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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