Novel Poly(vinyl alcohol)-Degrading Enzyme and the Degradation Mechanism

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ABSTRACT: A novel poly(vinyl alcohol) (PVA) degradation pathway was demonstrated such that the hydroxy group of PVA was first dehydrogenated into the corresponding carbonyl group to form the β-hydroxy ketone moiety which was followed by the aldolase-type cleavage to produce the methyl ketone and the aldehyde terminals by the PVA-assimilating strain, *Alcaligenes faecalis* KK314. Both the biodegradation steps of dehydrogenation and subsequent aldolase-type cleavage were catalyzed by the same protein having 67 kDa as the holoenzyme and apoenzyme of PVA dehydrogenase, respectively.

Introduction

Poly(vinyl alcohol) (PVA) is the only carbon−carbon backbone polymer that is biodegradable under both aerobic[1−7] and anaerobic conditions.[8] PVA is produced and widely used in the industrial field, and recently, PVA has attracted attention as a water-soluble biodegradable polymer as well as a biodegradable segment in the polymer chain. There may be some ways to develop biodegradable polymer-related PVA, such as incorporation of a vinyl alcohol block into a nonbiodegradable functional polymer chain,[5,9−13] grafting functional oligomers into the PVA chain,[14] and modification of the PVA chain.[15−17]

The microbial degradation of PVA was first reported since more than 60 years ago.[1] However, extensive studies on PVA degradation were only started at the beginning of the 1970s. In 1973, the PVA-assimilating microbial strain, *Pseudomonas* O-3, was isolated by Suzuki et al.[2] The PVA-degrading enzyme of this strain was isolated and analyzed.[18,19] Also, a microbial strain, *Pseudomonas* sp., was isolated and analyzed for the enzymatic degradation of PVA by Watanabe et al.[3,20] and Sakai et al.[21−25] Symbiotic microbial strains, *Pseudomonas putida* VM15A and *Pseudomonas* sp. VM15C, were obtained and analyzed for their roles with respect to the PVA degradation mechanism by Shimao et al.[4,26,27] The proposed biodegradation mechanism of PVA using some *Pseudomonas* strains are illustrated in Figure 1. Sakai et al. have shown the sequential reaction of secondary alcohol oxidase (SAO) via a β-hydroxy ketone to produce a β-diketone which was cleaved by β-diketone hydrolase.[23,24] Shimao et al. have suggested the cooperation of pyrroloquinoline quinone (PQQ)-dependent PVA dehydrogenase (PVADH) and a hydrolase during the course of PVA degradation.[27] Cloning and characterization of the gene encoding PQQ-dependent PVADH of *Pseudomonas* sp. VM15C were carried out by the same authors.[28] Hatanaka et al. also isolated the PQQ-dependent PVADH.[29,30] PVADH was quite different from the SAO reported by Morita et al. such that PVADH recognizes two hydroxy groups on the 1,3-diol of PVA and directly converts the 1,3-diol to a β-diketone.[21] On the other hand, the secondary alcohol oxidase has been shown to convert the 1,3-diol on PVA into a β-diketone by the sequential oxidation of hydroxy groups.[24] However, a more extensive analysis of the enzymatic chain-cleavage reaction will be needed. It is not clear whether the β-hydroxy ketone moiety of the PVA can be cleaved by the enzyme other than the β-diketone during the course of the biodegradation.

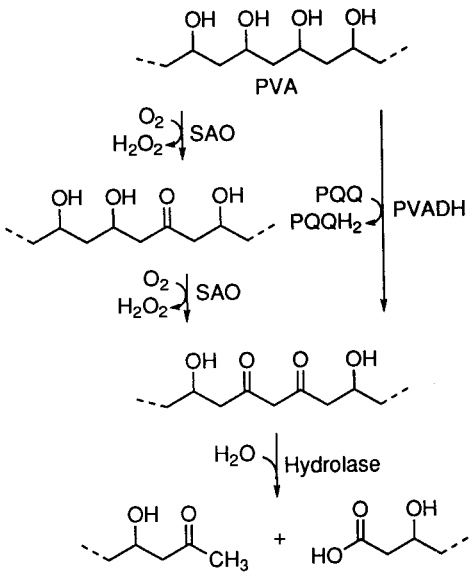

Figure 1. Reported enzymatic degradation mechanism of PVA.

This paper describes the novel PVA degradation mechanism that the β-hydroxy ketone is responsible for during the PVA degradation by the enzyme from *Alcaligenes faecalis* KK314. It was also revealed that the dehydrogenation of PVA and the subsequent aldolase-type cleavage occurred by the same protein.

Results and Discussion

Microbial Degradation of PVA. The PVA-assimilating bacterial strain, *Alcaligenes faecalis* KK314, obtained from river water was used as the PVA-degrading enzyme source.[31] As a reference, the bacterial strain, *Pseudomonas* sp. 113P3, which was isolated as a PVA-assimilating microbe by Hatanaka et al., was used.[29,30] The growth curves of *A. faecalis* and *Pseudo-*

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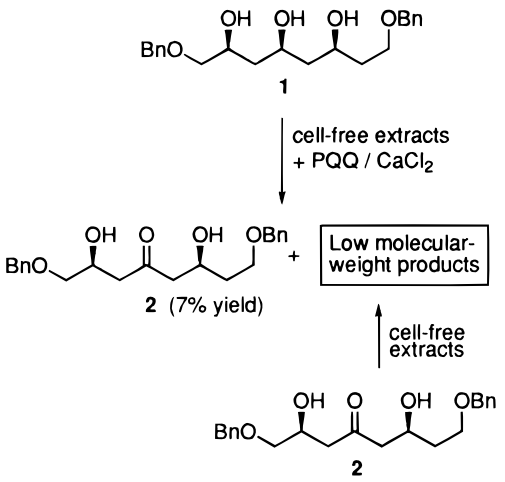


Figure 2. Reaction of isotactic-type trimer **1** and β-hydroxy ketone **2** with cell-free extracts with/without PQQ.

monas sp. 113P3 on PVA-23000 showed similar tendencies such that both microbial strains rapidly grew during a 3 day cultivation time. It is reported that the PVA oxidase was secreted in the incubation media by some *Pseudomonas* sp.[21,26] However, no oxidase activity against PVA was detected in the incubation media of *A. faecalis*. Contrary to this, the PVADH activity was detected in the incubation media of *A. faecalis*.

Isolation of PVADH Using Isotactic-Type Vinyl Alcohol Trimer. The PVA degradation mechanism and its related enzyme were analyzed using the isotactic-type vinyl alcohol trimer **1** as the model compound illustrated in Figure 2. **1** was incubated with the cell-free extracts in the presence of PQQ and $CaCl_2$, and it was found that a small amount of β-hydroxy ketone **2** (7% yield), which was equivalent to the dehydrogenation product of **1**, was present among the lower-molecular-weight degradation products. It was also found that the isolated **2** was further degraded by the treatment of the cell-free extracts irrespective of the presence of PQQ and $CaCl_2$ to produce the same low-molecular-weight degradation products as that obtained by the treatment of **1** with the cell-free extracts containing PQQ and $CaCl_2$. These results indicated that the biodegradation might have occurred via the production of the β-hydroxy ketone **2** with subsequent chain cleavage. Therefore, to clarify these mechanisms, PVADH was purified using **1** and β-hydroxy ketone degrading enzyme was purified using compound **2**.

The enzyme, which catalyzed the dehydrogenation, was isolated from the cell-free extracts of *A. faecalis* using trimer **1** as the screening substrate. The purification of PVADH from the cell-free extracts of *A. faecalis* is summarized in Table 1. The enzyme could be purified to a homogeneous state by a three-step fractionation, two repeated anion exchange chromatographies (DEAE-Sepharose FF) and hydrophobic column chromatography (Butyl-Toyopearl 650), from the cell-free extracts. By using these procedures, PVADH was purified 135-fold. The homogeneity and the molecular weight of the purified PVADH were examined by SDS-PAGE and GPC. The SDS-PAGE was carried out using a 10% acrylamide gel, and the authentic protein standards were used for the calibration. GPC was carried out using a TSK gel G-3000SW column (TOSOH Co., Ltd., Tokyo, Japan) in 0.1 M phosphate buffer (pH 7.2, 1 M = 1 mol dm^{-3}) containing 0.3 M NaCl with UV detection at both 220 and 410 nm, using the authentic protein standards. The purified PVADH gave a single protein band corresponding to a molecular weight of about 67 kDa on SDS-PAGE as shown in Figure 3. The same molecular weight of the purified PVADH was obtained by the GPC measurement. These results indicate that the PVADH of *A. faecalis* was a monomeric protein with a molecular weight of about 67 kDa. This molecular weight was in accordance with that obtained from the PVA-assimilating microbe, *Pseudomonas* sp. 113P3, by Hatanaka et al.[30]

It was confirmed that the trimer **1** was converted to β-hydroxy ketone **2** by the PVADH from *A. faecalis* with PQQ and $CaCl_2$. That is, **1** (10 mg) was incubated with the PVADH (100 mU/mL) containing 10 nM PQQ and 1 mM $CaCl_2$ in 10 mM phosphate buffer (pH 7.2) at 37 °C for 2 days. After the reaction, the reaction mixture was extracted with ethyl acetate (20 mL), washed with saturated aqueous NaCl (20 mL), dried over Na_2SO_4, and concentrated in vacuo to give crude product with some degradation products. Purification by column chromatography (2 g of silica gel, 1:1 chloroform−ethyl acetate) gave **2** (R_f 0.33, 1:1 chloroform−ethyl acetate). The ^1H NMR and ^{13}C NMR spectra of **2** were in complete agreement with those of the chemically synthesized authentic standard. However, 2- or 6-carbonylated product of **1** and β-diketone were not detected in the enzymatic reaction mixture. It was also confirmed that without PQQ conversion of **1** to **2** did not practically occur.

Isolation of β-Hydroxy Ketone-Cleaving Enzyme. The enzyme, which catalyzed the cleavage reaction, was isolated from the cell-free extracts of *A. faecalis* using β-hydroxy ketone **2** as the screening substrate. The isolation and purification procedures were the same as those described for the PVADH. A 67 kDa protein as determined by both SDS-PAGE and GPC was isolated from the cell-free extracts. Using this isolated enzyme, the reaction products were analyzed. A mixture of **2** (10 mg) and the purified enzyme (100 mU/mL as PVADH) in 10 mM phosphate buffer (pH 7.2, 5 mL) was incubated at 37 °C for 2 days. After the reaction, the reaction mixture was extracted with ethyl acetate (20 mL). The extracts were washed with saturated aqueous NaCl (20 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo to give crude products. Purification by column chromatography (2 g of silica gel, 5:1 chloroform−ethyl acetate) gave the methyl ketone **3** (R_f 0.42, 2:1 chloroform−ethyl acetate). After the extraction by ethyl acetate, the aqueous layer was acidified to pH 3 by 1 M HCl and then extracted with ethyl acetate (20 mL). The extracts were washed with saturated aqueous NaCl (20 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo to give the crude product. Isolation by column chromatography (2 g of silica gel, 8:1 chloroform−methanol) gave the carboxylic acid **5** (R_f 0.06, 2:1 chloroform−ethyl acetate). These products were identified by ^1H NMR, ^{13}C NMR, HPLC, and ESI-MS.

The reaction of **2** and previously isolated PVADH were then analyzed with and without PQQ and $CaCl_2$. It was also found that **2** was cleaved by the PVADH to produce the methyl ketone **3** (R_f 0.42, 2:1 chloroform−ethyl acetate) and carboxylic acid **5** (R_f 0.06, 2:1 chloroform−ethyl acetate) irrespective of PQQ and $CaCl_2$ as determined by TLC (2:1 chloroform−ethyl acetate). These two spots on the TLC were isolated by silica gel column chromatography, and their structures were

Table 1. Purification of PVA Dehydrogenase of *Alcaligenes faecalis* KK314

purification step	vol (mL)	protein concn (mg/mL)	total activity (U)	total protein (mg)	specific activity (mU/mg)	yield (%)	purification (fold)
cell-free extracts	135	8.7	35.1	1179	30	100	1
DEAE Sepharose FF (1)	56	0.5	13.7	29	471	39.0	16
DEAE Sepharose FF (2)	21	0.5	9.2	11	828	26.3	28
Butyl-Toyopearl 650	14	0.03	2.4	0.6	4025	6.9	135

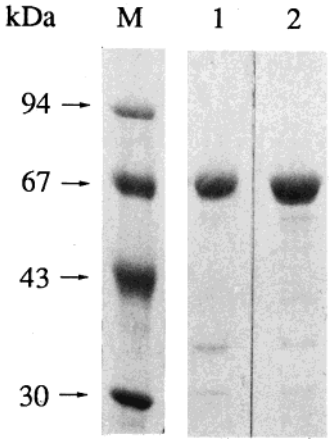


Figure 3. SDS-PAGE of PVADH from *A. faecalis* and *Pseudomonas* sp. stained with Coomassie blue: lane M, molecular weight marker; lane 1, *A. faecalis*; lane 2, *Pseudomonas* sp.

analyzed by ^{1}H NMR, ^{13}C NMR, and ESI-MS to confirm the structures of **3** and **5**. The ^{1}H NMR, ^{13}C NMR, ESI-MS, and MS/MS spectroscopies of **3** and **5** completely agreed with those of the chemically synthesized authentic standards. Under these conditions, it was confirmed that no degradation product occurred without enzyme. This indicated that this degradation reaction occurred exclusively by the enzyme.

It was also found that the aldehyde **4** was readily dehydrogenated by the enzyme to the corresponding carboxylate **5**. That is, a mixture of the aldehyde **4** (10 mg), the PVA degrading enzyme (100 mU/mL as PVADH), PQQ (10 nM), and $CaCl_2$ (1 mM) in 10 mM phosphate buffer (pH 7.2, 5 mL) was incubated at 37 °C for 2 days. After the reaction, the pH of the mixture was adjusted to 3 by 1 M HCl. The mixture was extracted with ethyl acetate, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give the crude product. Purification by column chromatography (1 g of silica gel, 3:1 hexanes−ethyl acetate) gave the carboxylic acid **5** (R_f 0.06, 2:1 chloroform−ethyl acetate). ^{1}H NMR and ^{13}C NMR spectra completely agreed with the authentic standards.

Degradation Mechanism of *β*-Hydroxy Ketone. There may be two routes to the cleavage of **2** into **3** and **5** as shown in Figure 4. By route 1, direct cleavage occurs and produces the methyl ketone **3** and an aldehyde **4** as the primary products. The latter may be simultaneously dehydrogenated by the enzyme to produce the carboxylic acid **5**. By route 2, the formation of the *β*-diketone may be a key intermediate which may be enzymatically or spontaneously hydrolyzed to **3** and **5**. Consequently, the detection of an aldehyde or *β*-diketone will be a decisive factor as to which route is actually followed. However, the concentration of the aldehyde **4** was low because of the rapid enzymatic transformation of **4** into **5**. To confirm which route occurred by the enzyme, enzymatic reaction products were analyzed using LC/MS which might allow to the more sensitive detection of **4**. A fluorometric method for the determination of **4** using 1,3-cyclohexanedione with HPLC and subsequent ESI-MS were carried out. That is, the enzymatic reaction mixture was reacted with 1,3-cyclohexanedione at 80 °C for 1 h, and the fluorescent decahydroacridine derivatives formed were directly determined by HPLC on an ODS column followed by ESI-MS. At the same time, the chemically synthesized **4** was also reacted with 1,3-cyclohexanedione for identification and calibration. It was found that a single peak ascribed to the decahydroacridine derivative of **4** appeared on the HPLC chromatogram. This fraction was further analyzed by ESI-MS to confirm having a mass of 352 (*m*/*z*) corresponding to the mass of the decahydroacridine derivatives of **4**. The MS/MS for this peak of 352 (*m*/*z*) produced a 244 (*m*/*z*) peak corresponding to the debenzyloxy form of the decahydroacridine derivative of **4**. These behaviors of the decahydroacridine derivative of **4** were in complete agreement with those of the authentic standard. As the result, it was confirmed that **4** was produced by the enzymatic reaction of **2**. Also, no *β*-diketone was detected in the enzymatic reaction mixture.

As a conclusion, the 67 kDa protein could catalyze the cleavage of the *β*-hydroxy ketone **2** into a methyl ketone **3**, an aldehyde **4**, and a carboxylate **5** as shown in Figure 5. Also, **4** was found to be quickly transformed into **5** by the enzyme. Therefore, **5** should be regarded as the secondary product from **4**. On the other hand, no *β*-diketone was detected in the reaction mixture. On the basis of these results, this enzyme worked as an aldolase which catalyzed the reaction of the *β*-hydroxy ketone **2** into the methyl ketone **3** and aldehyde **4**. Surprisingly, this enzyme catalyzed the dehydrogenation of the vinyl alcohol trimer **1** and PVA in the presence of PQQ and $CaCl_2$ into the *β*-hydroxy ketone **2** and low-molecular-weight PVA, respectively. Though the two enzymes having the same molecular weight of 67 kDa were isolated using the separate screening substrates, they were identical proteins having the same dehydrogenase and aldolase functions. In the presence of PQQ and $CaCl_2$, both proteins, which were obtained by separate procedures, showed the same activities as PVADH and aldolase. On the other hand, without PQQ, the two proteins only showed the same activity as aldolase. Further purification of each protein using isoelectric focusing and gel filtration revealed that the protein was a single one. As a conclusion, it was regarded that the two proteins were identical. Therefore, in this report, a single protein fraction of 67 kDa isolated from *A. faecalis* and having both dehydrogenase activity in the presence of PQQ and aldolase activity was only called the PVA-degrading enzyme. That is, the enzyme protein is the apoenzyme of PVADH which requires PQQ. More exactly, the apoenzyme of PVADH acted as aldolase. Further analyses of this enzyme with respect to the amino acid sequences will be published elsewhere.

Aldol Reaction of Methyl Ketone and Aldehyde by the PVA-Degrading Enzyme from *A. faecalis*. It was confirmed that the aldol condensation reaction

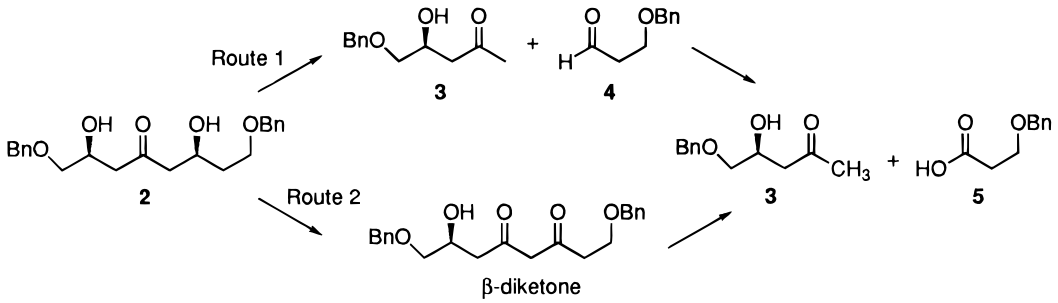


Figure 4. Possible enzymatic degradation route of β-hydroxy ketone **2**.

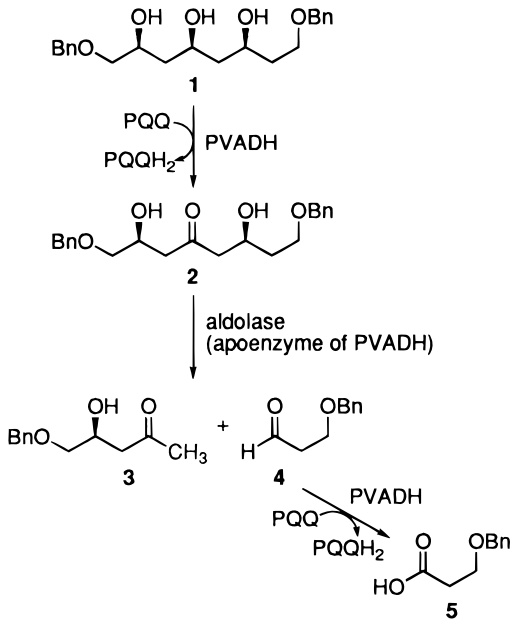


Figure 5. Enzymatic reaction of isotactic-type vinyl alochol trimer **1** using the purified PVADH (67 kDa) from *A. faecalis*.

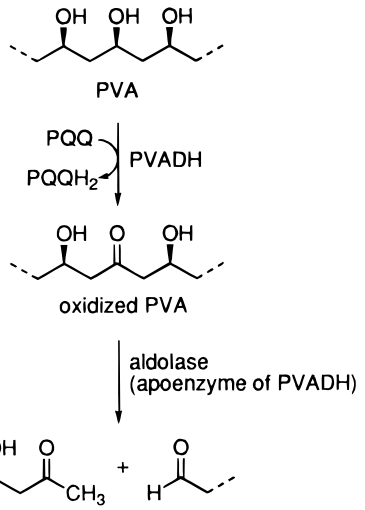


Figure 6. PVA degradation mechanism by the PVADH (67 kDa) from *A. faecalis*.

occurred between methyl ketone **3** and aldehyde **4** to produce the β-hydroxy ketone **2** by the enzyme as the reverse reaction of aldolase. That is, **3** (129 mg, 48 mM) and **4** (102 mg, 48 mM) were incubated with the enzyme (apoenzyme of PVADH: 100 mU/mL as PVADH) in 10 mM phosphate buffer (pH 7.2) at 37 °C for 2 days. After the reaction, the reaction mixture was extracted with ethyl acetate, washed with saturated aqueous NaCl, dried over Na_2SO_4, and concentrated in vacuo to give the crude products. Purification by column chromatography (silica gel, 1:1 acetonitrile−H_2O) gave **2** (R_f 0.48, 2:3 hexanes−ethyl acetate). The ^{1}H NMR and ^{13}C NMR spectra of **2** completely agreed with those of the chemically synthesized authentic standard. It was also confirmed that **3** and **4** were unchanged without the enzyme. Under the same reaction conditions except that the carboxylic acid **5** was used instead of **4**, no reaction was observed. This might indicate that the reaction product was the aldehyde **4**, not the carboxylic acid **5** by the degradation of **2** by the enzyme.

Enzymatic Degradation of PVA by the PVA-Degrading Enzyme from *A. faecalis*. On the basis of these results using the model compounds, it is suggested that the biodegradation of PVA occurs first by the dehydrogenation to produce the β-hydroxy ketone moiety which is cleaved by the aldolase-like reaction to produce the lower-molecular-weight fragments of PVA as shown in Figure 6. To confirm the degradability of the partially oxidized PVA containing the β-hydroxy ketone moieties by the enzyme, chemically synthesized partially oxidized PVA was reacted with PVADH with/without PQQ and $CaCl_2$. A mixture of the partially oxidized PVA (oxi-PVA-5500, 1 mg/mL), the enzyme (100 mU/mL as PVADH), PQQ (10 nM), and $CaCl_2$ (1 mM) in 10 mM phosphate buffer (pH 7.2) was stirred at 37 °C for 2 days. The mixture was analyzed using GPC and MALDI-TOF MS before and after the reaction. Under the same conditions, PVA was incubated with PVADH with/without PQQ and $CaCl_2$. These results are shown in Figure 7.

It was confirmed that the significant decrease in molecular weight of the partially oxidized PVA (oxi-PVA-5500) was due to PVADH irrespective of the presence of PQQ and $CaCl_2$. This indicated that the aldolase-like reaction occurred at the β-hydroxy ketone moieties of the oxidized PVA by the enzyme. On the other hand, in the case of PVA, a molecular weight reduction occurred only when PQQ and $CaCl_2$ were present in the reaction mixture. That is, with the addition of PQQ and $CaCl_2$, the enzyme exhibited activities as PVADH, and PVA was partially dehydrogenated to produce the β-hydroxy ketone moiety which was cleaved by the same enzyme as aldolase to decrease the molecular weight of PVA. It was confirmed that PVA and oxidized PVA were unchanged without the enzyme under these conditions. Similar tendencies were observed for the PVA degradation having various molecular weights between M_n 2200 and 90 000.

Comparison of Degradation Mechanism of PVA by PVADH from *Pseudomonas* sp. The degradation mechanism of PVA was reported by Hatanaka et al. such that PVA was first dehydrogenated by PVADH from *Pseudomonas* sp. 113P3 to produce the β-diketone

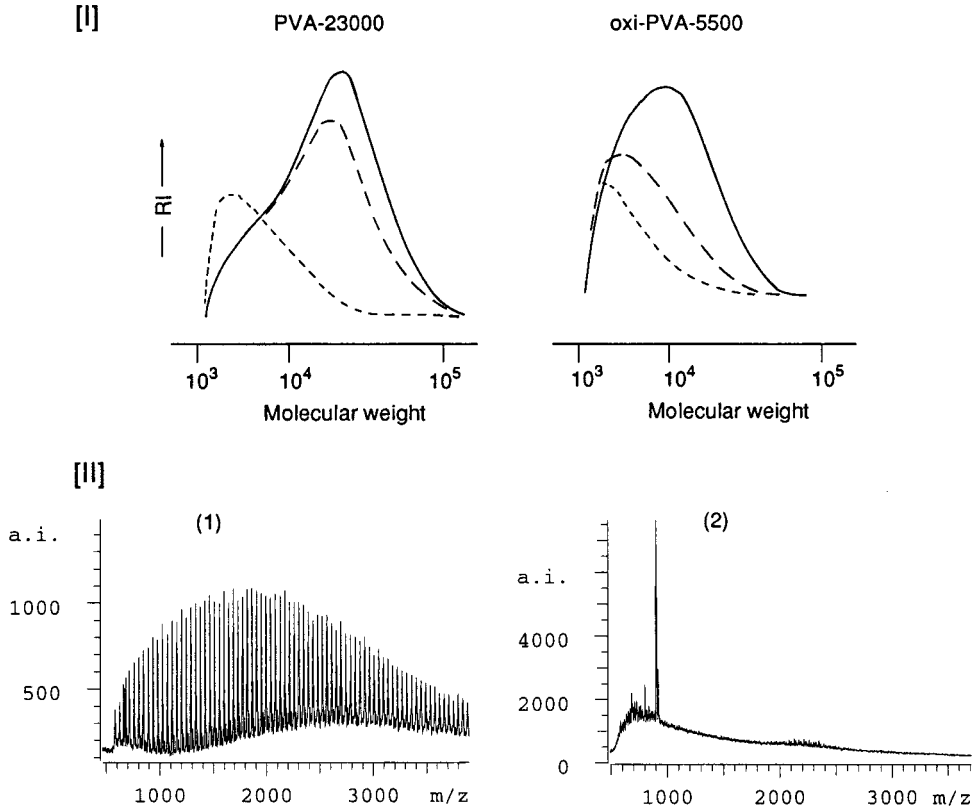


Figure 7. [I] GPC profile changes of PVA-23000 and oxi-PVA-5500 by the 2 day treatment with PVADH from *A. faecalis*: (—) initial, (– – –) enzyme, (- - -) enzyme + PQQ/$CaCl_2$. [II] MALDI-TOF mass spectrum of PVA-2200 by the 2 day treatment with PVADH from *A. faecalis* and PQQ/$CaCl_2$, recorded in reflector mode using DHBA as matrix. (1) PVA-2200, (2) after 2 day incubation with PVADH and PQQ/$CaCl_2$.

which was hydrolytically cleaved.[29,30] To compare the nature of PVADH obtained from *Pseudomonas* sp., PVA and low-molecular-weight model compounds were used for the evaluation. The PVADH was isolated from the cell-free extracts of *Pseudomonas* sp. according to the literature.[30] The PVADH was purified 284-fold from the cell-free extracts by DEAE-Sepharose FF column chromatography, precipitation by ammonium sulfate and Sephadex G-75 column chromatography. The specific activity was 7860 mU/mg of protein. The PVADH gave a single protein band corresponding to a molecular weight of 67 kDa on SDS-PAGE as shown in Figure 3. It was found that PVADH from *Pseudomonas* sp. catalyzed the dehydrogenation of trimer **1** in the presence of PQQ and $CaCl_2$ to produce the β-diketone (enol mixture) in addition to the β-hydroxy ketone. The reaction conditions were the same as those using PVADH from *A. faecalis*. The identification was carried out by ^{1}H NMR after the purification by column chromatography. On the other hand, without PQQ and $CaCl_2$, the incubation of β-hydroxy ketone **2** and PVADH from *Pseudomonas* sp. produced no products, such as β-diketone and the degradation products. This indicated that PVADH from *Pseudomonas* sp. could not cleave the β-hydroxy ketone **2** by an aldolase-like reaction. It was also confirmed that PVADH from *Pseudomonas* sp. could catalyze the dehydrogenation of PVA with PQQ and $CaCl_2$ to produce β-diketone moieties as determined by UV spectroscopy.[25,32] However, no molecular weight reduction was observed by PVADH from *Pseudomonas* sp.

Discussion of Biodegradation Mechanism of PVA. It has not yet been clarified whether the β-hydroxy ketone or β-diketone was produced as the dehydrogenation product or during the oxidase reaction of PVA, which was further enzymatically cleaved to yield a low-molecular-weight fraction. In this report, by the treatment of PVA with PVADH from *A. faecalis*, the formation of the β-hydroxy ketone was detected, and no β-diketone was detected in the enzymatic reaction mixture as determined by UV spectroscopy.[25,32] The β-hydroxy ketone formation was also supported by the experimental results using the low-molecular-weight model compounds and PVADH. The aldol condensation of the methyl ketone **3** and aldehyde **4** supported the β-hydroxy ketone route by the enzymatic degradation. It was also confirmed that randomly oxidized PVA containing β-hydroxy ketone moieties were cleaved by the aldolase-like reaction to decrease the molecular weight of the polymer by the apoenzyme of PVADH from *A. faecalis*. This aldolase-like cleavage was further confirmed using the model compound. Though this enzyme has both dehydrogenase activity in the presence of PQQ and aldolase activity, the produced aldehyde was partially transformed into a carboxylate. As a conclusion, the enzymatic degradation mechanism of PVA by *A. faecalis* could be summarized as shown in Figure 6.

PVA degrading enzymes have previously been isolated by some research groups as PVA oxidase, PVA dehydrogenase, oxidized PVA hydrolase, and β-diketone hydrolase. They were all isolated as the separated protein that occurred either in the microbial cells or in the incubation media. The PVA cleaving enzyme having both dehydrogenase activity and aldolase activity has not yet been reported. However, on the basis of the biodegradation process, it will be preferable and feasible for the PVA cleaving enzyme to simultaneously carry out both the dehydrogenation of PVA and subsequent

cleavage by the same protein. Thus, the PVA-degrading enzyme from *A. faecalis* is novel with respect to having unique catalytic activities for both the dehydrogenation with PQQ and subsequent aldolase-like cleavage reaction on the same protein.

Conclusions

A novel PVA degradation pathway was demonstrated such that the hydroxy group of PVA was first dehydrogenated into the corresponding carbonyl group which was followed by the aldolase-type cleavage to produce the methyl ketone and aldehyde terminals by the PVA-assimilating strain, *Alcaligenes faecalis* KK314. The dehydrogenase was found to be a monomeric protein having 67 kDa, requiring PQQ and $CaCl_2$ for its catalytic activity. The apoenzyme of PVADH acted as aldolase.

Experimental Part

Materials. PVA (98% hydrolyzed) having number-average molecular weight of M_n = 23 000 and molecular weight distribution of $M_w/M_n = 2.6$ (PVA-23000) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pyrroloquinoline quinone disodium salt (PQQ) was kindly supplied by Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan).

Measurements. The weight-average molecular weight (M_w), number-average molecular weight (M_n), and molecular weight dispersion (M_w/M_n) were measured by a gel permeation chromatography (GPC) using GPC columns (TSKgel G5000PW + G2500PW, TOSOH Co., Ltd., Tokyo, Japan) with 0.1 M phosphate buffer/0.3 M NaCl as eluent at a flow rate of 0.8 mL/min. The GPC system was calibrated with poly(ethylene oxide) standards (M_n, 3000−996 000; M_w/M_n, 1.02−1.10), purchased from TOSOH Co., Ltd., with a refractive index detector. ^{1}H NMR spectra were recorded with a JEOL model GSX-270 (270 MHz) spectrometer (JEOL Ltd., Tokyo, Japan). ^{13}C NMR spectra were recorded with a JEOL model GSX-270 Fourier transform spectrometer operating at 67.5 MHz with complete proton decoupling. Infrared (IR) spectra were measured using a JASCO Fourier transform spectrometer model FT/IR-5000 (JASCO Ltd., Tokyo, Japan). The matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was measured with a Bruker Proflex mass spectrometer. The spectrometer was equipped with a nitrogen laser. The detection was in the reflector and linear mode. 2,5-Dihydroxybenzoic acid (DHBA) was used as the matrix, and positive ionization was used. The liquid chromatography (LC)/mass spectroscopy (MS) was carried out using a Finnigan LCQ LC/MS system (Finnigan Corp., CA). The electrospray ionization (ESI) was used for the measurements.

Microorganisms and Growth Conditions. The isolated PVA-assimilating microbe, *Alcaligenes faecalis* KK314,[31] which was obtained from river water by an enrichment culture technique, was maintained on agar slants using a 0.5% PVA-23000 concentration of the culture medium and used throughout this work. The composition of the culture medium was as follows: 0.1% NH_4NO_3, 0.02% KH_2PO_4, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.005% peptone, 0.005% yeast extract, 1 μg/mL (final concentration) PQQ, and 1.5% agar. The initial pH was adjusted to 7.5.

The PVA-assimilating microbe, *Pseudomonas* sp. 113P3, which was isolated from activated sludge from a dye works in the city of Kurashiki, was kindly supplied by Kuraray Co., Ltd. (Osaka, Japan). The culture medium was the same as that of *A. faecalis*.

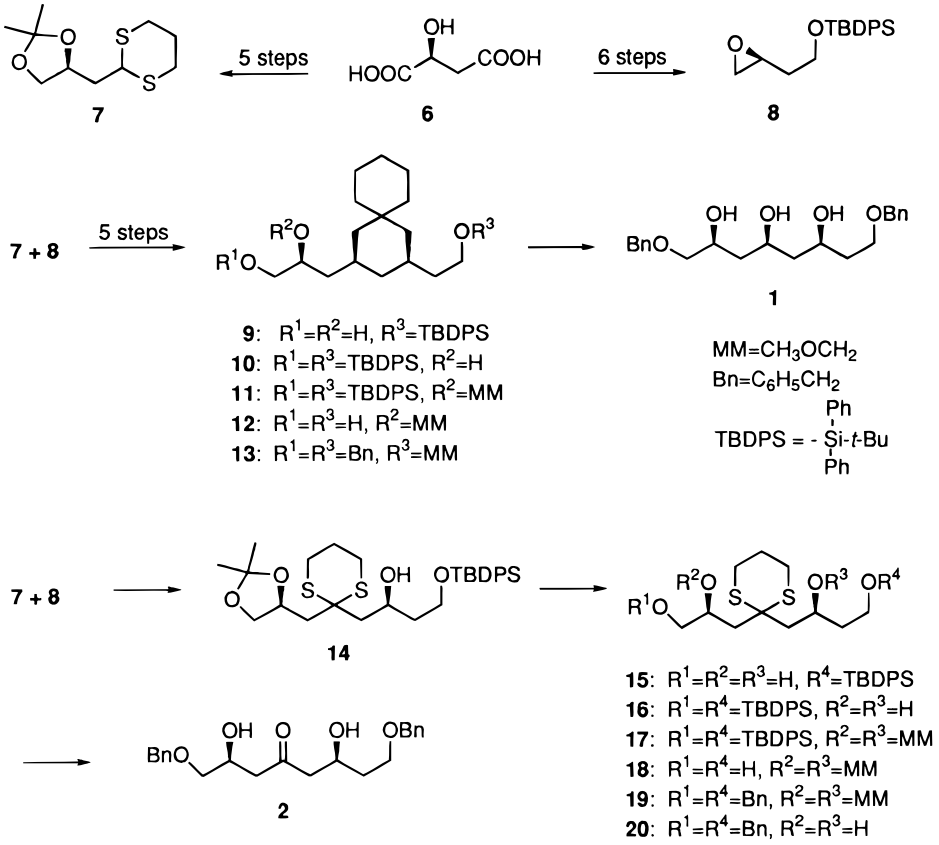
Purification of the PVA-Degrading Enzyme of *A. faecalis*. (1) Preparation of Cell-Free Extracts. The basal medium for cell cultivation was the same as that for the slants without agar using a 0.2% KH_2PO_4, 1.0% K_2HPO_4, and 1.0% PVA-23000 concentration. An antifoaming agent (0.01%) was added before shaking. Cultivation was carried out with 200 mL of the culture medium in a 500 mL shake flask at 30 °C with reciprocal shaking for 5 days. Cells were harvested by centrifugation at 13 000*g* and 4 °C for 25 min to obtain wet cells from a 1600 mL culture broth. The cells were suspended in 160 mL of distilled water containing 1 mM EDTA·2Na, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100 (for purification of PVADH, Tween 80 was used instead of Triton X-100) and divided into three portions in a 50 mL beaker. The cell suspension was then disrupted with an ultrasonic oscillator (19 kHz, 125 W, 50% pulse) for 30 min at 0 °C. The cell debris was removed by centrifugation at 20 000*g* for 20 min at 4 °C to obtain the supernatant (135 mL). The supernatant was dialyzed against 10 mM phosphate buffer (pH 7.2) at 4 °C using a dialyzing tube (Seamless Cellulose Tubing, UC36-32-100, Viskase Sales Corp.) to obtain the cell-free extracts for enzymatic reaction. For further purification of PVADH, the supernatant was dialyzed by using Tris-HCl buffer (pH 8.5).

(2) Purification of PVADH. The cell-free extracts (135 mL) of *A. faecalis* were first charged to a DEAE-Sepharose FF (Pharmacia, Upsala, Sweden) column (130 mL) which was equilibrated with 10 mM Tris-HCl buffer (pH 8.5). After the column was washed with the same buffer to reduce the absorption at 280 nm, PVADH was eluted with a linear concentration gradient of NaCl in the Tris-HCl buffer (0−0.25 M NaCl). Each fraction was monitored at 280 and 410 nm. The active fractions were collected and dialyzed against 10 mM phosphate buffer (pH 7.2) overnight. The dialyzed fraction was again charged to the same DEAE-Sepharose FF column and eluted with a linear gradient of NaCl in the Tris-HCl buffer (0−0.25 M NaCl) in the same procedure with the first run. The active fractions that showed the highest PVADH activities were collected and dialyzed against 10 mM phosphate buffer (pH 7.2) overnight. The dialyzed fraction was further purified using the Butyl-Toyopearl 650 (TOSOH Co., Ltd.,) column which was equilibrated with a 10 mM phosphate buffer containing 0.4 M ammonium sulfate. That is, the dialyzed fraction (23 mL) was charged to the column. PVADH was then eluted with a linear concentration gradient of ammonium sulfate in the 10 mM phosphate buffer (0.4−0 M ammonium sulfate). The active fractions were collected and dialyzed against 10 mM phosphate buffer (pH 7.2) and used as the PVADH.

Enzyme Assay of PVADH. PVADH activity was assayed by measuring the rate of reduction of phenazine ethosulfate (PES) by PVA-23000, as determined by the decrease in A_{600} resulting from the coupled reduction of 2,6-dichlorophenolindophenol (DCPIP) according to the method of Shimao et al.[27] and Hatanaka et al.[30] with bovine serum albumin as the standard.[33] A mixture (1.0 mL) of 7.5 mg of PVA-23000, 1 mM PES, 0.1 mM DCPIP, 1 mM KCN, 0.5% Tween 80, 1 mM $MgCl_2$, 500 μM $CaCl_2$, 1 μM PQQ, and 50 mM phosphate buffer (pH 7.2) was preincubated at 30 °C for 15 min, and then the enzyme solution (0.5 mL) in a total volume of 1.5 mL was added to start the reaction. The initial rate of reaction was measured by the decrease in absorbance at 600 nm resulting from the coupled reduction of DCPIP, the value of 1.91×10^4 being referred to as the molecular absorption coefficient of DCPIP. One unit of the enzyme was defined as the amount that reduced 1 mM substrate per minute under the assay conditions. The specific activity of the enzyme was expressed as units per milligram of protein. Protein concentration was measured by Lowry et al.[34]

Detection of the Aldehyde by LC/MS. Aldehyde **4** produced by the enzymatic reaction was analyzed by a fluorometric method using 1,3-cyclohexadione (CHD) with HPLC and followed by ESI-MS and MS/MS. The CHD reagent was prepared by dissolving ammonium sulfate (5 g), glacial acetic acid (5 mL), and CHD (0.25 g) in about 25 mL of water and making the volume up to 50 mL with water.[35,36] The enzymatic reaction mixture (4 mL) and the CHD reagent (1 mL) were reacted at 80 °C for 1 h and then directly injected (20 μL) into the HPLC column (Inertsil ODS-2, 4.6 mm i.d. × 250 mm, GL Science Co., Tokyo, Japan) with acetonitrile−water (1/1.5 v/v) as the eluent at a flow rate of 0.5 mL/min with a fluorescent detector (excitation, 385 nm; emission, 445 nm). In a similar way, chemically synthesized **4** (2.5 mg) in phosphate buffer

Scheme 1



(pH 7.2, 1 mL) was reacted with the CHD reagent (3 mL) at 80 °C for 1 h for HPLC analysis as the standard.

Low-Molecular-Weight Model Compounds. Detailed chemical preparation of the compounds and their analytical data are deposited as Supporting Information. Only the outlines of the syntheses are shown below.

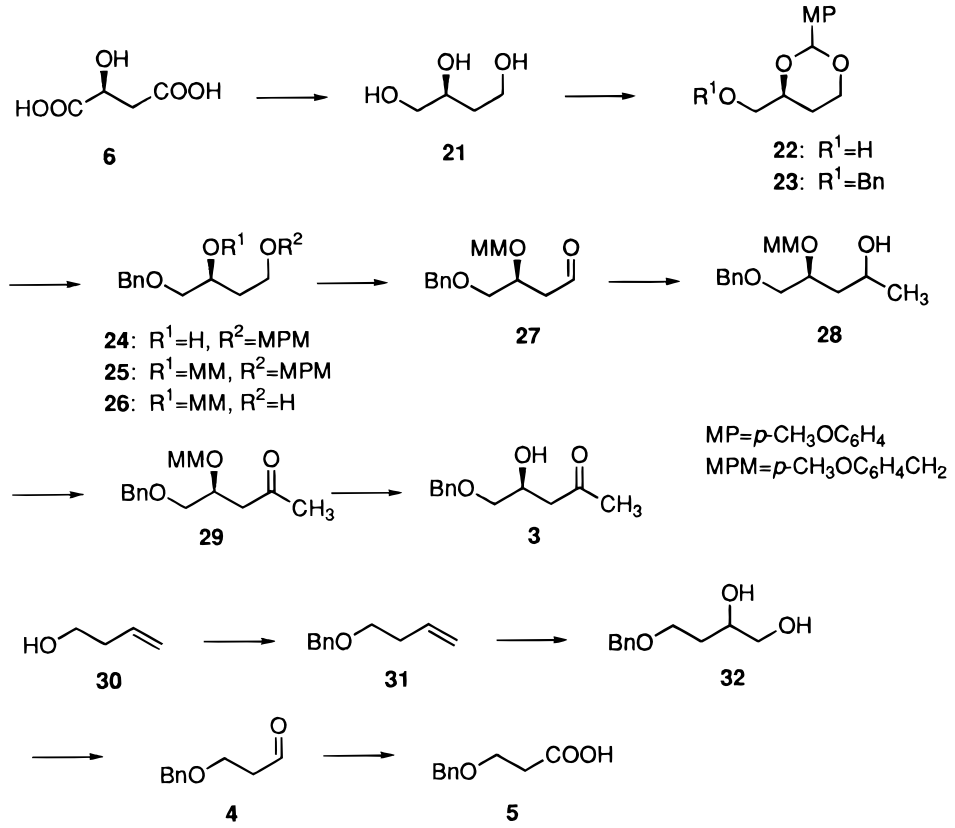
(1) Preparation of Vinyl Alcohol Trimer [(*2S,4S,6S*)-1,8-Bis(benzyloxy)-2,4,6-octanetriol] (1). The isotactic type vinyl alcohol trimer **1** was synthesized from **13** which was prepared from l-malic acid (**6**) according to the literature (Scheme 1).[37−42] The selective silylation of the primary alcohol **9** by *tert*-butyldiphenylsilyl chloride gave **10**, which was followed by the protection using chloromethyl ethyl ether which gave **11**. Desilylation of **11** by tetrabutylammonium fluoride gave **12**. Finally, the benzylation of **12** by benzyl bromide yielded **13**, which was converted to **1** under acidic conditions. ^{1}H NMR ($CDCl_3$): δ 1.41−1.90 (6H, m, H-3, H-5, H-7), 2.55−3.12 (3H, br, OH), 3.35−3.50 (2H, m, H-8), 3.60−3.75 (2H, m, H-1), 4.00−4.19 (3H, m, H-2, H-4, H-6), 4.51 (2H, s, $-CH_2Ph$), 4.55 (2H, s, $-CH_2Ph$), 7.22−7.40 (10H, m, ArH).

(2) Preparation of Monoketone [(2S,6S)-1,8-Bis(benzyloxy)-2,6-dihydroxy-4-octanone] (2). The monoketone **2** was synthesized by the reaction of **7** and **8** which were prepared from L-malic acid (Scheme 1). To a solution of **7** in dry THF was added *n*-butyllithium in hexane. To this mixture, **8** in THF was added and then stirred. The reaction was quenched with saturated aqueous NH_4Cl. Purification by column chromatography gave **14** as a yellow oil. **14** in 75% aqueous acetic acid was stirred and azeotropically concentrated in vacuo using toluene to give the crude yellow oil of **15**. To a solution of **15** and imidazole in dry DMF was added *tert*-butyldiphenylchlorosilane and then stirred. Purification by column chromatography gave **16** as a yellow oil. To a solution of **16** in dry dichloromethane were added diisopropylethylamine and chloromethyl methyl ether and then stirred. Purification by column chromatography gave **17** as a yellow oil. To a solution of **17** in dry THF was added 1 M tetrabutylammonium fluoride in THF and then stirred. Purification by column chromatography gave **18** as a colorless oil. To a solution of **18** in dry DMF was added NaH and stirred. To this mixture, benzyl bromide was added and then stirred. The reaction was quenched with ethanol. Purification by column chromatography gave **19** as a colorless oil.

19 in 50% aqueous acetic acid was stirred and then concentrated azeotropically in vacuo using toluene to give the crude product. Purification by column chromatography gave **20** as a colorless oil. To a solution of **20** in 80% acetonitrile aqueous solution were added calcium carbonate and methyl iodide and then stirred. Purification by column chromatography gave **2** as white crystals. ^{1}H NMR: δ 1.76 (2H, m, H-7), 2.58 (1H, dd, H-5, J = 17.6 and 6.4 Hz), 2.59 (1H, dd, H-3, J = 17.2 and 9.2 Hz), 2.66 (1H, dd, H-3′, J = 17.2 and 9.2 Hz), 2.69 (1H, dd, H-5′, J = 17.6 and 6.4 Hz), 2.99 (1H, d, OH-2, J = 4.2 Hz), 3.38 (1H, d, OH-6, J = 3.2 Hz), 3.43 (1H, dd, H-1, J = 9.6 and 5.6 Hz), 3.48 (1H, dd, H-1′, J = 9.6 and 4.6 Hz), 3.65 (2H, m, H-8), 4.26 (1H, m, H-2 H-6), 4.48, 4.54 (each 2H, s, $-CH_2Ph$), 7.26−7.39 (10H, m, ArH).

(3) Preparation of Methyl Ketone [(4S)-5-Benzyloxy-4-hydroxy-2-pentanone] (3). The methyl ketone **3** was synthesized from **29**, which was prepared from L-malic acid (Scheme 2). To a solution of the 2 M borane−methyl sulfide (BMS) THF complex and trimethyl borate was added L-malic acid in dry THF and stirred. The reaction was quenched by adding methanol, and the mixture was then concentrated in vacuo to give a crude oily product of **21**. To a solution of **21** in dry dichloromethane was added anisaldehyde dimethylacetal and then camphorsulfonic acid and stirred. Triethylamine was then added in order to make the pH of the mixture slightly alkaline and concentrated in vacuo to give a crude product. Purification by column chromatography gave **22** as a colorless oil. To a solution of **22** in dry THF was added 60% NaH and then stirred. To this mixture, benzyl bromide was added and stirred. The reaction was quenched with ethanol. Purification by column chromatography gave **23** as white crystals. To a solution of **23** in dry dichloromethane was added diisobutylaluminum hydride in hexane. To this was added methanol and saturated aqueous NH_4Cl with stirring. Ethyl acetate and

Scheme 2



saturated aqueous potassium sodium tartarate tetrahydroxides were added and stirred. Purification by column chromatography gave **24** as an oil. To a stirred solution of **24** in dry dichloromethane was added diisopropylethylamine (35.5 mL, 21.0 mmol) and then chloromethyl methyl ether and stirred. Purification by column chromatography gave **25** as a colorless oil. A solution of **25** and ceric ammonium nitrate in acetonitrile−water was stirred. Purification by column chromatography gave **26** as a colorless oil. To a solution of oxalyl chloride in dry dichloromethane was added a mixture of DMSO and dry dichloromethane and stirred. To this solution **26** in dry dichloromethane was dropwise added at −78 °C and stirred to give the crude product **27**. To a stirred suspension of small pieces of magnesium in dry diethyl ether was added methyl iodide and stirred until the magnesium was dissolved. To this solution **27** in dry diethyl ether was added. Purification by column chromatography gave **28** as a colorless oil. To a solution of oxalyl chloride in dry dichloromethane was added a mixture of DMSO and dry dichloromethane and stirred. To this solution, **28** in dry dichloromethane was added and stirred. The mixture was neutralized with triethylamine. Purification by column chromatography gave **29** as a colorless oil. **29** in 50% aqueous acetic acid was stirred and then azeotropically concentrated using toluene to give the crude oil. Purification by column chromatography gave **3** as a colorless oil. ^{1}H NMR: δ 2.17 (3H, s, H-1), 2.62 (1H, dd, H-3, J = 5.6 and 16.2 Hz), 2.78 (1H, dd, H-3′, J = 7.2 and 16.2 Hz), 3.01 (1H, d, OH, J = 4.2 Hz), 3.44 (1H, dd, H-5, J = 6.2 and 9.6 Hz), 3.48 (1H, dd, H-5′, J = 4.6 and 9.6 Hz), 4.26 (1H, m, H-4), 4.56 (2H, s, $-CH_2Ph$), 7.28−7.37 (5H, m, ArH).

(4) Preparation of Aldehyde 4 and Carboxylic Acid 5. The aldehyde **4** and carboxylic acid **5** were synthesized from 3-buten-1-ol (**30**) (Scheme 2).

4-Benzyloxypropanal (4). To a solution of **30** in dry THF was added NaH, and then benzyl bromide was added and stirred. The reaction was quenched with ethanol. Purification by column chromatography gave **31** as a colorless oil. To a solution of **31** in dry acetone was added dropwise 10% potassium permanganate and then stirred. The reaction was quenched with saturated aqueous sodium sulfite. Purification by column chromatography gave **32** as a colorless oil. To a solution of **32** in dry acetone was added dropwise sodium metaperiodate in water and then stirred. Purification by column chromatography gave **4** as a colorless oil. ^{1}H NMR: δ 2.69 (2H, dt, H-2, J = 2.4 and 6.2 Hz), 3.81 (2H, t, H-3, J = 6.2 Hz), 4.52 (2H, s, $-CH_2Ph$), 7.23−7.38 (5H, m, ArH), 9.78 (1H, t, CHO, J = 2.4 Hz).

4-Benzyloxypropanoic Acid (5). To a solution of **4** in dry THF was added dropwise 10% potassium permanganate and then stirred. The reaction was quenched with saturated aqueous sodium sulfite, and then the mixture was filtered. The pH of the filtrate was adjusted to 3−4 by 1 N HCl. Purification by column chromatography gave **5** as a colorless oil. ^{1}H NMR: δ 2.66 (2H, t, H-2, J = 6.2 Hz), 3.65 (2H, t, H-3, J = 6.2 Hz), 4.53 (2H, s, $-CH_2Ph$), 7.23−7.37 (5H, m, ArH).

Partially Oxidized PVA. Partially oxidized PVA (oxi-PVA-5500) was basically prepared according to the method of Huang et al.[17] PVA (1.0 g) was dissolved in 10 mL of water in an autoclave with heating. To this was added 0.1 mL of glacial acetic acid and stirred for 15 min, and then 2.5 mL of aqueous sodium hypochlorite solution was slowly dropwise added and stirred at 25 °C for 3 h. After the reaction, the reaction mixture was neutralized with 1 M NaOH and dialyzed against distilled water for 1 day and then lyophilized to obtain the oxidized PVA with an oxidation degree of 4.7 mol % as determined by ^{1}H NMR in a 345 mg yield. The molecular weight as determined by GPC was M_n 5500 and M_w/M_n 1.7. The oxidation degree was determined by comparison of the ^{1}H NMR spectral integration intensities for the δ = 2.72−2.81 ppm peak (CH_2CO) corresponding to the methylene protons adjacent to the carbonate group in the oxidized PVA with the corresponding methylene protons of PVA at δ = 1.59−1.93 ppm. ^{1}H NMR (D_2O): δ 1.59−1.93 (CH_2CHOH), 2.72−2.81 (CH_2CO), 3.91−4.13 (CH).

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Supporting Information Available: Preparation and analytical results of the model compounds and partially oxidized PVA. This material is available free of charge via the Internet at http://pubs.acs.org.

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