

Study of Microbial Polyhydroxyalkanoates Using Two-Dimensional Fourier-Transform Infrared Correlation Spectroscopy

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ABSTRACT: The premelting behavior of bacterially synthesized polyester poly(3-hydroxybutyrate-co-3-hydroxyhexanoate), abbreviated as P(HB-co-HHx), was investigated by two-dimensional Fourier-transform infrared (2D FTIR) correlation spectroscopy. The temperature-dependent dynamic spectra were measured over a temperature range of 25–300°C. We focused our study on the thermally induced intensity fluctuations of bands for C=O (1700–1760 cm⁻¹), C—H (2910–3010 cm⁻¹) and C—O—C groups (1220–1310 cm⁻¹) stretching vibrations. Changes of crystalline conformation due to the thermal perturbation could be detected by the intensity and location variations of those characteristic bands responding to the variations of dipole moments. 2D correlation analysis indicated that the appearance of fully amorphous component did not happen simultaneously with the disappearance of crystalline component, suggesting that there was an intermediate state between ordered crystalline and amorphous states in P(HB-co-HHx). © 2001 John Wiley & Sons, Inc. *J Appl Polym Sci* 82: 934–940, 2001

Key words: polyhydroxyalkanoates; PHA; poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); P(HB-co-HHx); 2D FTIR; premelting; biodegradable polymer

INTRODUCTION

Polyhydroxyalkanoates, abbreviated as PHA, are synthesized by many bacteria as inclusion bodies¹ [Fig. 1(A) and (B)], it has received a lot of attentions as biodegradable materials.^{1,2} Fourier-transform infrared spectroscopy has been applied to qualitatively study PHA in an uninvasive way.³ PHA

shows its strong characteristic band near 1730 cm⁻¹ due to the C=O stretching vibration, and the accompanying bands near 1280 cm⁻¹ and 1165 cm⁻¹ are attributed to the C—O—C group.³ The melting behavior of PHA has been very interesting for many PHA researchers, as it related to many potential applications for this polymer.^{1,2,4,5}

Two-dimensional infrared (2D IR) correlation spectroscopy was originally proposed by Noda in 1986.⁶ The synchronous and asynchronous 2D IR correlation spectra are constructed from perturbation-induced time-dependent fluctuations of IR signals. The appearance of synchronous and asynchronous peaks represents the similarity and dissimilarity of the basic trends of dynamic inten-

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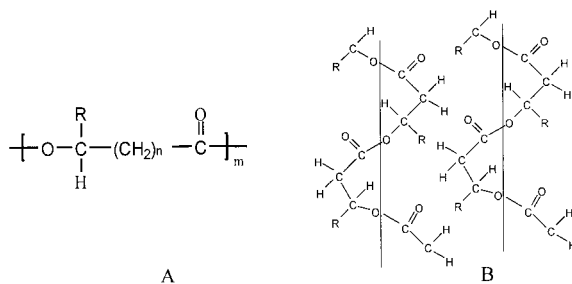


Figure 1 Molecular structure (A) and proposed structure model (B) of polyhydroxyalkanoate (PHA).

sity variations observed at two different wavenumber, respectively.⁷

Some features of the 2D FTIR spectra distinguish the advantages for 2D correlation analysis over the conventional one-dimensional FTIR spectroscopy. Autopeaks located at the diagonal positions in synchronous 2D spectra represent the extent of the dynamic variations of IR signals affected by the external perturbation.⁸ Cross-peaks in synchronous 2D spectra are especially useful in identifying the various inter- or intramolecular interactions because of the synchronism between the two variations at different wavenumbers.^{9,10} Asynchronous spectra are utilized to enhance the spectral resolution of highly overlapped bands, for if the IR intensities of overlapped bands vary in different ways, respectively, they could be clearly distinguished by the presence of asynchronous crosspeaks.⁷ Another powerful advantage of 2D analysis is that the sign of crosspeaks in synchronous and asynchronous spectra can provide the sequential relationship between the responses of different system components to the external stimulus.^{11,12}

In 1993, a more general 2D correlation spectroscopy was introduced, which enabled one to produce 2D correlation spectra from various intensity variations of spectra with arbitrary and complex functional forms as well as simple sinusoid waveform with a fixed frequency.¹³ The spectral intensity fluctuation may be induced by physical variables such as temperature, pressure, concentration, and composition.¹³ This led to a much wider range of applications of 2D correlation analysis. 2D approach has been very successful in studies of several research fields including the study of biological samples in recent years.^{14–16}

In this article, temperature-dependent melting behavior of P(HB-co-HHx) was studied. 2D FTIR correlation analysis was expected to detect the changes in the regions of C=O and C—H stretch-

ing mode, and be useful for investigating the structural variation during the heating process.

BACKGROUND

The generalized 2D correlation analysis of temperature-dependent FTIR spectra of PHBHHx samples is carried out as follows. For a series of temperature-dependent IR spectra $A(\nu, T)$ observed as a function of wave number ν and temperature T in a range between T_{\min} and T_{\max} , the orthogonal pair of 2D correlation spectra, $\Phi(\nu_1, \nu_2)$ and $\Psi(\nu_1, \nu_2)$, are given by

$$\begin{aligned} & \Phi(\nu_1, \nu_2) + i \Psi(\nu_1, \nu_2) \\ &= \frac{1}{\pi (T_{\max} - T_{\min})} \int_0^{\infty} \tilde{Y}_1(\omega) \cdot d\omega. \quad (1) \end{aligned}$$

The term $\tilde{Y}_1(\omega)$ is the temperature domain Fourier transform of the IR spectral intensity variations $A(\nu_1, T)$ observed at some chosen wave number ν_1 , and $\tilde{Y}_2^*(\omega)$ is the conjugate of the Fourier transform of $A(\nu_2, T)$ observed at a different wave number ν_2 . The IR spectral intensity variations analyzed by the 2D correlation technique is usually mean centered around the temperature-averaged intensity to emphasize the spectral changes selectively induced by the effect of temperature. The Fourier frequency ω represents the individual Fourier frequency component of the variation of $A(\nu, T)$ measured along the temperature axis T .¹³

The real part of the 2D correlation $\Phi(\nu_1, \nu_2)$ is called the synchronous spectrum. It represents the overall similarity or coordinated feature of the temperature-dependent variations in IR absorption intensities measured at two different wave numbers, ν_1 and ν_2 . The asynchronous spectrum $\Psi(\nu_1, \nu_2)$ or the imaginary part of the 2D correlation, on the other hand, represents the dissimilarity or independently varying trends of IR intensities as the temperature is changed. While the generalized 2D correlation analysis in this study is carried out for temperature variations, because of the historical close association between the 2D correlation spectroscopy and statistical time-series analysis, terminologies implying some level of temporal variations, like synchronous and asynchronous spectra, are still used.

The temperature-based 2D correlation spectra have the following useful properties. A synchro-

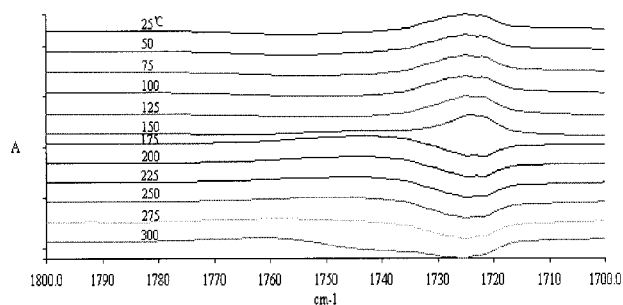


Figure 2 Dynamic IR spectra corresponding to the temperature-dependent FTIR spectra of P(HB-co-HHx) in the spectral region of 1800–1700 cm^{-1} over the temperature range of 25 to 300°C.

nous 2D IR correlation spectrum is symmetric with respect to the main diagonal line ($\nu_1 = \nu_2$). Autopeaks appearing at the diagonal position represent the overall extent of temperature-induced changes in IR signals. Crosspeaks at off-diagonal positions of a synchronous spectrum represent simultaneous changes of signals at two different wave numbers, suggesting a coupled or related origin of intensity variations. If the sign of a crosspeak is positive, the IR intensities at corresponding wave numbers are increasing or decreasing together. If negative, one is increasing while the other is decreasing. In an asynchronous 2D IR correlation spectrum, crosspeaks develop if the spectral intensity varies independently or out of phase with each other. The sign of a crosspeak becomes positive if the intensity changes at ν_1 occur before (i.e., at lower temperatures than) ν_2 , and negative if the intensity changes at ν_1 occur after (i.e., at higher temperatures than) ν_2 . This sequence is reversed if $\Phi(\nu_1, \nu_2) < 0$.¹¹

METHODS

P(HB-co-HHx) was accumulated in cells of strain *Aeromonas hydrophila* 4AK4, which was supplied by Procter & Gamble Co., Cincinnati, OH. Purified PHA was extracted by hot chloroform from freeze dry cells and precipitated by ethanol, followed by a vacuum dry process. NMR detection showed that the fraction of 3-hydroxyhexanoate in the sample of P(HB-co-HHx) was ca. 10%. Purified PHA film on the KRS-5 (cocrystal of TlBr and TlI) window for FTIR scan was prepared by chloroform solution casting.

The FTIR spectra were obtained using a FTIR Spectrum 2000 spectrometer (Perkin-Elmer,

USA) at the scanning condition of 50 scans, a resolution of 4 cm^{-1} . An optical path difference (OPD) velocity of 0.5 cm/s , a gain of 8, KRS-5 as the window material, a deuterium-triglycine-sulfate (DTGS) detector in the region of 4000 to 400 cm^{-1} equipped with a controlled heating device were used for these studies.

Differential scanning calorimetry (DSC-7, Perkin-Elmer) measurement was performed from –50 to 180°C at a heating rate of 20°C/min.

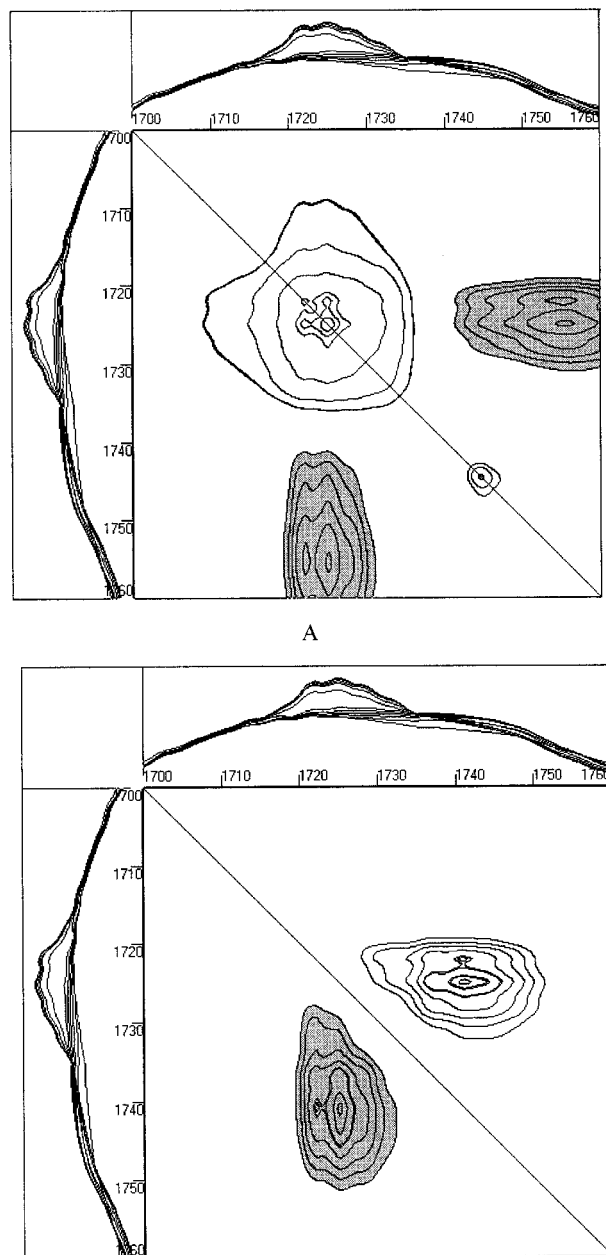


Figure 3 Synchronous (A) and asynchronous (B) correlation spectra of P(HB-co-HHx) in the region of 1700–1760 cm^{-1} .

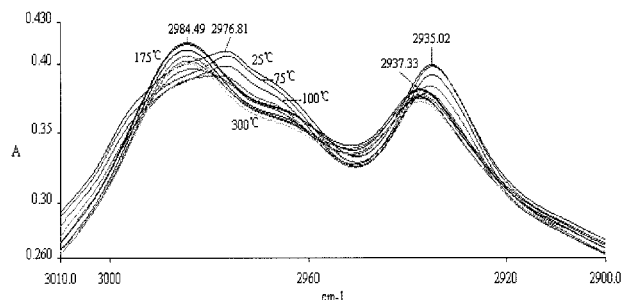


Figure 4 Temperature-dependent FTIR spectra in the stretching region of C—H groups over a temperature range from 25 to 300°C.

RESULTS AND DISCUSSION

Temperature-dependent FTIR spectra of bacterially synthesized P(HB-*co*-HHx) in the region of 1800–1700 cm^{-1} were obtained from 25 to 300°C, with an increment of 25°C. The heating processes were carried out from room temperature to temperatures above the polymer melting point. During the course of measurement, the polymer was converted from crystalline to amorphous phase. Band assignments could be made as follows: Band of C=O group around 1720 cm^{-1} was identified as the characteristic band of polyhydroxyalkanoates (PHA) including P(HB-*co*-HHx). To accentuate the changing features of one-dimensional spectra, different spectra termed dynamic spectra by Noda¹³ were constructed using all the average spectral data collected from the entire heating process (Fig. 2).

The synchronous and asynchronous 2D IR spectra in the spectral region between 1700 and 1760 cm^{-1} were shown in Figure 3(A) and (B), respectively. As mentioned above, the features in this region mainly revealed the characteristics of C=O stretching vibration in the copolymer. The autopeak located at 1745 cm^{-1} is expected to be the characteristic band of amorphous component of P(HB-*co*-HHx), compared to the band near 1720 cm^{-1} , which is due to the crystalline phase of the polymer [Fig. 3(A)]. Crosspeaks between the 1745 cm^{-1} band and the 1720 cm^{-1} band in the region were not found, and the intensity of the crossregion was negative, indicating that variations of these two bands occurred asynchronously and on opposite direction.

Asynchronous correlation spectrum could be more important to detect the sequential order of the occurrence of events induced by the external perturbation. The sequence of different spectral

intensity variations could be determined by the signs of the crosspeaks in both synchronous and asynchronous 2D correlation spectra.¹⁵ Figure 3(B) is the contour map of 2D asynchronous correlation spectrum in the same region of Figure 3(A). A pair of crosspeaks was observed at the spectral coordinates between 1720 and 1745 cm^{-1} . The signs of these crosspeaks pointed out that the intensity variation of the band at 1745 cm^{-1} related to the amorphous phase followed the

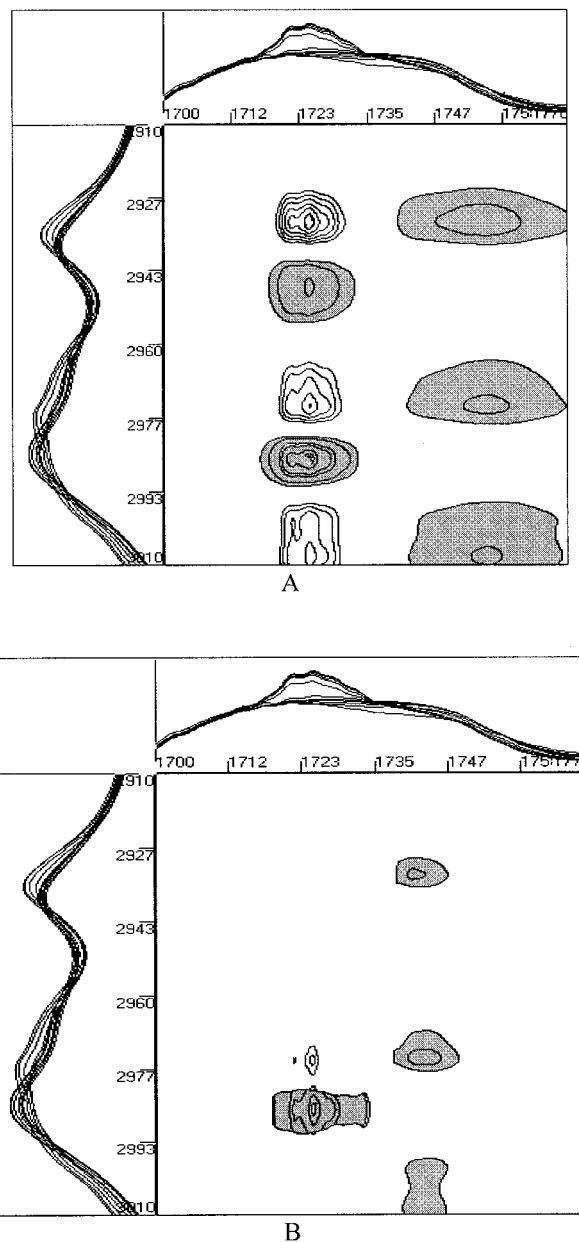


Figure 5 Synchronous (A) and asynchronous (B) 2D correlation spectra between the C=O and C—H stretching vibration regions.

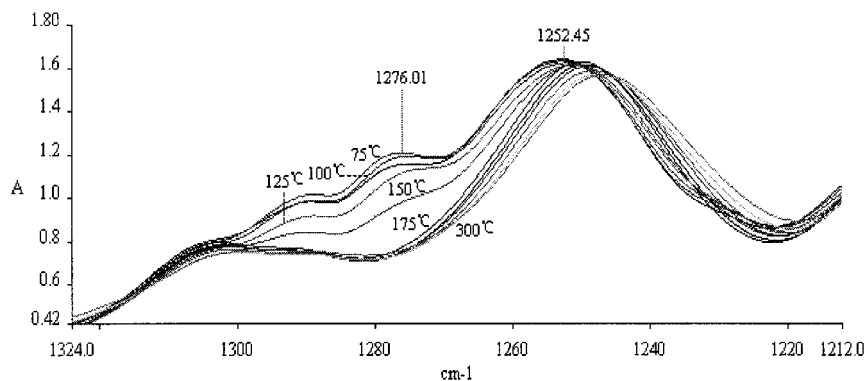


Figure 6 IR spectra in the region corresponding to the C—O—C group in temperature ranging from 25 to 300°C.

intensity decrease of the band at 1720 cm^{-1} corresponding to the ordered crystalline component. As the system temperature raised from 25 to 300°C, the IR intensity at 1720 cm^{-1} showed a decrease, indicating the highly ordered crystalline phase was diminishing. This disappearance of crystals did not result in the immediate appearance of fully amorphous liquid-like structure. The existence of much less ordered but not completely molten intermediate structure has been described as the premelting process in many polymers.¹² Following the intermediate state, the amorphous contribution increased at a higher temperature.

The shift of spectral bands to a higher wave number was closely related to the altered local orientation distribution of electric dipole-transition moments in the system, which was induced from the temperature-dependent variations of local environment of submolecular structures. The increase in temperature resulted in variations of dipole moments for the C=O and C—H stretching bands. In the crystalline structure of this polymer, the hydrogen atom of the CH₂ and CH₃ groups located closer to the oxygen atom of other molecules' C=O groups than that in the amorphous structure because of the highly ordered and probably helix configuration of the crystals [Fig. 1(B)], this can resemble the structural model proposed for poly- β -hydroxybutyrate or PHB.¹⁷ With the change of the microenvironment, the dipole moments for these bands were enhanced, resulting in increased wave numbers for the frequency value of the band maximums.

The structural basis mentioned above could be reasonably investigated by the synchronous and asynchronous spectra using 2D correlation analysis. The temperature-induced IR signal variations in C—H stretching region was shown in

Figure 4. The spectra could be divided into two sets, one was recorded from 25 to 125°C, and was represented by bands located at 2935 and 2976 cm^{-1} . Another included spectra recorded from 150 to 300°C in which the above peaks shifted to higher wave numbers, namely, 2937 and 2984 cm^{-1} . This shift could be attributed to the formation of amorphous structure. In the synchronous 2D correlation spectrum [Fig. 5(A)], the cross-peaks at the spectral coordinates between C—H and C=O spectral region showed the similarity of these two spectral variations. Positive peaks located at (1725 cm^{-1} , 2932 cm^{-1}), (1725 cm^{-1} , 2974 cm^{-1}), and (1746 cm^{-1} , 2985 cm^{-1}), respectively, indicated that the C—H groups and C=O groups vibrated with coupling, and thus the dipole moments could vary simultaneously. More information obtained from the asynchronous spectrum could prove the existence of a premelting process [Fig. 5(B)]. Three negative crosspeaks including (1725 cm^{-1} , 2985 cm^{-1}), (1741 cm^{-1} , 2932 cm^{-1}), and (1741 cm^{-1} , 2974 cm^{-1}) and their positive intensity in synchronous corresponding region indicated that the crystalline phase in the sample disappeared first. Apparently, only after going through the intermediate state, the fully amorphous phase appeared at a higher temperature.

The thermally induced intensity variations of P(HB-co-HHx) in the range from 1310 to 1220 cm^{-1} could be observed in Figure 6. The bands in this spectral range were assigned to be the characteristic band of C—O—C group in the polyester. The decreasing IR absorption at 1280 cm^{-1} was attributed to the phase transition (crystalline-amorphous) of this polymer and the disappearance of highly ordered and probably helix confor-

mation of polyester backbone as the temperature was raised above the melting point.

The intensity of IR signal at 1280 cm^{-1} corresponding to the helix conformation was simultaneously varying with the decrease of band at 1720 cm^{-1} , which was revealed by the positive synchronous cross peak (1725 cm^{-1} , 1277 cm^{-1}) constructed from dynamic spectra in two different spectral ranges [Fig. 7(A)]. In the asynchronous 2D correlation spectrum [Fig. 7(B)] the intense positive crosspeak at spectral coordinates between 1740 cm^{-1} and 1277 cm^{-1} indicated that the helix conformation of crystals disappeared before the appearance of fully amorphous liquid like structure. This phenomenon confirmed the existence of premelting process. In other words, an intermediate state to amorphous structure was identified by the asynchronicity of 2D correlation analysis [Fig. 7(B)].

Differential scanning calorimetry (DSC) has been appended to verify the premelting behavior of P(HB-co-HHx). The DSC measurement of the sample between -50 to 180°C showed a small and wide endothermic pre melting peak around 60°C (pointed out by the arrow) associated to the motion of the polymer chain (Fig. 8). The melting point of this sample was determined at 121.5°C , which was revealed by the intense endothermic peak.

SUMMARY

In this article, the bacterially synthesized biopolymers P(HB-co-HHx) have been studied using generalized 2D FTIR correlation spectroscopy. The existence of so-called premelting process, which could not be investigated by the conventional one-dimensional FTIR spectroscopy, has successfully been detected. Bands in the regions of $1700\text{--}1760\text{ cm}^{-1}$ and $2910\text{--}3010\text{ cm}^{-1}$ were attributed to the stretching vibration of C=O and C—H groups, respectively. The electric dipole-transition moments for these two groups were enhanced as the polymer changed from crystalline to amorphous states, resulting in the shift of peaks of the bands to higher wave numbers. IR absorption approximately at 1280 cm^{-1} was assigned to the helix conformation of polymer chain in crystals, indicating the crystallinity in the system.

2D correlation spectra revealed the asynchronous relationship between disappearance of the crystals and appearance of the amorphous structure. Dynamic IR signals corresponding to the

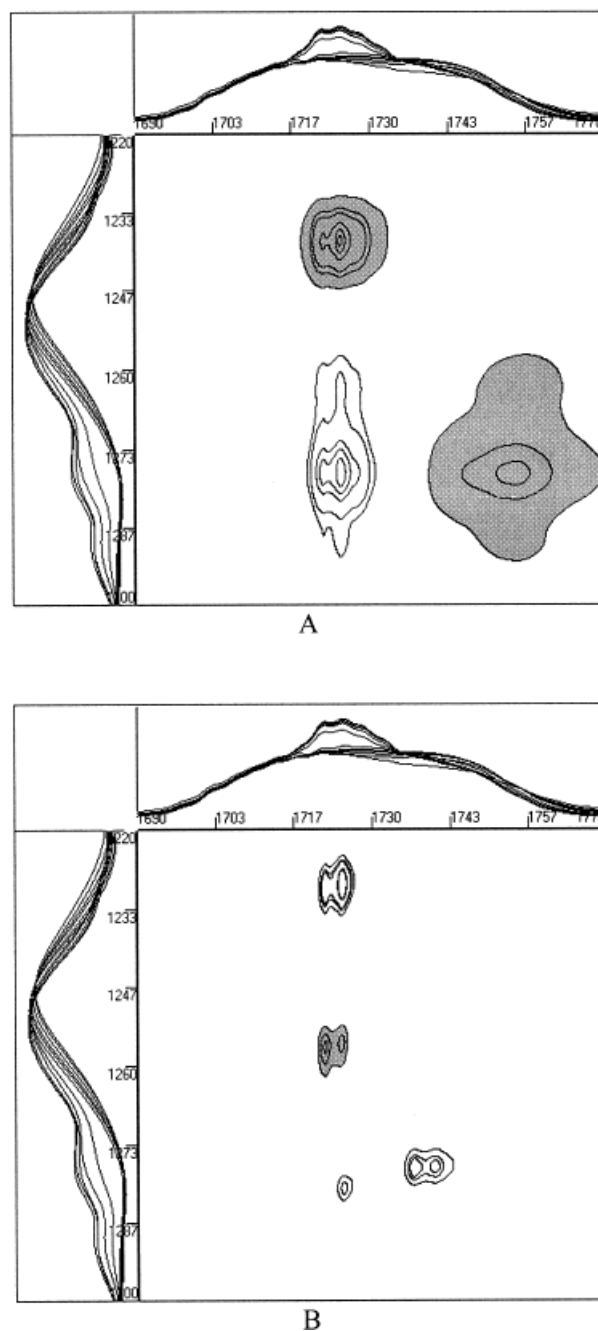


Figure 7 The synchronous (A) and asynchronous (B) 2D correlation spectra of P(HB-co-HHx) between the two regions of C=O and C—O—C groups.

crystalline and amorphous C=O stretching mode formed crosspeaks in asynchronous but not in synchronous 2D spectra, indicating the existence of intermediate state. C—H stretching vibration in the crystals and in the amorphous structure varied synchronously with the variation of band at 1720 and 1745 cm^{-1} , respectively, and the

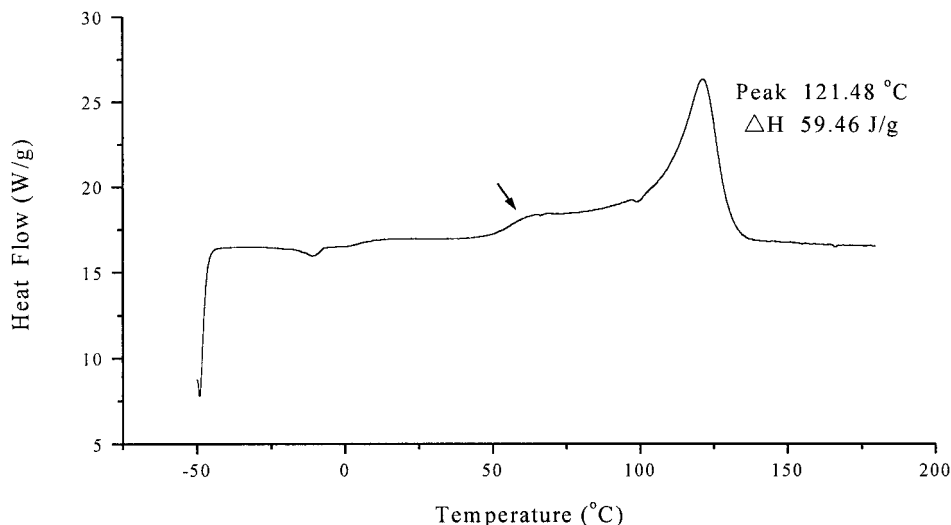


Figure 8 Differential scanning calorimetry (DSC) scan of P(HB-co-HHx) from -50 to 200°C .

asynchronous crosspeaks constructed from crystalline C—H groups and amorphous C=O groups as well as from amorphous C—H groups and crystalline C=O groups proved the interaction of dipole moments between these two groups, conforming to the characteristic of submolecular structures. The helix structure of polymer chain in the crystals diminished simultaneously with the gradual decrease of the crystalline C=O band, revealed by the synchronous crosspeak at 1280 and 1720 cm^{-1} wave numbers and the asynchronous crosspeak at 1280 and 1745 cm^{-1} .

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