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FOOD CHEMICAL CONTAMINANTS

Characterization of Partially Transesterified Poly(β-Hydroxyalkanoate)s by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOFMS) was used for the characterization of a partially transesterified poly(β-hydroxyalkanoate) (PHA), a polymer produced by the bacterial strain *Alcaligenes eutrophus* with saponified vegetable oils as the sole carbon sources. The transesterification was carried out separately under acidic and basic conditions to obtain PHA oligomers weighing <10 kDa. The intact oligomers were detected in their cationized forms, $[M + Na]^+$ and $[M + K]^+$, by MALDI–TOFMS. A composition analysis, using the MALDI–TOF spectra, indicated that the oligomers obtained via acid catalysis contained a methyl 3-hydroxybutyrate end group, and those obtained by base catalysis had a methyl crotonate (olefinic) end group. In addition to hydroxybutyrate (HB), the oligomers were found to contain a small percentage of hydroxyvalerate, which was independently confirmed by gas chromatography/mass spectrometry. In comparison, analysis of a commercial PHA polymer, transesterified under identical conditions, showed only the presence of HB, i.e., a pure poly(HB) homopolymer.

Poly β-hydroxyalkanoates (PHAs) are a class of naturally occurring, linear homochiral polyesters that function as intracellular carbon and energy storage material (1). They are produced by a variety of bacteria under an excess carbon source and nutrient-limiting conditions such as the absence of nitrogen, phosphorus, or sulfur. Because the type of PHA monomer depends on the bacterial strain as well as the substrate used for its accumulation (2, 3), several results are possible. The most common types of PHAs are poly β-hydroxybutyrates (PHBs) and poly β-hydroxyvalerates (PHVs). The copolymers, P (HB-co-HV) in general, are used in a wide range of both performance and commodity applications because of their thermoplastic and biodegradable properties (2, 4). They offer one of the best bioremediation solutions for single-use disposable items. The depolymerized oligomeric PHAs, like their monomeric counterparts, may also be used as chiral precursors for pharmaceutical products (5). Consequently, the study of PHAs, including their structural characterization, will have an impact on their industrial and commercial utilization, particularly in relation to environmental chemical pollution.

The PHA polymer in our study was produced by the bacterial strain *Alcaligenes eutrophus* with saponified vegetable oils of vernonia and soybean as the carbon and energy sources (6). The PHA polymer was transesterified under both acid- and base-catalyzed conditions to obtain PHA oligomers. In a recent paper, we reported the analysis of these PHAs by nuclear magnetic resonance spectrometry (NMR) and gel permeation chromatography (6). Mass spectrometry (MS) techniques such as gas chromatography (GC)/MS, fast atom bombardment/MS (7), and pyrolysis/MS (8) have been used by others for the qualitative and quantitative analysis of PHAs. Also, Montaudo et al. (9) reported the matrix-assisted laser desorption/ionization time-of-flight MS (MALDI–TOFMS) analysis of 700–2000 Da (7-mer to 22-mer) range HB/HV cooligomers obtained by using acid-catalyzed transesterification. In this paper, we report the characterization of the acid- and base-catalyzed PHAs by using MALDI–TOFMS.

Experimental

Materials

A. eutrophus strain 17699 was obtained from the American Type Culture Collection (Manassas, VA) and was subcultured in trypticase soy broth. Stock cultures were maintained on

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Table 1. Relationship between concentration of sodium methoxide and size of resulting oligomers

PHBs concn, M	Sodium methoxide concn, M	Oligomer size
1.2×10^{-2}	7.0×10^{-6}	70-mer
1.2×10^{-2}	8.3×10^{-6}	50-mer
1.2×10^{-2}	9.7×10^{-6}	35-mer
1.2×10^{-2}	1.1×10^{-5}	25-mer

trypticase soy agar at 4°C with transfer every 14 days. The fermentation medium and process were described previously (6).

PHA Isolation

The lyophilized cells, obtained from the fermentation process, were accurately weighed and suspended in 200 mL chloroform and refluxed for 3 h; the hot mixture was filtered through a Whatman cellulose extraction thimble (33 × 94 mm, single thickness; Aldrich Chemical Co., Milwaukee, WI). The chloroform was then evaporated to give a transparent polymeric film, which was purified by washing 3 times with methanol (15 mL) and dried in air.

Solvents and Reagents

(**a**) *Dichloromethane*.—Reagent grade; Chemical Abstracts (CAS) Service Registry No. 75-09-2; Aldrich Chemical Co. (Milwaukee, WI).

(**b**) *Tetrahydrofuran* (*THF*).—Liquid chromatography (LC) grade; CAS 109-99-9; Aldrich Chemical Co.

(**c**) *Methanol*.—99.8%, reagent grade; CAS 67-56-1; Aldrich Chemical Co.

(**d**) *Sodium methoxide*.—25% (w/w) in methanol; CAS 124-41-4; Aldrich Chemical Co.

(**e**) *Commercial PHB*.—CAS 26063-00-3; Aldrich Chemical Co.

(**f**) *Chloroform*.—LC grade; Aldrich Chemical Co.

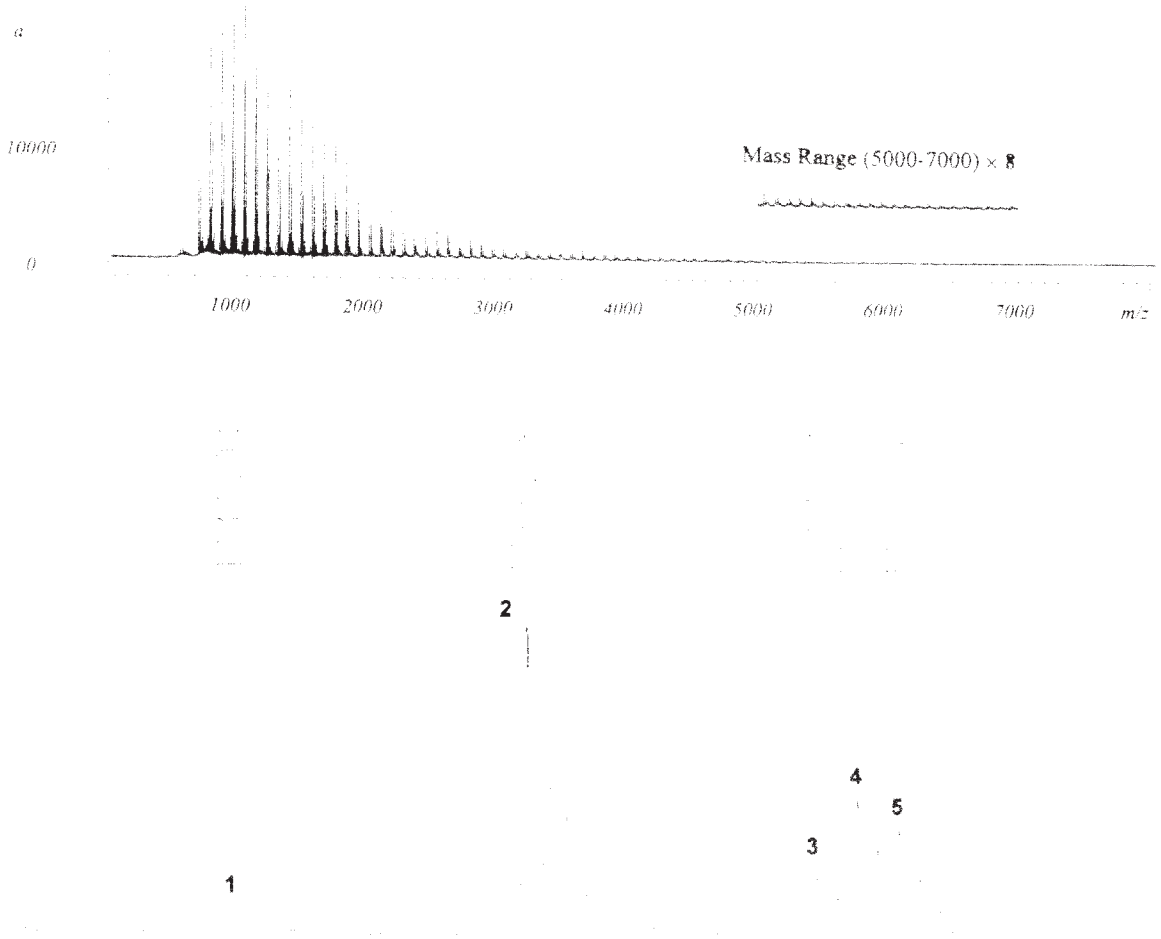

Figure 1. (Top) Positive ion MALDI–TOF mass spectrum (reflectron mode) of partially transesterified (base-catalyzed) PHA, showing cationized molecules. (Bottom) Expansion of the spectrum between *m/z* 1130 and 1185. Peak assignments: 1 = $[CH_3CH{=}CHC(O)\{HB\}_{12}OH{-}Na]^+$, 2 = $[CH_3CH{=}CHC(O)\{HB\}_{12}OCH_3{-}Na]^+$, 3 = $[CH_3CH{=}CHC(O)\{HB\}_{11}\{HV\}_1OCH_3{-}Na]^+$, 4 = $[CH_3CH{=}CHC(O)\{HB\}_{12}OH{-}K]^+$, 5 = $[CH_3CH(OH)CH_2C(O)\{HB\}_{12}OCH_3{-}Na]^+$.

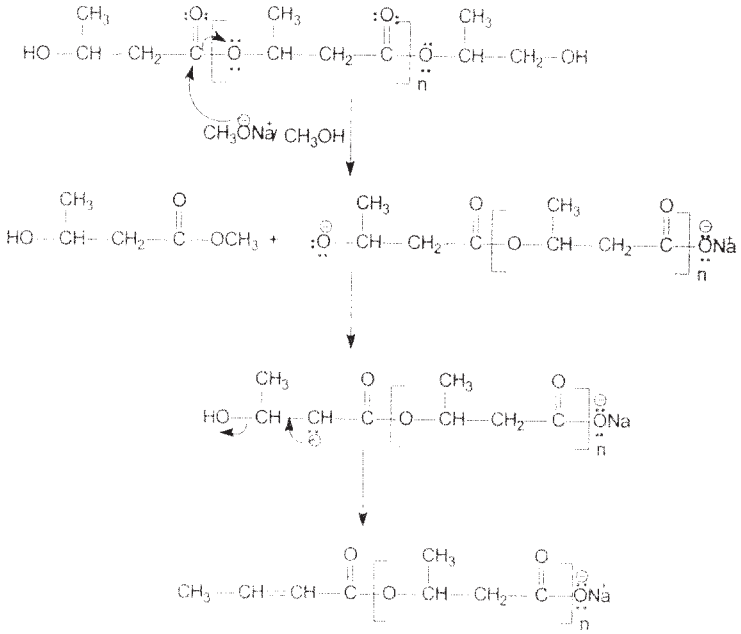

Figure 2. Proposed mechanism for the formation of oligomers with an olefinic end group during the base-catalyzed transesterification of PHBs.

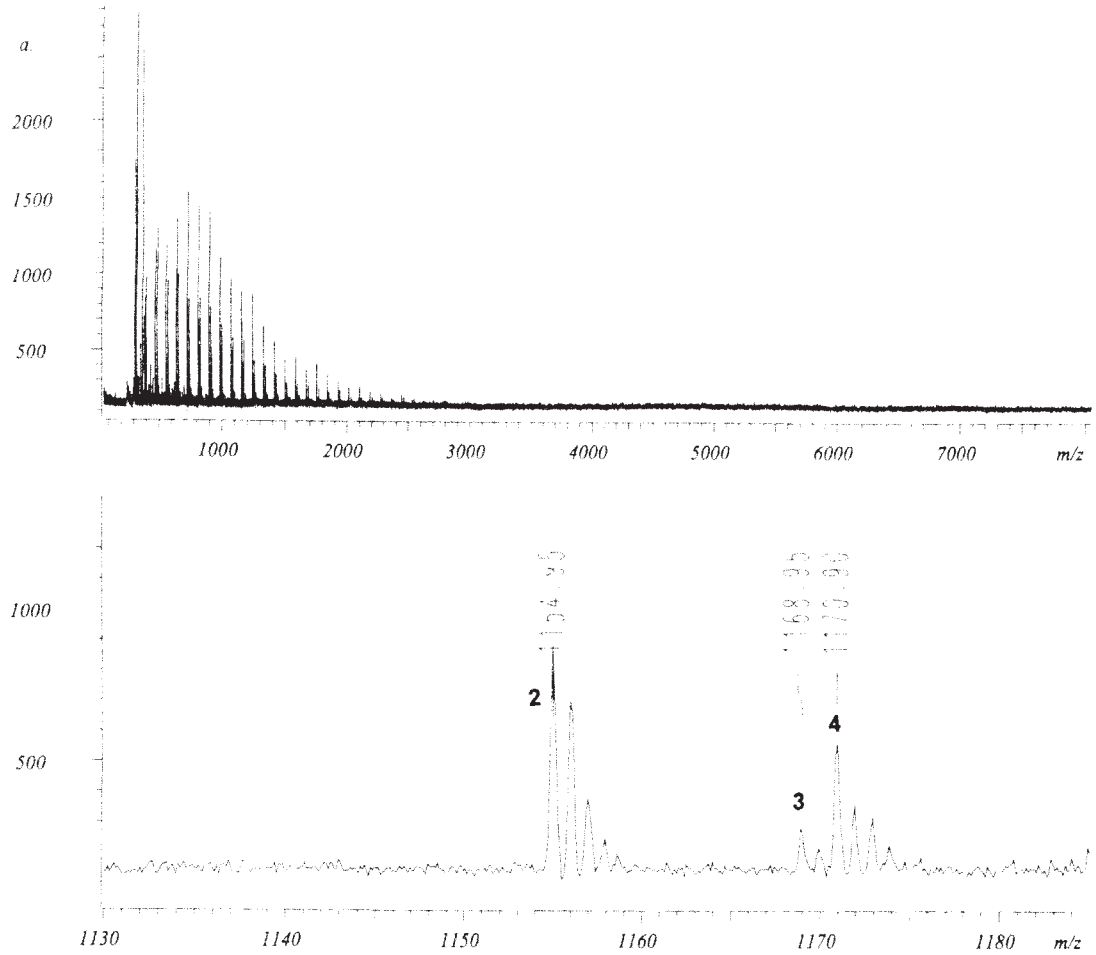

Figure 3. (Top) Positive ion MALDI–TOF mass spectrum (reflectron mode) of partially transesterified (base-catalyzed) PHA. The sample was methylated before transesterification. (Bottom) Expansion of the spectrum between *m/z* 1130 and 1185. Note the absence of peak 1 (*see* Figure 1 for peak assignments).

(**g**) *Diethyl ether*.—99.9%, LC grade; CAS 60-29-7; Aldrich Chemical Co. or Sigma (St. Louis, MO).

(**h**) *1-Methyl-3-nitro-1-nitrosoguanidine* (*MNNG*).—CAS 70-25-7, used in the methylation step; Aldrich Chemical Co. or Sigma.

(**i**) *Matrixes for MALDI analysis.*—2,5-Dihydroxybenzoic acid (DHB), 99%, CAS 490-79-9; and dithranol, 97%, CAS 1143-38-0 (Aldrich Chemical Co.) were used as obtained.

Instrumentation

(**a**) *GC/MS*.—GC/MS data were obtained by using a Hewlett-Packard 5890 Series II Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA) directly interfaced to a Finnigan SSQ single-quadrupole mass spectrometer (Finnigan MAT, San Jose, CA). A 1 μL sample (1 μg/μL) was injected on column, with helium as the carrier gas. A constant helium flow of 35 cm/s was maintained by electronic pressure control, through a DB-5 column, 30 m, 0.32 mm id, 0.25 μm film thickness (J&W Scientific, Folsom, CA). The GC program was as follows: hold at 60°C for 3 min, increase to 100°C at 20°C/min, increase from 200 to 300°C at 15°C/min, and hold at 300°C, for a total of 32 min. For electron ionization (EI) analysis, the source pressure was approximately 25 mtorr, and the mass spectral scan range was 15–500 amu. The chemical ionization (CI) source pressure was approximately 8000 mtorr, isobutane was the CI gas, and the scan range was 100–600 amu. The source temperature was 100°C for both EI and CI.

(**b**) *MALDI–TOFMS*.—All measurements were performed on a Bruker Reflex III MALDI–TOF mass spectrometer (Bruker Instruments, Billerica, MA) with 25 kV acceleration and detection in the positive ion high-resolution reflectron mode. The samples were ionized by irradiation with the output of a nitrogen laser (337 nm, 5 ns) with power levels slightly above the threshold (approximately 10^6–10^7 W/cm^2). The optical path contained a right angle beam-steering prism, a variable attenuator, and a 305 mm focal-length quartz lens placed approximately 300 mm from the target. Ions generated from the samples were accelerated to a potential of 25 kV by using a 3-stage acceleration source. An electrostatic ion guide was used to assist in the nominal 2 m field-free flight path. A hybrid, discrete dynode electron multiplier, followed by dual

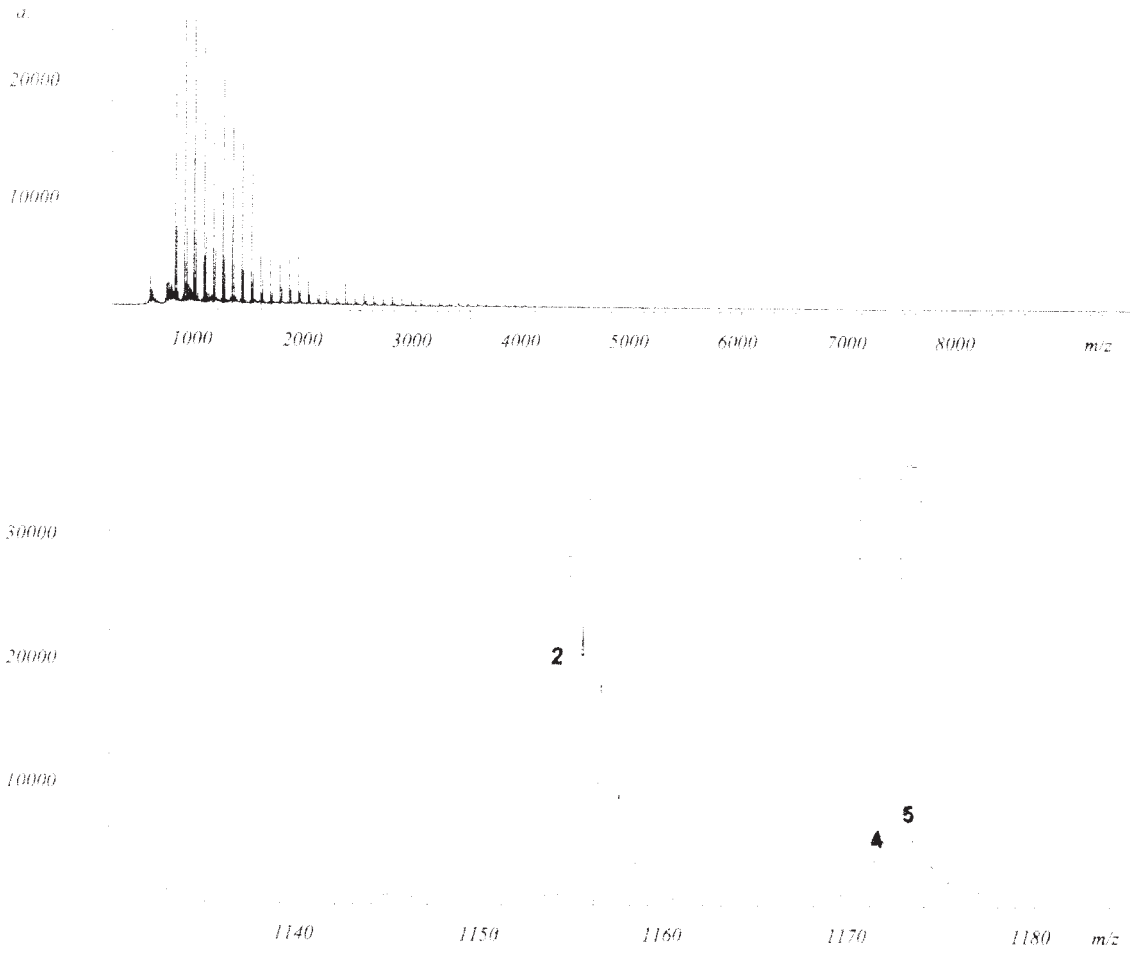

Figure 4. (Top) Positive ion MALDI–TOF mass spectrum (reflectron mode) of partially transesterified (base-catalyzed) commercial PHB. (Bottom) Expansion of the spectrum between *m/z* 1130 and 1185. Note the absence of peaks 1 and 3 (*see* Figure 1 for peak assignments).

microchannel plates, was used for detection. Ions below *m/z* 700 were removed with pulsed deflection, and 100–200 transients were summed to generate a mass spectrum. Four independent MALDI–MS runs were made for each sample to evaluate reproducibility.

MALDI Sample Preparation

Sample preparation is extremely important in MALDI–MS for obtaining reproducible results, and thus similar procedures must be followed. The matrix used was either dithranol or DHB at a concentration of 10 mg/mL in THF. A 1 mg/mL polymer solution (in chloroform) was mixed with 1 mL THF. Then the dithranol solution and polymer solution were mixed in a small sample tube in a 5:2 ratio (matrix:sample). A 1 μL aliquot of this mixture was deposited onto the stainless steel sample holder. The solvent was allowed to air-dry before the sample plate was loaded into the MALDI ion source.

Calibration for MALDI Experiments

The MALDI–TOF mass spectrometer was calibrated by using the synthetic polymer mixtures of polyethylene glycols 1000, 5050, and 10 200 having peaks over the mass range of interest. The calibration file was generated by employing 7 peaks from the mass range of interest. The mass accuracy of the peaks generated by MALDI–TOFMS was approximately 0.02%.

Base-Catalyzed Transesterification

A series of solutions, 7.0×10^{-6} to 1.1×10^{-5}M, of sodium methoxide in methanol were reacted with 1 mg/mL solutions of polymer in chloroform, and each reaction mixture was vortexed for 2 min at room temperature. The reactions were then allowed to proceed for 5 min, after which 10 μL aliquots were taken for MALDI analysis. The commercial PHB was similarly transesterified and analyzed.

Acid-Catalyzed Transesterification

For the acid-catalyzed transesterification, 7.0×10^{-4} to 8.0×10^{-4}M solutions of sulfuric acid in methanol were reacted with 1 mg/mL solutions of polymer in chloroform at 50°C for 1 h, in a modification of a previously described procedure (9), and the reaction mixtures were cooled to room temperature. Then 1 mL water was added to each mixture to induce phase separation. The lower chloroform layer was separated, washed with 1 mL water twice, and dried with magnesium perchlorate. Aliquots were taken as before to perform the GC/MS and MALDI–TOFMS analyses.

Methylation of the Polymer by Diazomethane

Diazomethane is a carcinogen and can explode unaccountably as a gas and in solution. To minimize these hazards, diazomethane generation and the subsequent methylation were performed in situ by using a modified diazomethane generator assembled with a Clear-Seal Joint®. A stock solution of polymer (1 mg/mL in chloroform) was prepared, and 2 mL was placed in the outer reaction vessel of the generator, which was placed in an ice bath. The inner reaction vessel containing 100 mg MNNG was placed inside the cooled outer reaction vessel containing the sample solution. A 10% sodium hydroxide solution was then added dropwise to the MNNG, from a syringe with a narrow gauge to prevent diazomethane escape via the screw-cap opening, for the generation of 1.02 mmol diazomethane. In the event of pressure buildup, the syringe was left in place to act as a relief valve. The vessel was then removed from the ice and allowed to stay at room temperature for 10 min, and then the excess diazomethane was reacted with 10 μL concentrated acetic acid. The product was subsequently partially transesterified before MALDI–TOFMS analysis.

Results and Discussion

Base-Catalyzed Transesterification

Base-catalyzed transesterfication is not commonly used for characterizing PHBs, but it has been applied to the analysis of triacylglycerols (10). Our rationale for using base catalysis was to create a milder reaction condition so as to preserve the end groups of the parent polymer. Table 1 shows the relationship between the concentration of the sodium methoxide and the size of the resulting oligomers. The results show that the size of the oligomers was very much dependent on the concentration of the base used.

Figure 1 (top) shows the MALDI–TOF mass spectrum of partially transesterified (base-catalyzed) PHA. The spectrum is dominated by a series of ion peaks that correspond to $[M + Na]^+$ and $[M + K]^+$ species of intact oligomers. Note that no salt was added in the sample preparation, but is present as an impurity in the matrix, solvent, or glassware. Overall, the spectrum shows a high abundance of low-molecular weight oligomers, and the abundance decreases gradually as the molecular weight increases. Ions were observed for oligomeric species from 7-mer to 70-mer, with the lower mass limit being defined by the matrix interference, and the upper limit, by the signal-to-noise ratio. No fragmentation of the oligomer adduct ion was observed.

Figure 1 (bottom) shows an expanded view around 1 low-mass oligomer. A closer examination shows the spectrum to be made up of clusters of isotopically resolved peaks of the same oligomer containing different end groups. The nominal separation between these clusters, 86 amu, matches the expected PHA repeat unit [-$OCH(CH_3)CH_2CO$-]. The signal identified as peak 5, starting from *m/z* 1173, is assigned to the oligomer with an intact hydroxyl (OH) functionality, i.e., $[CH_3CH(OH)CH_2C(O)\{HB\}_{12}OCH_3–Na]^+$. This peak also has a component of an isotope at *m/z* 1171. Also in Figure 1 (bottom), the ion at *m/z* 1155 (peak 2) is assigned to

Table 2. Comparison of different reaction conditions for acid-catalyzed transesterification

PHB concn, M	Sulfuric acid concn, M	Time and temp.	Results
5.8×10^{-2}	2.8×10^{-5}	3 h, 100°C	Monomer (ref. 11)
5.8×10^{-2}	4.5×10^{-5}	40 h, room temp.	22-mer (ref. 7)
1.2×10^{-2}	7.5×10^{-5}	1 h, 50°C	100-mer

the sodiated dodecamer with an olefinic end group, $[CH_3CH=CHC(O)\{OCH(CH_3)CH_2C(O)\}_{12}OCH_3—Na]^+$. The corresponding potassium adduct of this oligomer is represented by the less-intense peak 4, starting at *m/z* 1171. The presence of the dehydrated end group was contrary to our expectation because base-catalyzed transesterification was specifically used to create milder reaction conditions (relative to acid-catalyzed transesterification). Figure 2 shows the proposed mechanism for the formation of oligomers with an olefinic end group during the partial transesterification of a PHA. The driving force for the formation of an olefinic end group is believed to be due to the intermediate alkoxide anion, which subsequently leads to the formation of an enolate ion, leading to a stable α,β-unsaturated olefinic end group. A mechanism involving decarboxylation is also probable; however, this is not supported by our data, which show a significantly lower intensity for the oligomer with a carboxyl end group. Thus, the weak signal, identified as peak 1, at *m/z* 1141 is assigned to a sodiated dodecamer with olefinic and carboxyl end groups, $[CH_3CH=CHC(O)\{OCH(CH_3)CH_2C(O)\}_{12}OH–Na]^+$. In this oligomer, the acid end group is believed to originate mostly from the parent polymer because it would not be methylated during the transesterification reaction. To confirm this, the polymer was methylated, before transesterification, with diazomethane and then transesterified with base as a catalyst. MALDI–TOFMS analysis (*see* Figure 3) of the reaction product showed the absence of an ion that corresponds to peak 1.

Another unexpected signal, identified as peak 3, at *m/z* 1169, was atttributed to the presence of valerate units in the polymer backbone. Our observation of this peak led us to undertake further analysis using a commercial PHB homopolymer. In one experiment, the commercial PHB homopolymer was transesterified under the same experimental conditions used previously. Its MALDI spectrum (*see* Figure 4) showed that peak 3 was absent. The absence of this peak suggests that the PHA synthesized in our laboratory may be an HB/HV copolymer. Further studies using ^{13}C NMR were not able to detect the valerate, probably because of its low concentrations; this result is consistent with earlier findings (6). We also note the absence of peak 1 (Figure 4) due to the much greater molecular weight of the commercial PHB, compared with that of the PHA synthesized in our laboratory.

In another experiment, a GC/MS analysis was performed on a completely base-hydrolyzed polymer. The results of this analysis showed, in addition to the methyl β-hydroxybutyrate,

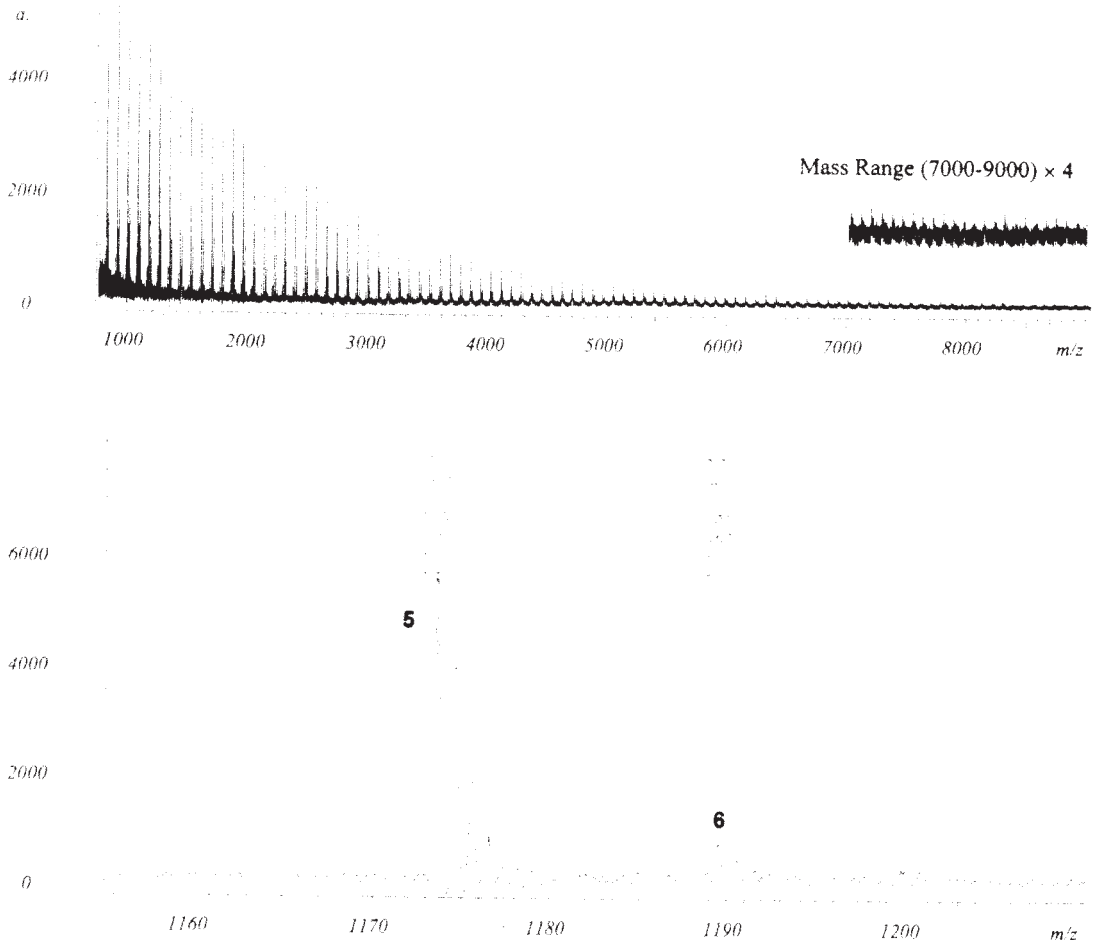

Figure 5. (Top) Positive ion MALDI–TOF mass spectrum (reflectron mode) of partially transesterified (acid-catalyzed) commercial PHB, showing cationized molecules. (Bottom) Expansion of the spectrum between *m/z* 1130 and 1185. Peak assignments: 5 = $[CH_3CH(OH)CH_2C(O)\{HB\}_{12}OCH_3–Na]^+$ and 6 = $[CH_3CH(OH)CH_2C(O)\{HB\}_{12}OCH_3–K]^+$.

a trace amount of methyl β-hydroxyvalerate, a further indication that the cluster peak 3 in Figure 1 (bottom) could therefore be attributed to a copolymer HB/HV, i.e., $[CH_3CH=CHC(O)\{OCH(CH_3)CH_2C(O)\}_{11}\{OCH(CH_2CH_3)CH_2C(O)\}OCH_3–Na]^+$.

Acid-Catalyzed Transesterification

Table 2 compares our reaction conditions with those of previously reported schemes of acid-catalyzed transesterification (7, 11). The sulfuric acid concentration, temperature, and time were critical in the outcome of the reaction. Overall, our transesterification shows that the PHA polymer can be effectively broken down to large-size oligomers, which have the potential for synthesizing other key chemical compounds.

The MALDI–TOF mass spectrum of partially transesterified PHA, with acid as a catalyst, is shown in Figure 5 (top). Similar to the base-catalyzed mass spectrum, this mass spectrum contains a series of ion peaks that correspond to $[M + Na]^+$ and $[M + K]^+$ species of intact oligomers ranging from 7-mer to 100-mer; Figure 5 (bottom) shows an expanded view of 1 low-mass oligomer. The most abundant ion, peak 5, at *m/z* 1173 is assigned to the sodiated dodecamer with a methyl β-hydroxybutyrate end group, $[CH_3CH(OH)CH_2C(O)\{HB\}_{12}OCH_3–NA]^+$. The corresponding potassium adduct of the same oligomer is peak 6. This result is quite a contrast to the MALDI spectrum of base-catalyzed transesterification, because no oligomeric species with a dehydrated end group are observed.

Conclusions

We have used high-resolution MALDI–TOFMS to analyze a partially transesterifed PHA polymer. The PHA was synthesized by using the bacterial strain *A. eutrophus* in a saponified oil medium. Transesterification was carried out under basic and acidic conditions to obtain oligomers up to 10 kDa (100-mer). The MALDI–TOFMS analysis showed that the base-catalyzed reaction, despite being relatively mild, resulted in dehydrated end groups (olefinic), whereas the acid-catalyzed oligomer end groups showed no dehydration. MALDI–TOFMS results for the base-catalyzed transesterification revealed a carboxyl end group in the parent polymer. An interesting finding was the presence of trace amounts of HB/HV cooligomer in the sample, further confirmed by GC/MS. When a commercial PHB homopolymer was transesterified under the identical conditions and analyzed, no HV component was observed; this result suggests that the PHA polymer synthesized in our laboratory was actually P(HB/HV). Thus, saponified vegetable oils could be used for biosynthesis of P(HB/HV), a conclusion that can be further tested by investigating a series of saponified vegetable oils as carbon sources for *A. eutrophus*.

Acknowledgments

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