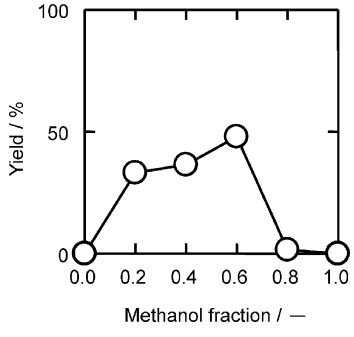

Fig. 2 The effect of methanol fraction in polymerization medium on polymer yield

Chemical analysis of the prepared polymer

The polymer products obtained by enzyme-catalyzed polymerization in methanol/phosphate buffer solution were partly soluble in THF at room temperature. A yellowish transparent solution of the polymer was obtained by heating in a thermostatic bath at 308 K; no crystallization was observed upon cooling to room temperature.

The weight-averaged molecular weight of the synthesized polymer was measured by GPC, and the obtained chromatogram is shown in Fig. 3, together with the chromatogram of the monomer. As can be seen, the main peaks of the polymer and the monomer solutions appeared at 12.32 min and 14.59 min, respectively. This implies that the oxidative reaction of *p*-hydroxycinnamic acid catalyzed by horseradish peroxidase produced poly(*p*-hydroxycinnamic acid), the weight-averaged molecular weight of which was found to be 1,451.

The ^{1}H-NMR spectra of *p*-hydroxycinnamic acid and the obtained polymer are shown in Fig. 4. The multiple peaks seen around 6.7–8 ppm are due to aromatic protons, and the peak around at 6.3 ppm corresponds to olefin proton. The ^{1}H-NMR spectrum of the obtained polymer exhibits broad peaks. It is regrettable that these two ^{1}H-NMR spectra give no useful information on the coupling.

Figure 5 shows the IR spectra of *p*-hydroxycinnamic acid and the obtained polymer. The peaks at 3,400 cm^{-1}, 1,700 cm^{-1} correspond to the O-H bond and C = O bond, respectively. Furthermore, these two spectra show peaks at 1,600 cm^{-1}, 1,500 cm^{-1} and 1,450 cm^{-1} due to the aromatic C = C bond. Comparing the IR spectrum of the obtained polymer with that of the monomer, it was found that the IR spectrum of *p*-hydroxycinnamic acid had a peak at 1,300 cm^{-1} due to its phenolic C-OH bond, which disappeared in the obtained polymer. In addition,

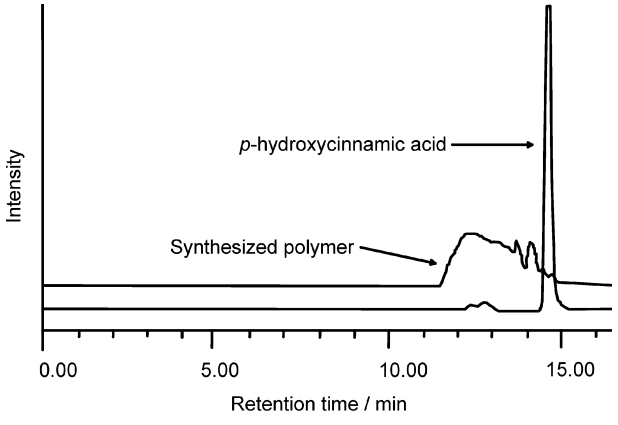

Fig. 3 Gel permeation chromatography chart of *p*-hydroxycinnamic acid and synthesized polymer by enzyme-catalyzed polymerization in methanol/phosphate buffer solution

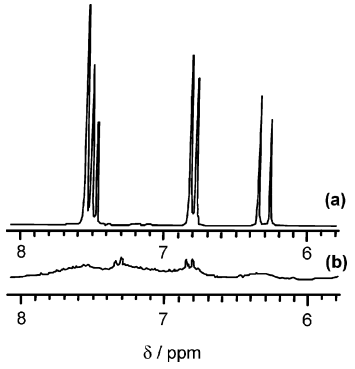

Fig. 4a, b ^{1}H-NMR spectra of **a** *p*-hydroxycinnamic acid, and **b** the obtained polymer

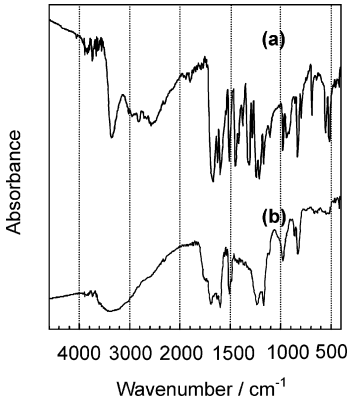

Fig. 5 IR spectra of *p*- hydroxycinnamic acid (*a*) and the obtained polymer (*b*)

the peak at 3,400 cm^{-1} of the obtained polymer became broader, implying that the hydrogen bonding of the hydroxyl group became stronger due to the formation of the high molecular weight and/or the network formation.

Preparation of poly(*p*-hydroxycinnamic acid) microspheres

Dispersion polymerization is an effective procedure for preparing polymer microspheres; polymerization is initiated in a homogeneous solution of the monomer and water-soluble polymer. Water-soluble polymers provide the nucleation site for the formation of polymer microspheres and play an important part in the dispersion of growing microspheres [10]. For in situ formation of polymer microspheres by enzyme-catalyzed polymerization, the paramount object is the selection of water-soluble polymer. In this study, the following water-soluble polymers were examined; poly(vinyl alcohol) (of which the degrees of polymerization and saponification were ca. 500 and 88.2 mol%, respectively), poly(methyl methacrylate), poly(ethylene glycol), polyethyleneimine, poly(styren-alt-maleic acid, sodium salt), poly(vinyl methyl ether), poly(vinyl methyl ether-alt maleic acid), polyvinylpyrrolidone, poly(acrylic acid), and poly(acrylic acid, sodium salt).

Figure 6 presents SEM photographs of the polymer precipitates prepared with poly(ethylene glycol), poly (vinyl alcohol), polyethyleneimine and polyvinylpyrrolidone as water-soluble polymers. The concentration of water-soluble polymer was 10% wt. A SEM photograph of the polymer precipitate prepared without a water-soluble polymer is also shown.

Polymer precipitates prepared without a water-soluble polymer showed no definite morphology and had diameters around 10 μm. With the exception of poly(vinyl alcohol), when water-soluble polymers were adopted as dispersion stabilizers, coagulum or polymer precipitate showing no definite morphology were obtained. Their diameters were larger than 10 μm. Uyama et al. succeeded in the preparation of polyphenol microspheres with poly(vinyl methyl ether) as a water-soluble polymer [3, 6]. However, for dispersion polymerization of *p*-hydroxycinnamic acid, poly(vinyl methyl ether) was not found to be a useful stabilizer. Despite slight coagulation, it is clear from Fig. 4 that fine polymer microspheres were prepared in the case of poly(vinyl alcohol). The diameter of the polymer microspheres was around 1 μm.

Figure 7 shows the effect of poly(vinyl alcohol) concentration on the formation of polymer microspheres. It was clear that the diameter of polymer microspheres became smaller as poly(vinyl alcohol) concentration increased up to 8% wt of poly(vinyl alcohol) concentration. The minimum polymer microsphere diameter was a few hundred nanometers. This tendency was similar to that for polyphenol microspheres proposed by Uyama et al. [3]. The observed behavior is quite similar to that in dispersion polymerization of vinyl monomers such as styrene and methyl methacrylate. In dispersion polymerization, the added soluble polymer works as dispersion stabilizer by adsorption on the precipitated particle nuclei and/or formation of graft-polymer, providing stable particle dispersion. This tendency indicates that an increase in poly(vinyl alcohol) concentration reduced the agglomeration of unstable particle nuclei and caused the effective production of the smaller microspheres. However, further addition of poly(vinyl alcohol) induced coagulation; the critical factor seemed to be the increase in solution viscosity. In all, poly(vinyl alcohol) was found to be the most effective water-soluble polymer for producing reactive poly(*p*-hydroxycinnamic acid) microspheres.

In conclusion, enzyme-catalyzed polymerization of *p*-hydroxycinnamic acid was carried out with horseradish

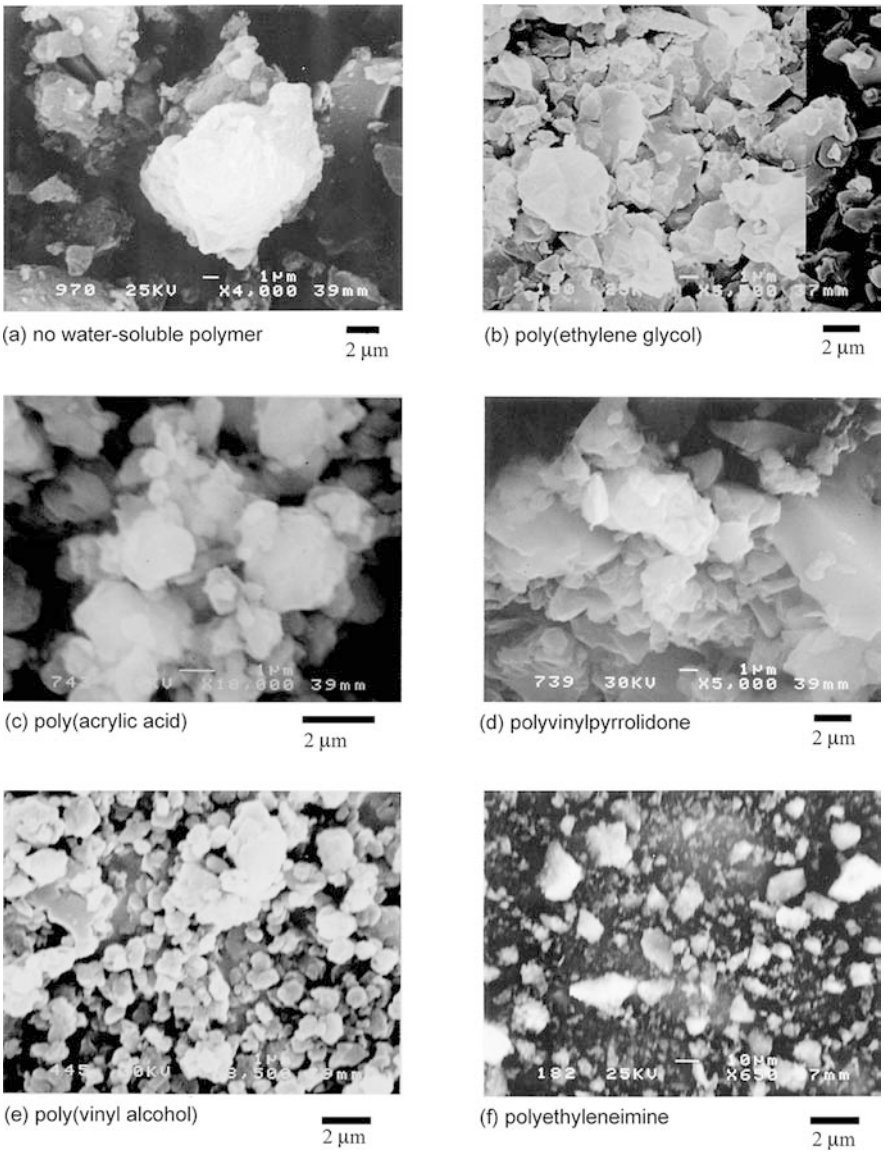

Fig. 6a–f Scanning electron microscopy (SEM) photographs of obtained polymer precipitates by enzyme-catalyzed polymerization of *p*-hydroxycinnamic acid with water-soluble polymer as a dispersion stabilizer

peroxidase as an enzyme and hydrogen peroxide as an oxidant. Moreover, we investigated the synthesis of reactive polymer microspheres of poly(*p*-hydroxycinnamic acid). The following results were obtained.

1. The mixture solution of methanol/phosphate buffer solution was the most suitable for enzyme-catalyzed polymerization of *p*-hydroxycinnamic acid using horseradish peroxidase as catalyst. The methanol fraction was found to be a significant parameter on polymer yield. The highest polymer yield was observed at 60% methanol.
2. The NMR spectrum of the obtained polymer was almost the same as that of the monomer; the FT-IR spectrum indicated the existence of carboxyl groups in the obtained polymers. The weight average molecular weight of poly(*p*-hydroxycinnamic acid) was found to be 1,451.
3. Dispersion polymerization of *p*-hydroxycinnamic acid was carried out in a mixture solution of methanol and phosphate buffer solution with a water-soluble polymer. After testing several such polymers as stabilizing media, poly(vinyl alcohol) was found to be the most effective in producing reactive poly(*p*-hydroxycinnamic acid) microspheres.

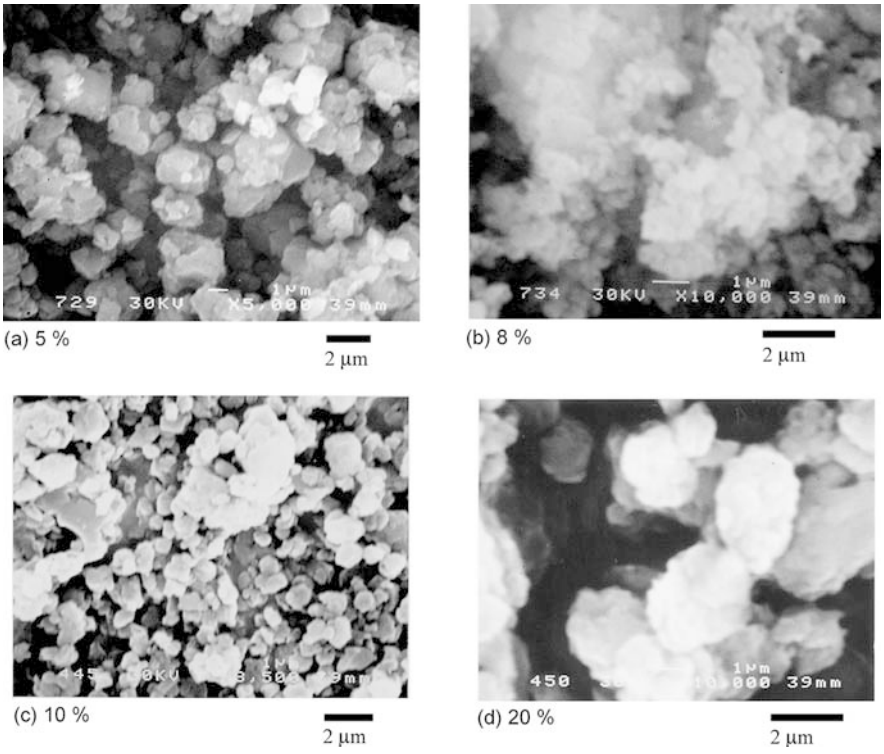

Fig. 7 SEM photographs of poly(*p*-hydroxycinnamic acid) microspheres prepared under various poly(vinyl alcohol) concentrations; the degrees of polymerization and saponification of poly(vinyl alcohol) were ca. 500 and 88.2 mol%, respectively

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