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Characterization of short-chain poly3-hydroxybutyrate in baker's yeast

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Abstract

A short-chain poly3-hydroxybutyrate including four comonomers, originating from a complex with calcium polyphosphate, was isolated from commercial baker's yeast cells (*Saccharomyces cerevisiae*) and characterized as the second complexed poly(3-hydroxyalkanoate) (cPHA) in eukaryotes. The number-average molecular weight of 4982.5 Da with a polydispersity index of 1.11 was much lower than that of beet cPHA previously isolated. End-group analysis suggested that at least 60% of the molecules form the cyclic structures. Here, the organism-dependent structural diversity of cPHAs was completely established. It was also found that a change of culture medium influences the molecular weight but not the polydispersity of baker's yeast cPHA.

Keywords: *Saccharomycs cerevisiae*; Baker's yeast; Time-of-flight MALDI MS; Number-average molecular weight; Weight-average molecular weight; Polydispersity; Short-chain poly3-hydroxyalkanoate; cPHA

1. Introduction

In nature, poly(3-hydroxyalkanoate)s (PHAs) are classified into low-molecular-weight PHA of less than 20 kDa and high-molecular-weight PHA of approximately 50–5000 kDa (Lemoigne, 1926). The former, called complexed PHA (cPHA), is a ubiquitous constituent of both prokaryotic and eukaryotic cells (Reusch and Sadoff, 1983; Reusch, 1989, 1992; Reusch et al., 1992). The latter, called storage PHA (sPHA), is synthesized and stored as granules in cytosols only by certain prokaryotes (Doi, 1990). cPHAs are widely distributed in various cell fractions in complex with other biomacromolecules, such as proteins and inorganic polyphosphates (polyPs). Most of them are complexed covalently with proteins; a small fraction of them are non-covalently complexed with calcium polyP (Reusch and Sadoff, 1983; Reusch, 1989, 1992; Reusch et al., 1992, 2002; Seebach et al., 1994; Seebach and Frits, 1999; Huang and Reusch, 1996). There is evidence that these polyP-complexes form ion channels in plasma membranes and play a role in the acquisition of competence in *Escherichia coli* (*E. coli*) (Reusch et al., 1986, 1995; Seebach et al., 1996; Das et al., 1997, 1999).

Seebach et al. have identified poly(3-hydroxybutyrate)s (PHBs) by ^{1}H NMR spectroscopy in the partially purified cPHAs from eukaryotes of spinach, beef-heart mitochondria, and human aortae and from a prokaryote of *E. coli*. Also, the (*R*) configuration of the 3-hydroxybutyric acid (3-HB) units in cPHAs from *E. coli* and spinach and the presence of 3-hydroxyvalerate (3-HV) comonomer in cPHA from *E. coli* have been shown (Seebach et al., 1994). To date, only two cPHAs have been purely isolated. One is from the prokaryote *E. coli*, designated as *E. coli* cPHA. Wild *E. coli* cells do not synthesize sPHAs under normal growth conditions. However, genetically competent *E. coli* cells rapidly synthesize short-chain PHB in their membranes. Reusch et al. have isolated the native cPHA-polyP complex from such cells and proposed its whole structure (Reusch and Sadoff, 1988; Seebach et al., 1994),

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the polyester part of which was short-chain PHB of degree of polymerization (n) = 140 with 14 mol% 3-HV comonomer. Very recently, Suzuki et al. have characterized the polyP-complex-derived cPHA from the eukaryote of sugar beet (*Beta vulgaris L.*), designated as beet cPHA (Suzuki et al., 2005). It had the slightly shorter chain PHB of $n = 106$ and a much lower 3-HV content than *E. coli* cPHA. These results confirmed the organism-dependent structural diversity of cPHAs.

The aim of this work is to chemically characterize an alternate, eukaryotic cPHA to further confirm the organism-dependent structural diversity of cPHAs. To this aim, baker's yeast (*Saccharomyces cerevisiae*) was chosen among eukaryotic organisms excluding plants (Reusch, 1989, 1992; Reusch et al., 1992). In this paper, the isolation and structural determination of baker's yeast cPHA derived from a polyP-complex are described.

2. Results and discussion

2.1. Isolation of cPHA from commercial baker's yeast cells

Yeast cPHA was isolated from commercial cakes made using baker's yeast. Seebach et al. have extracted a variety of cPHAs using dried $CHCl_3$ for prolonged periods or $CHCl_3$–MeOH (10:1) for 5–10 min (Seebach et al., 1994). However, Suzuki et al. employed the drastic condition of a boiling $CHCl_3$–MeOH (10:1) for 2 days to efficiently extract cPHA from beets, and showed that no partial methanolysis and/or no formation of the cyclic molecules *via* intramolecular transesterification did not occur in the purified beet cPHA. Also, it was shown that a change of extraction solvent (only $CHCl_3$) did not influence the chain length and 3-HV content (Suzuki et al., 2005). Therefore, the isolation method employed here was performed as we described previously for beet cPHA (see Experimental). Namely, it involved the lyophilization of the yeast cells, the washing of the cells by methanol extraction, the extraction of cPHA using hot solvents, and the purifications of cPHA by precipitation, partition between the solvents, and gel-permeation chromatography. All isolation steps were monitored by ^{1}H NMR spectroscopy. Two signals characteristic of 3-HB units were used: two double-doublets due to H-2 at ca. δ 2.5 ppm and a multiplet due to H-3 at ca δ 5.26 ppm. Pure cPHA (designated as yeast cPHA-1), 2.6 mg, was obtained from 850 g dry wt of the cells. This cPHA-1 is the second cPHA purified from eukaryotic organisms.

2.2. Identification of monomer units

Fig. 1 shows the (a) ^{13}C and (b) ^{1}H NMR spectra of cPHA-1. Only the signals due to 3-HB units were observed in both spectra (also see PHB in Table). However, Fig. 2a that has an expanded ^{1}H NMR spectrum of Fig. 1b, shows a number of significant signals due to the comonomers and free-end hydroxyl groups. Four comonomers, namely, 3-HV, 4-hydroxybutyrate (4-HB), 3-hydroxypropanoate (3-HP), and crotonate (CA), were identified. Table 1 summarizes these assignments. Double-pulse field gradient selective echo 2D total correlation spectroscopy and homonuclear proton-decoupling experiments using a 600 MHz ^{1}H NMR spectrometer unequivocally confirmed these assignments (data not shown). The existence of 3-HB, 3-HV, and CA (very small) units was also confirmed by GC-MS of cPHA-1 ethanolyzates (see Experimental). This cPHA-1 with the four comonomers including 3-HV identified in *E. coli* and beet cPHAs enable us to reinvestigate the structure of beet cPHA. The expanded spectrum of beet cPHA is shown in Fig. 2c. The signals due to 4-HB units at δ 4.11 and 1.94 ppm and CA units at δ 1.87 ppm are clearly observed, except those due to 3-HV units previouly identified (also see 4-HB, CA, and 3-HV in Table 1). Therefore, it was confirmed that beet cPHA includes 4-HB and CA together with 3-HV, as a comonomer.

The amount of each comonomer in cPHA-1 was estimated from the corresponding signal intensities and compared with the theoretical values of the known {^{13}C}H-satellite signals: e.g. H-3 at δ 5.1 and 5.4 ppm (each equivalent to ca. 0.0055H) or H-4 at δ 1.14 ppm (equivalent to approximately 0.011H). As results, 3-HV units were 1.03 mol%, but others were less than 0.1 mol% (4-HB: 0.06 mol%, 3-HP: 0.07 mol%, CA: 0.02 mol%). Thus, the chemical structure of cPHA-1 was determined to be PHB including ca. 1 mol% 3-HV and a small amount of 4-HB, 3-HP, and CA comonomers. Notably, yeast cPHA-1 has a ca. 10-fold higher 3-HV content than beet cPHA.

The comonomer content of beet cPHA, estimated similarly, was almost identical to that of yeast cPHA-1 (4-HB: 0.1 mol%, CA: 0.01 mol%).

2.3. Determination of molecular weight by MALDI MS

Fig. 3a shows a typical time-of-flight MALDI MS spectrum of cPHA-1, recorded in the linear and positive-ion modes. Two distinct peak series having an 18 mass difference were observed. Of the series, the peak groups greater than ca. m/z 3500 were due to linear PHBs cationized by Na^+ ions (s), whereas the peak groups smaller than ca. m/z 3500, as will be described later, were due to the fragmented species that correspond to linear PHBs (s) and those having terminal CA groups (c). The number-average molecular weight M_n and the weight-average molecular weight M_w were calculated from the masses and their intensities of the molecular-related peaks of the linear PHBs in the ca. m/z 3500–10000 region, using the usual definitions (Cowie, 1991). The ratio of the two averages, M_w/M_n, denotes the polydispersity index *PDI*. Using each given equation, M_n: 4982.5 Da, M_w: 5524.9 Da, and *PDI*: 1.11 were obtained. M_n corresponds to a chain length of $n = 57.4$. This result means that cPHA-1 is much shorter in chain length than beet cPHA (Suzuki et al., 2005). *PDI* is slightly broad compared with that of beet cPHA

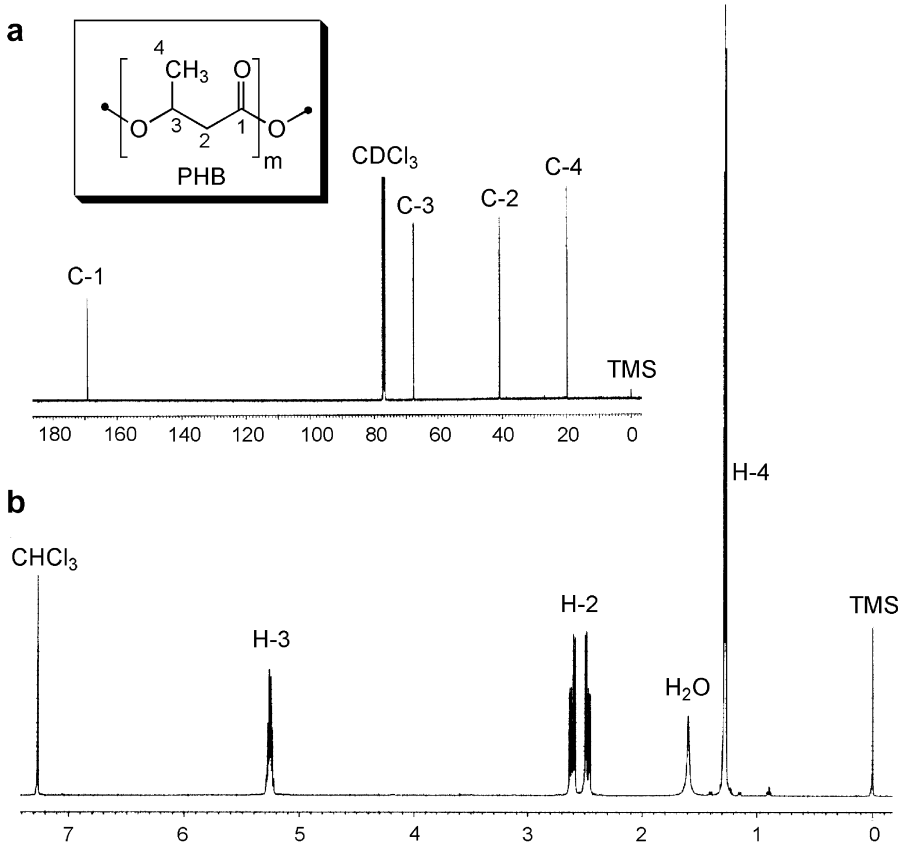


Fig. 1. NMR spectra in $CDCl_3$ of cPHA isolated from commercial baker's yeast cells (*Saccharomyces cerevisiae*). (a) 125 MHz ^{13}C NMR spectrum and (b) 500 MHz ^{1}H NMR spectrum of yeast cPHA-1. δ ppm: 169.1 (C-1), 40.8 (C-2), 67.6 (C-3), 19.8 (C-4).

(*PDI*: 1.01), but is sufficiently narrow (Seebach et al., 1994). The peak at *m/z* 4959.3, one of the largest magnitudes of molecular-related ions, is comparable to that for a chain length of $n = 57$ (calculated $[M + Na]^+$: *m/z* 4948.2).

2.4. Determination of end-group structures

In Fig. 2a, the three signals characteristic of the terminal 3-HB units having free 3-hydroxyl groups were observed at δ 4.18, 3.10, and 1.22 ppm (Suzuki et al., 2005). These assignments are shown in 3-HB of Table 1. The 3-HB content was estimated from the integrals of the H-3 or 3-OH signals. They were 0.0033H and 0.0036H equivalents, which respectively corresponded to 18.9% of the molecules for H-3 and 20.6% of the molecules for 3-OH when the chain length is $n = 57.4$. In other words, the terminal hydroxyl groups are ca. 80% masked.

On the other hand, the content of each free form at the end carboxyl and hydroxyl groups of cPHA-1 was estimated by quantitative ^{31}P NMR spectroscopy (Spyros et al., 1997). The ^{31}P NMR spectrum of the cPHA-1 phosphite ester (202 MHz, $CDCl_3$) showed three signals assigned to phosphites attached to carboxyl groups at δ 135.58 and 135.62 ppm and to a hydroxyl group at δ 146.41 ppm. The two signals at δ 135.58 and 146.41 ppm were identified to be phosphites attached to the free carboxyl and hydroxyl groups in linear cPHA-1 because of their chemical shifts (Spyros et al., 1997). Their content corresponded to 3.6% of the molecules for the carboxyl groups and 17.5% of the molecules for the hydroxyl groups. The latter value is almost identical to that estimated using the ^{1}H NMR spectroscopic data in Fig. 2a, 18.9–20.6%. The major, alternate carboxylated-phosphite signal at δ 135.62 ppm that corresponded to 21.0% of the molecules was not assigned, because no masking compounds were detected as described above. When such unassignable compounds (e.g. hydroxyalkanoic acids or alkanedioic acids) attach to either the terminal carboxyl or hydroxyl groups of cPHA-1, the total amounts of their masking groups are estimated to be 75.4% (100% minus 3.6% minus 21.0%) of the molecules for the carboxyl ends or 60.1% (100% minus 18.9% minus 21.0%) of the molecules for the hydroxyl ends. These values strongly suggested that at least ca. 60% of cPHA-1 molecules are cyclic species although, at present, there is no direct evidence about them.

2.5. Isolation of cPHA from cultured baker's yeast cells

As described above, cPHA-1 obtained from commercial baker's yeast has marked differences in chemical structure from beet cPHA as follows; (i) much shorter chain length, (ii) slightly broader polydispersity, (iii) existence of cyclic molecules, and (iv) much higher 3-HV content. Here, we

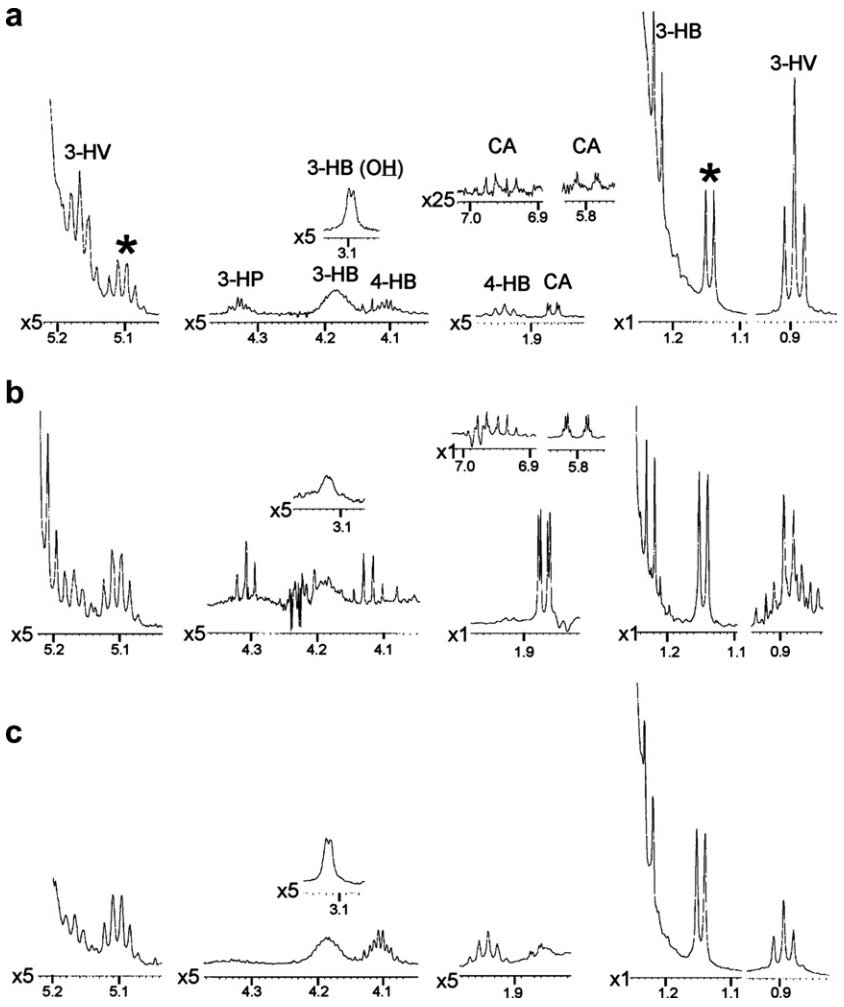
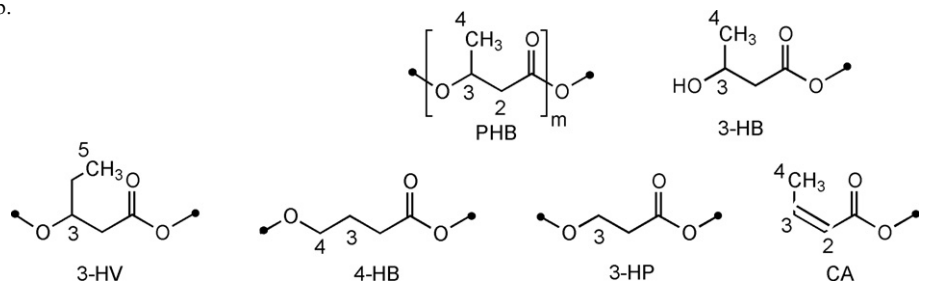


Fig. 2. Expanded ^{1}H NMR spectra (500 MHz, $CDCl_3$) of cPHAs between δ 0.5 and 5.5 ppm. (a) Yeast cPHA-1 isolated from commercial baker's yeast cells, (b) yeast cPHA-2 isolated from cultured, fresh baker's yeast cells, (c) beet cPHA. Signals (∗) represent the $\{^{13}C\}H$-satellite peaks.

Table 1
Assignments of signals in ^{1}H NMR spectrum of commercial baker's yeast cPHA

Co-monomer	Chemical shift (6 ppm, J = Hz)					
	PHB	3-HB	3-HV	4-HB	3-HP	CA
H-2	2.46 and 2.60 two dd (15.6,6.0 and 15.6,7.3)	nd	nd	nd	nd	5.80 dq (15.6, 1.8)
H-3	5.26 m	4.18 m 3.1 br.d (3-OH)	5.17 m	1.94 m	4.33 m	6.95 dq (15.6,6.9)
H-4	1.27d/(6.5)	1.22d/(6.4)	nd	4.11 m	nd	1.87dd/(6.9,1.8)
H-5	–	–	0.89 t (7.4)	–	–	–

nd: not assigned because of overlap.



isolated cPHA from cultured, fresh baker's yeast cells to particularly confirm whether molecular weight and its polydispersity vary with applied culture medium. If a chain shortening in cPHA-1 occurs before the isolation, the cPHA obtained from the fresh baker's yeast cells should have both a higher molecular weight and a smaller polydis-

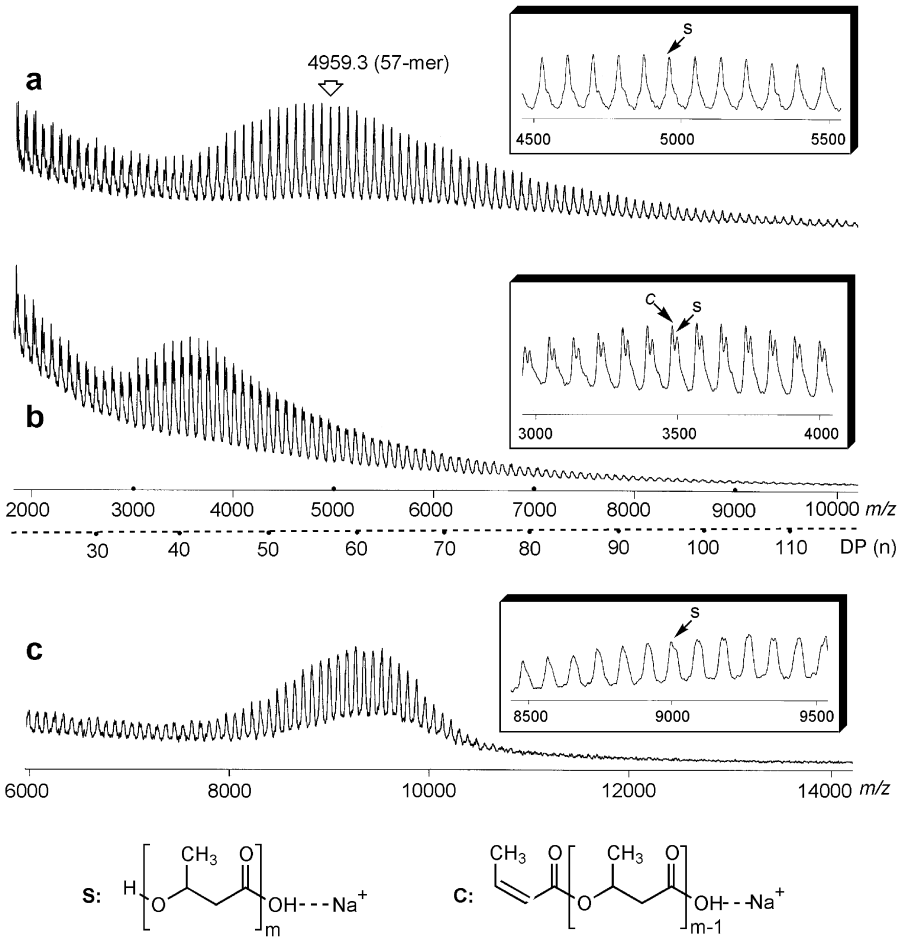


Fig. 3. Time-of-flight MALDI MS spectra of cPHAs, recorded in linear and positive-ion modes using DHBA as matrix. (a) Yeast cPHA-1, (b) yeast cPHA-2, (c) beet cPHA. Insets show expanded portions of their spectra. s: linear PHBs, c: PHBs having crotonate end groups. DP: degree of polymerization (n).

persity than cPHA-1. Commercial baker's yeast is commonly produced using a culture medium containing sugars and inorganic ammonium and phosphate salts. In our baker's yeast culture, a modified YM medium containing glucose, peptone, and malt and yeast extracts was used. The jar-fermented yeast cells (ca. 150 g dry wt) that were obtained from 30 l of culture broth yielded ca. 700 μg of cPHA (designated as yeast cPHA-2).

Fig. 3b shows the MALDI MS spectrum of cPHA-2. The molecular weight and its polydispersity were calculated using the molecular-related peaks in the ca. m/z 2500–8000 region; M_n is 4448.8 Da equivalent to a chain length of $n = 51.2$, *PDI*: 1.11. Unexpectedly, M_n slightly decreased, whereas *PDI* remained unchanged. These results clearly indicate that no chain shortening occurs in the cPHA-1 sample and that the shortage of the monomer substrates under the applied culture medium decreases the molecular weight of cPHA-2. Fig. 2b shows the expanded ^{1}H NMR spectrum of cPHA-2. The comonomer content of cPHA-2 greatly differs from that of cPHA-1. 3-HV content decreased to 0.40 mol% from 1.03 mol%, and CA content markedly rose to 0.43 mol% from 0.02 mol% that corresponded to 22.0% of the molecules. No 3-HP and 4-HB comonomers were detected. The amount of the free-hydroxyl end groups similarly estimated corresponded to 15.2% of the molecules, being slightly lower than that of cPHA-1, 18.9%. These results clearly show that the culture condition influences not only the molecular weight, but also the amount of comonomers and terminal free-hydroxyl groups. That is, it seems that such structural diversity between the two yeast cPHAs depends on the amount of supplied substrates. In particular, the remarkable increase in the CA content of cPHA-2 was prominent and gave rise to the characteristic MALDI MS spectrum described below (see Fig. 3b). The carboxyl-end structures of cPHA-2 remain unknown because the very low amount of cPHA-2 makes the quantitative ^{31}P NMR experiment using a phosphite derivative impossible. Therefore, it is not clear whether cPHA-2 includes also the same cyclic molecules as cPHA-1.

There is the greatest difference in the MALDI MS spectra between the two yeast cPHAs. Namely, peaks equivalent to dehydration products of linear cPHA are observed in all mass region for cPHA-2 but only in the mass region lower than ca. m/z 3500 for cPHA-1. The linear cPHA

molecules with terminal CA-groups are equivalent to the cyclic species and to the dehydration products by fragmentation. Of these series, peaks greater than ca. *m*/*z* 2500 for cPHA-2 are suggested to be due to the original CA-terminated molecules, whereas peaks lower than ca. *m*/*z* 2500 for cPHA-2 and ca. *m*/*z* 3500 for cPHA-1 are suggested to be due to the dehydration products of linear cPHAs by fragmentation, but not the cyclic species suggested in cPHA-1. These differences between two yeast cPHAs are proportional to the content of the linear cPHAs with terminal CA-groups. As described above, their content was high in cPHA-2 (ca. 22%) but low in cPHA-1 and beet cPHA (1% order). The mass spectral results of beet cPHA (Fig. 3c) supported this assignment. Now, cPHA-1 that includes the high amount of cyclic molecules is expected to give peaks equivalent to dehydration products in the mass region greater than ca. *m*/*z* 3500. However, these peaks were not observed. This fact appears to show an extraordinarily low ionization-efficiency of the cyclic polyester molecules in MALDI MS using DHBA as a matrix. These considerations render the molecular weights calculated for yeast and beet cPHAs valid.

3. Concluding remarks

In this study, the characterization of cPHAs from baker's yeast cells has established the organism-dependent structural diversity of cPHAs, e.g. molecular weight and its polydispersity, terminal-group structures including cyclic forms, and comonomer content. The most interesting, structural characteristics is that cPHA-1 (probably also cPHA-2) includes the corresponding cyclic molecules. Nobody has ever proposed such cyclic structures for cPHAs. In an *in vitro* enzymatic polymerization of β-butyrolactone using hydrolases such lipases and PHB-depolymerases, productions of short-chain cyclic and linear PHB are known: the amount of cyclic polymers increased with increasing degree of monomer conversion (Matsumura et al., 1998; Suzuki et al., 2001).

The existence of the four comonomers including 3-HV in yeast cPHA-1 was a new finding. Also, it was found that a change of the culture medium directly influence the molecular weight, but not the polydispersity of baker's yeast cPHA.

4. Experimental

4.1. NMR spectroscopy

NMR spectra were recorded using JEOL ECP 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz, ^{31}P: 202 MHz) or JEOL ECA 600 (^{1}H: 600 MHz) in $CDCl_3$ (99.8% deuterium-atom enriched). Chemical shifts are referenced to the TMS peak of δ 0.00, the $CDCl_3$ peak of δ 77.0, and the bisphenol A peak of δ 138.57 ppm.

4.2. MALDI MS spectroscopy

MALDI MS data were recorded using a time-of-flight MALDI MS spectrometer (Voyager, Applied Biosystems) in the linear and positive-ion modes. Ions were generated using a nitrogen laser at 337 nm with a pulse duration of 3 ns. Static acceleration voltage was set at 20 kV. MS spectra were obtained from the 250 shots accumulated. For sample preparation, DHBA (0.5 mg) as a matrix and MeOH (10 μl) saturated with sodium tetrafluoroborate (Aldrich) were added to the cPHA sample (ca. 100 μg) in $CHCl_3$ (10 μl). For the analysis, the mixed solution (0.5–1.0 μl) was spotted on a sample slide and dried in air.

4.3. Isolation of cPHA from baker's yeast cells

Commercial cakes made using baker's yeast (Kaneka Co., Ltd, Japan, 6 kg) were lyophilized and powdered. The resulting powder (850 g dry wt) was washed twice with MeOH (each 2 l) at room temperature for 24 h. The collected cells were oven-dried at 50 °C overnight and subjected to extraction in a boiling solvent of $CHCl_3$–MeOH (10:1) for 2 day. The concentrated filtrate was precipitated with hexane and then with MeOH. The precipitate was partitioned between $CHCl_3$ and H_2O (2:1, 30 ml). The $CHCl_3$ layer was collected and concentrated. The residue was similarly precipitated with MeOH–$CHCl_3$ (10:1, 80 ml). The precipitate was subjected to preparative GPC. The GPC conditions were as follows: column, Sephadex LH-20 (φ 5 cm × 50 cm); solvent, $CHCl_3$; and flow rate, 2.5 ml/min. The sample, dissolved in $CHCl_3$ (1.7 ml), was injected into the GPC column. The eluate was collected and the combined cPHA fractions were concentrated. The precipitation of this residue with MeOH–$CHCl_3$ (9:1, 50 mL) yielded cPHA-1 (2.6 mg).

4.4. Culture of baker's yeast cells

Commercial baker's yeast was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of a modified YM medium (pH 6.2) consisting of 3% glucose, 5% peptone, 3% yeast extract, and 3% malt extract. Incubation was carried out on a shaker at 30 °C for 2 day. The seed culture of the six flasks containing the same medium was obtained after further repeated inoculations (*x*2). The three seed cultures were poured into each of two 20 l jar fermentors containing the same medium (each 15 l); incubation was carried out at 30 °C for 27 h. The cells were collected and lyophilized. cPHA from the dried cells (ca. 150 g dry wt) was isolated by the same method described above, yielding ca. 700 μg of cPHA-2.

4.5. GC-MS analysis of cPHA-1 ethanolyzates

A mixture of cPHA-1 (ca. 50 μg), $CHCl_3$ (250 μl), conc. HCl (100 μl), and EtOH (850 μl) was heated at 100 °C for 4 h in a capped tube. An aqueous solution (1.7 ml) consist-

ing of 0.65 N NaOH and 0.9 M NaCl was added to the cooled reaction mixture. The bottom layer was carefully collected and subjected to GC–MS using a gas chromatograph HP 6890 series coupled to a mass spectrometer HP 5973 at 70 eV with a He flow rate of 1 ml/min. HP-5MS column (φ 0.25 mm × 30 m, 0.25 μm film thickness, G&L Science) was used. The column oven temperature was programmed as follows: 40 °C for 4 min, increasing at 2 °C/min up to 90 °C and at 15 °C/min up to 300 °C, and being maintained 300 °C for 10 min. MS (70 eV, Retention time: min): Et CA (8.40 min); *m/z* 114 $(M)^+$, Et 3-HB (13.10 min); *m/z* 131 $(M)^+$-H, Et 3-HV (19.30 min); *m/z* 145 $(M)^+$-H.

4.6. ^{31}P NMR spectroscopy of cPHA P-1 phosphite ester derivative

A sample (100–150 nM equivalent) was dissolved in $CDCl_3$ (0.6 ml). A stock solution [60 μl; bisphenol A (5.0 mg) and chromium acetylacetone (3 mg) in dry pyridine (5 ml)] and then 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (10 μl, Aldrich) was added to the sample solution. The mixture was transferred to NMR tube, and, after 24 h, analyzed using a 202 MHz ^{31}P NMR spectrometer, focusing on ^{31}P nuclei. The parameters used for quantitative ^{31}P NMR measurement are as follows: 45° pulse of 6.775 μs, proton-gated decoupling, and 20 s of relaxation delay.

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