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A THEMOGRAVIMETRIC ANALYSIS FOR POLY(3-HYDROXYBUTYRATE) QUANTIFICATION

Sei Kwang Hahn and Yong Keun Chang*

Department of Chemical Engineering and BioProcess Engineering Research Center
KAIST, 373-1 Kusong-dong, Yusong-gu, Taejon, 305-701, Korea

SUMMARY

A simple and quick thermogravimetric analysis method has been suggested for poly(3-hydroxybutyrate) [PHB] quantification. During the analysis, a rapid thermal degradation of PHB occurred in the range of 250 to 320 °C. From the gravimetric change during the thermal degradation, we could quantify PHB contents of various samples. Due to the simultaneous thermal degradation of cellular materials, the PHB contents were estimated slightly higher than those by gas chromatographic analysis. We have proposed a way to compensate for such errors using a linear correlation to allow accurate determination of PHB contents.

INTRODUCTION

Poly(3-hydroxybutyrate) [PHB] is a promising microbial thermoplastic which has properties comparable to polypropylene as well as a good biodegradability (Doi 1990). Therefore, there is a considerable interest in using PHB for packaging purposes in order to reduce the environmental impact of human garbage. PHB can also be used in the biomedical applications such as antibiotics, drug delivery, medical suture, and bone replacement (Holmes 1985).

In order to detect and quantify PHB, several methods have been suggested including gas chromatographic (Braunegg et al. 1978), turbidimetric (Williamson and Wilkinson 1958), spectrophotometric (Ward and Dawes 1973), and density gradient separation methods (Nickerson 1982). Among these methods, the gas chromatographic analysis was considered to be reliable and accurate, which was originally developed by Braunegg et al. (1978). In this method, methanolysis was carried out for more than 3 hours at 100 °C. The resulting methylester-derivatives of monomers dissolved in chloroform layer were analyzed with a gas chromatographe (GC).

As well known, poly(3-hydroxyalkanoate) [PHA] including PHB is thermally unstable above 180 °C. The thermal degradation was proved to be due to random chain scissions at ester groups (Morikawa and Marchessault 1981). The chain scission occurs by the widely accepted six-membered ring ester decomposition process.

Pyrolysis methods including thermogravimetric analysis have been extensively used for identification and characterization of various synthetic and biological polymers (Reiner 1965; Morikawa and Marchessault 1981). During the thermogravimetric analysis, PHA is rapidly degraded in the range of 250 to 320 °C. Based on this property, we have developed a simple and quick analytical method for the determination of PHB content.

MATERIALS AND METHODS

Production of PHB-containing biomass. For the production of PHB, we used *Alcaligenes eutrophus* (NCIMB 11599) and a recombinant *Escherichia coli* strain, XL1-Blue, harboring pSYL104 which had been kindly provided by prof. S. Y. Lee (KAIST, Korea). Fed-batch culture of *A. eutrophus* in a minimal medium was carried out with on-line glucose concentration control as previously described (Kim et al. 1994). Details on the stable high-copy-number plasmid pSYL104 containing the *A. eutrophus* PHA biosynthesis genes are described elsewhere (Lee et al. 1994a). Fed-batch culture of XL1-Blue (pSYL104) was carried out in a semi-defined medium employing the pH-stat feeding strategy as described previously (Lee et al. 1994b). After the fermentation, the cells were harvested by centrifugation at 4,000 × g and 25°C for 15 minutes and washed twice with distilled water. The resulting cell paste was freeze-dried and stored at 4 °C until needed.

PHB recovery. PHB was recovered from the cell powders by chloroform extraction, the dispersion treatment, and sodium dodecyl sulfate treatment. The details on the chloroform extraction and the dispersion treatment was given elsewhere (Hahn et al. 1995). In the case of SDS treatment, 5% (w/v) biomass was treated with 3% (w/v) SDS solution for 1 hour at 30 °C. The recovered PHB was rinsed with distilled water, centrifuged again, and then rinsed with acetone. Finally, recovered PHB was dried in a vacuum drying oven.

GC analysis of PHA. PHA content was determined by the methanolysis method of Braunegg et al. (1978). The amount of resulting methylester detected by a GC (Varian 3300) reflects PHA content of the sample. As an internal standard, *n*-butyric acid was used. PHA recovered by chloroform extraction was further purified by another round of extraction using the dispersion method, and used as the standard for PHA analysis. The purity of the standard PHA was checked by analyzing the residual protein content using the Lowry method (Lowry et al., 1951). Only trace amount of residual proteins was detected from the standard PHA. The PHA content was determined by triplicate experiments for each sample.

TG analysis of PHA. Thermogravimetric change of PHA sample was examined with a TG analyzer (TGA-Dupont 2000). The analysis was carried out under a nitrogen flow rate of 20 ml/min with a scanning rate of 10 °C/min.

RESULTS AND DISCUSSION

In order to develop a simple and efficient method for PHB quantification, various samples containing PHB from 37% (w/w) to 99% (w/w) were prepared as described in Table I. According to Huijberts et al. (1992), PHA contents detected by GC were thought not to be affected by the presence of non-PHB cellular materials (NPCM).

Figure 1 is the TGA thermogram for cell powder containing 37% (w/w) of PHB, which shows the way of PHB quantification by TG analysis. Along the thermogram curve line, two horizontal and a vertical tangential lines were drawn. The thermogram was composed of three parts, namely, before thermal-degradation, thermal-degradation, and after thermal-degradation of PHB. As temperature increased, the weight loss of NPCM also increased. The vertical distance between the two intersecting points, A and B, was regarded as the PHB content. In reality, however, PHB was overestimated due to the simultaneous thermal degradation of NPCM.

The TGA thermogram for cell powder containing 85% (w/w) PHB was shown in Figure 2. In this case, the weight loss before the thermal degradation of PHB was negligible. In addition, the rate of thermal degradation of NPCM was observed to be very slow after the thermal degradation of PHB. From the results, we speculated that the error caused by overestimation of PHB content would be negligible for the sample with such a high PHB content and that, with increasing PHB content, the value determined by TG analysis would approach to the real value of PHB content.

In order to investigate the possibility of distinguishing between PHB and other PHA, the thermal degradation of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [PHB/V] was carried out with a TGA. Unfortunately, however, it was not possible to distinguish various forms of PHA. As shown in Figure 3, there was only one peak observed for the PHB/V copolymer sample. PHA is believed to be thermally degraded by the widely accepted six-membered ring ester decomposition process. According to the mechanism, there are no effects of different R-groups on the thermal degradation of PHA (Morikawa and Marchessault 1981). This may be the reason why we could not distinguish between different PHAs using TG analysis.

For comparison, we also analyzed the PHB contents of all the samples with a GC and regarded them as the true values. As stated above, the PHB contents determined by TG analysis were estimated slightly higher than those by GC analysis. The results are summarized in Table I. Figure 4 shows the correlation of PHB contents determined by TG analysis and GC analysis, which is represented by $Y = 1.16 \times X - 15.27$ where Y is PHB content determined with a GC and X is PHB content determined with a TGA.

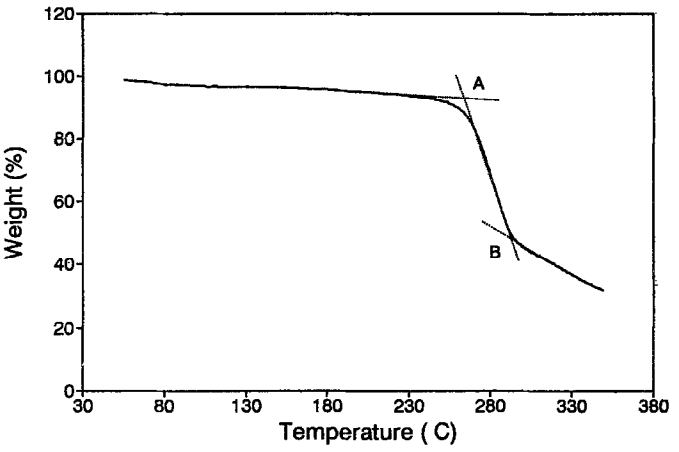

Figure 1. TGA thermogram of lyophilized cell powder containing 37% PHB.

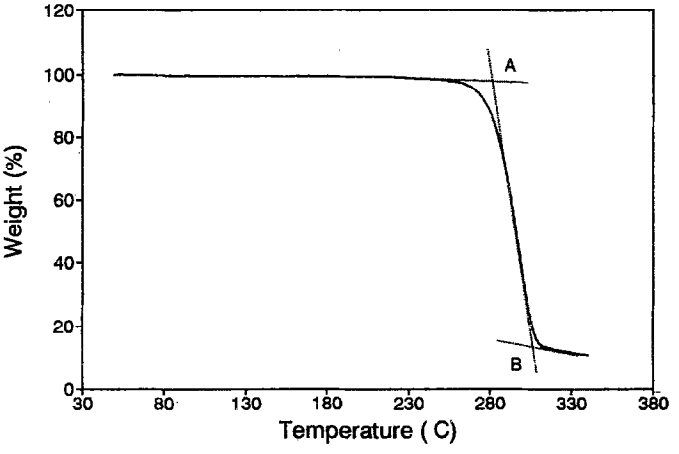

Figure 2. TGA thermogram of lyophilized cell powder containing 85% PHB.

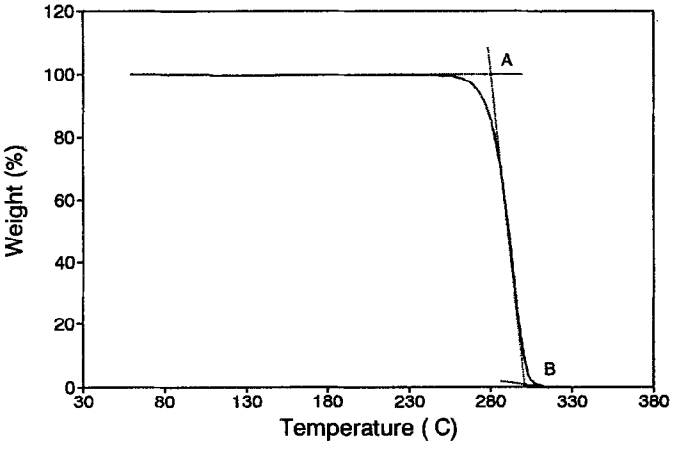

Figure 3. TGA thermogram of PHB/V copolymer (99% purity) purchased from Aldrich.

Table I. Samples used for PHB quantification by GC analysis and TG analysis.

Samples	TG analysis (%)	GC analysis (%)	Difference (%)
Lyophilized *A. eutrophus*	45	37	8
Lyophilized *A. eutrophus*	53	47	6
Lyophilized recombinant *E. coli*	65	59	6
Lyophilized *A. eutrophus*	67	62	5
Lyophilized *A. eutrophus*	86	85	1
PHB recovered by SDS treatment	95	95	0
PHB recovered by chloroform extraction	96.8	97	0.2
PHB recovered by dispersion treatment	98	98.3	0.3
PHB/V purchased from Aldrich	99	98.9	0.1

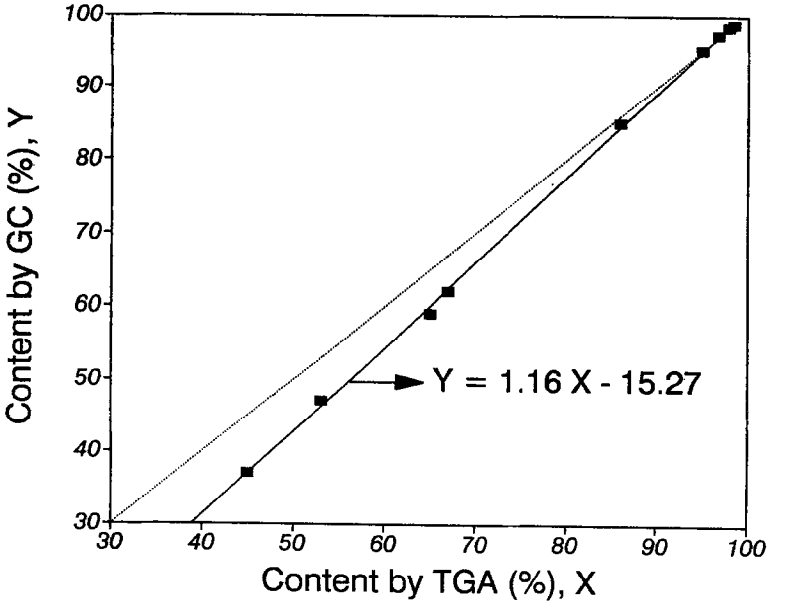

Figure 4. The correlation of PHB contents determined by TG analysis and GC analysis.

By using the above equation, PHB contents can be easily determined from TGA data with accuracy. Compared with GC method (Braunegg et al 1978), the proposed TGA method is a very simple, time-saving, and reproducible one. It took only 30 minutes for each sample, just loading a small amount of sample without any pretreatment steps. TGA data showed a good reproducibility with a negligible variance for triplicate analysis of each sample.

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