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Thermal evaluation of transgenic cotton containing polyhydroxybutyrate

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Abstract

Bacterial genes responsible for the synthesis of the aliphatic polyester compound, poly-D-(-)-3- hydroxybutrate (PHB) were introduced into cotton (*Gossypium hirsutum* L. Cv DP50) through particle bombardment. The resulting transgenic cotton contained PHB in the cotton fiber lumen. The presence of PHB granules in transgenic fibers resulted in measurable changes in thermal properties. The fibers exhibited better insulating characteristics than natural cotton. The rate of heat uptake was higher and cooling slower in transgenic fibers. Thus, the transgenic fibers had higher heat capacity and lower thermal conductivity. These results demonstrate that thermal properties of fibers can be enhanced by genetic engineering of cotton. Based on our preliminary studies of mixtures of PHB and cellulose powder, it appears that the biological composite of cellulose, PHB and, probably, bound water are necessary for the enhanced heat uptake of transgenic fibers. The heat uptake may be influenced by interactions of PHB within the fiber lumen. © 1998 Elsevier Science B.V.

Keywords: DSC; Genetic engineering; Heat uptake; Particle bombardment; Polyhydroxybutyrate; TG; Transgenic cotton

1. Introduction

Natural cotton in fiber and fabric form has long been the material of choice for manufactured clothing for providing intrinsic comfort and aesthetic appearance. However, significant advances in the quality of synthetic fibers as well as manufacturing innovations may erode the market share of cotton. Thus, overall improvements in textile quality and product diversification are important for the cotton industry. Superior dye binding, strength, thermal insulation, and length are valuable attributes in cotton textile applications [1]. In the past, improvements in cotton qualities and yield have been achieved through plant breeding [2]. However, due to the limitations of compatibility between varieties as well as the availability of desirable traits in existing varieties, new technologies are warranted for further improvements. This paper describes attempts to introduce traits from other sources into cotton through genetic engineering.

Cotton fibers are single cells that are formed by the expansion of epidermal cells of the ovule. A large vacuole (lumen) is present within the cell giving it an appearance of a hollow tube. Two major cell walls surround the lumen, the outer thin primary and the inner thick secondary wall. Cotton fiber is a biological composite of cellulose, small quantities of hemicellu-

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lose, pectins and proteins [3]. The major constituent of mature, unprocessed cotton is cellulose (89%), while all other components are minor (water 7%; protein 0.9%, wax, 0.7%; pectic and other organic acids, 1.4%, [3–5]). Cellulose is composed of several thousand units of β-(1–4)-D-glucopyranose units that form microfibrils which, in turn, are arranged in crystalline micelles interspersed with amorphous regions. The intrinsic properties of the constituents, as well as their interactions with one another, and their microstructure within the fiber play important roles in fiber attributes.

One of the genetic strategies, we have designed, to modify cotton fiber alters the genetic makeup of the plant so as to produce a new polymer in the fiber [6]. This may be accomplished by inserting gene encoding enzymes, necessary for the polymer synthesis, into cotton chromosomes. Some of the resulting transgenic plants may contain the inserted genes and may produce the polymer in the fiber during their development. These transgenic fibers may combine properties of cellulose and the new polymer. In order to test this concept, genes from *Alcaligenes eutrophus* which are responsible for the synthesis of polyhydroxybutyrate (PHB), an archetype polyhydroxyalkanoate (PHA), were modified and introduced into cotton [6].

PHAs are energy reserve compounds found in a variety of living organisms, including bacteria [7,8]. They are hydrophobic, biodegradable, thermoplastic compounds that are chemically and osmotically inert. The stereoregular group in the molecule can range from a β-methyl to a β-dodecanoate and their material properties vary with the functional groups. The PHB is synthesized from acetyl CoA through a three-enzyme pathway [7,8]. Acetyl CoA is a central molecule in the energy generation and biosynthesis of living organisms. It carrier an activated acetyl group and is a heat-stable co-factor which is required in many enzymatic reactions. It has been shown that PHB can be synthesized in plants from acetyl CoA by inserting two genes, acetoacetyl CoA reductase and PHA synthase [6,9].

We generated transgenic cotton by introducing two *A. eutrophus* genes corresponding to NADPH-dependent acetoacetyl CoA reductase and PHA synthase. The transgenic fibers contained granules of PHB in their lumen [6]. The identity of PHB in fibers was confirmed by HPLC analysis after the conversion of PHB to crotonic acid, by GC-MS analysis of ethyl ester derivatives and by electron microscopy studies [6,10,11]. These fibers containing PHB offer a unique opportunity to study the combined physical and chemical attributes of cellulose and PHB. Our preliminary investigations of these fibers indicated that the transgenic fibers differ in their properties from the control fibers [6]. Here, we detail an evaluation of their thermal properties.

2. Experimental

Experimental details of gene cloning, DNA manipulations and analysis of transgenic fibers are reported elsewhere [6]. Transgenic cotton was generated by the method of particle bombardment [12]. The technique employs an electric discharge gun, Accell^{R} to propel gold particles (1–3 μm) coated with DNA into cotton meristem cells [12]. The transformed plants are identified by histochemical testing of a marker gene activity, β-glucuronidase (GUS, [13]). Test samples were obtained from transgenic and control cotton plants grown in the greenhouse under identical conditions. PHB in fiber lumen was detected by transmission electron microscopy and epifluorescence microscopy. It was extracted from fibers with chloroform and analyzed by HPLC after acid hydrolysis [6,10]. Further confirmation of PHB was obtained by gas chromatography, followed by mass spectral analysis of ethyl ester derivatives [11]. Nearly 66% of the PHB granules in fibers were found to be in the molecular weight range of $0.6–1.8\times10^{6}$ Da by gel permeation HPLC [6].

Mature fibers were collected from plants and dried at 35°C for 48 h. A portion of the fiber was ground to a fine powder in a Spex freezer mill in liquid nitrogen. They were compared to microgranular (200 μm) cotton cellulose powder, that was obtained from Whatman. Other fibers were processed and spun into yarn by miniature spinning (Starlab VY-5 direct silver-to-yarn spinning frame: Starlab, Knoxville, TN) and knitted into cloth by a knitting machine (International Center for Textile Research & Development, Lubbock, TX). Unbleached and undyed fabric or fibers were used for thermal property measurements. Synthetic fabric made up of Thinsulate^{TM} material (3 M, St. Paul, MN) was obtained from 3 M.

2.1. Thermal analysis

The transgenic fiber or fabric samples along with samples of regular cotton were subjected to DSC and TG (TA Instruments model 910S and 951, respectively, New Castle, DE) analysis for comparison of their heat flow, thermal conductivity, specific heat and weight loss characteristics. Finally, thermal resistance values were measured and compared with those of a commercial Thinsulate™ product. All samples were run in open aluminum pans in static air at 10°C/min heating rate. Standard operating and measurement procedures [14] were followed for all thermal experiments, except that a modified DSC cell was used for thermal conductivity measurement using the following equation:

$$\lambda = qL/A(t_1 - t_2)$$

where λ is the thermal conductivity (W/m K), q the steady-state heat flow (W), L the heat flow path (m), A the area of sample (m^2), and $(t_1 - t_2)$ the temperature difference between the top and bottom of sample.

For the clarification of the results, cotton fiber should be considered as composed of 89% cellulose [3,15], whereas Thinsulate™ contains polyester materials in multiscrim-layered construction.

Heat uptake measurements by DSC involved thermal equilibration of a pair of empty sample and reference pans in the cell at the ambient temperature and zeroing out any heat flow value due to their mismatch prior to introduction of the sample into the equilibrated cell.

Total heat uptake was determined by integrating heat flow between the start of the experiment and the onset of decomposition of the sample. The DSC cell was calibrated by running an indium melt and entering a correction for both temperature and enthalpy values into the analysis software for each run. A general analysis software by TA Instruments was used for the integration. All samples were weighed by an analytical balance (10 μg accuracy).

Samples were conditioned at 50% RH under controlled laboratory humidity as measured by a psychrometer as well as by a standard humidity gage. Samples were conditioned at 90% RH in a temperature/humidity chamber (ELCONAP, Newark, NJ) containing a salt solution.

3. Results and discussion

Several lines of transgenic cotton that produce PHB were recovered from the transformation experiments [6]. Transmission electron microscopy studies of fibers revealed that PHB was present in granular form within the fiber lumen [6]. PHB levels in various cotton transformants were measured by analysis of crotonic acid and are shown in Table 1. Transgenic and control cotton were subjected to quantitative heat-flow measurements by DSC (Table 1). Two commercial cultivars, DP-50 and SG-125 were used as controls and showed heat uptake in the 618.8–623.5 J/g range. Repeat measurements of control DP-50 showed little variations and the small differences found may arise from differences in heat diffusion through different packing densities of ''loft'' samples. We also tested the heat uptake of bacterial cellulose. *Acetobacter xylinum* is a gram-negative bacterium that produces cellulose ribbons which intertwine to form pellicles. The cellulose produced by *A. xylinum* is pure and is not mixed with other polysaccharides or proteins as in cotton fibers [16,17]. The heat uptake of bacterial cellulose was 621.8 J/g, which is very similar to cotton fiber, indicating cellulose as the major contributor of heat uptake in cotton fibers (Table 1). Another cotton variety tested for heat uptake was green lint. The green lint is an experimental colored cotton variety and is known to have more wax in its fibers than DP-50 (~2% in green lint vs. ~0.6% in DP-50: R.A. Taylor, personal communication). Thus, the small increase (620.3 vs. 629.4 J/g) in heat uptake in green lint fibers may be due to the higher wax content which contributes to additional heat absorption due to melting (Table 1).

The heat uptake values for transgenic cotton, #7148-D, #6888-7, #8801, #12334, #D-5 and #7148-C were in the 627.5–695.3 J/g range (Table 1). Thus, heat uptakes of all transgenic fibers measured were higher than those for the controls. Typical DSC profiles of sample #8801 and SG-125 are shown in Fig. 1. The onset of the decomposition temperature for the transgenic samples was fractionally lower (Fig. 1). Though generally increased PHB levels also resulted in higher heat uptake values, the precise relationship cannot be determined at this time due to the limited number of samples as well as limited

Table 1
Heat uptake values of control and transgenic fibers. The PHB content in transgenic fibers was determined by extracting PHB by chloroform treatment of mature fibers after digestion with cellulase [4]. The extract was treated with sulfuric acid at 90°C for 45 min before being subjected to HPLC analysis on Aminex HPX-87H column using a Beckman System gold gradient HPLC system with variable wavelength detector. Quantitation was done by system Gold computer software. Heat uptake values were measured by DSC. Sample sizes were in the 9–19 mg range

Sample	Sample size/ mg	PHB/ μg/g	Heat uptake/ J/g	Average	%Heat uptake difference to DP-50
DP-50	(1) 10.06	—	(1) 618.8		—
	(2) 12.26	—	(2) 620.1		—
	(3) 11.38	—	(3) 623.5	620.3	—
	(4) 12.26	—	(4) 618.7		—
SG-125 (control)	(1) 11.31	—	(1) 622.0		—
	(2) 10.20	—	(2) 623.6	622.7	—
	(3) 10.41	—	(3) 622.6		—
Greem cotton (control)	(1) 9.99	—	(1) 632.2		+1.5%
	(2) 9.91	—	(2) 626.6	629.4	
#12334 (transgenic)	(1) 11.29	437	(1) 642.7	643.8	+3.8%
	(2) 9.21		(2) 644.8		
#7148 (transgenic)	(1) 11.20		(1) 695.3	692.7	+11.7%
	(2) 12.22	3440	(2) 692.0		
	(3) 10.10		(3) 690.7		
#6888-7 (transgenic)	14.21	30	627.5		+12%
#8801 (transgenic)	(1) 13.21	423	(1) 642.3	643.4	+3.7%
	(2) 10.18		(2) 644.4		
#D-5 (transgenic)	(1) 10.42	800	(1) 641.7	645.3	+4.0%
	(2) 11.00		(2) 648.8		
#7148-C (transgenic)	10.97	330	629.6		+1.5%
Bacterial PHB (Sigma)	10.61	100%	No intrinsic heat uptake		
Cellulose powder (Sigma)	10.57	—	360.9		
Acetobacter cellulose (control)	(1) 10.11	—	621.8	623.1	
	(2) 10.39	—	(2) 624.1		
DP-50 (granular)	(2) 11.95	—	614.4		
#7148-D granular (transgenic)	7.90	—	683.4		+10.2%
DP-50 Fabric (control)	(1) 9.80	—	617.8	616.0	
	(2) 10.60	—	(2) 614.2		
#7148-D Fabric (transgenic)	(1) 18.29	3440	(1) 695.4	694.5	+12.0%
	(2) 17.70		(2) 693.5		

sample size available. At low levels of PHB, we do not expect the measurements to be precise (Table 1).

Several measurements were conducted to evaluate the role of PHB in the higher heat uptake values of transgenic cotton. We attempted to asses the effect of increasing quantities of PHB, in cotton–PHB mixtures. DP-50 fiber was ground to granular form and mixed with PHB in the ratios of 1 : 1 or 1 : 5 (w/w) and subjected to DSC. Bacterial PHB alone showed no intrinsic heat uptake, whereas the heat uptake value of granular cotton was very similar to that of whole fiber (Fig. 2). The heat uptake values of DP-50:PHB at 1 : 1 or 1 : 5 ratio, were substantially decreased (400.3 and 293.0 J/g, respectively). As shown in Fig. 2, the 1 : 5 mixture showed distinct characteristics of individual components which are very different from the DSC profile of transgenic cotton. A characteristic transition of PHB, shown in Fig. 2, appears in all mixtures

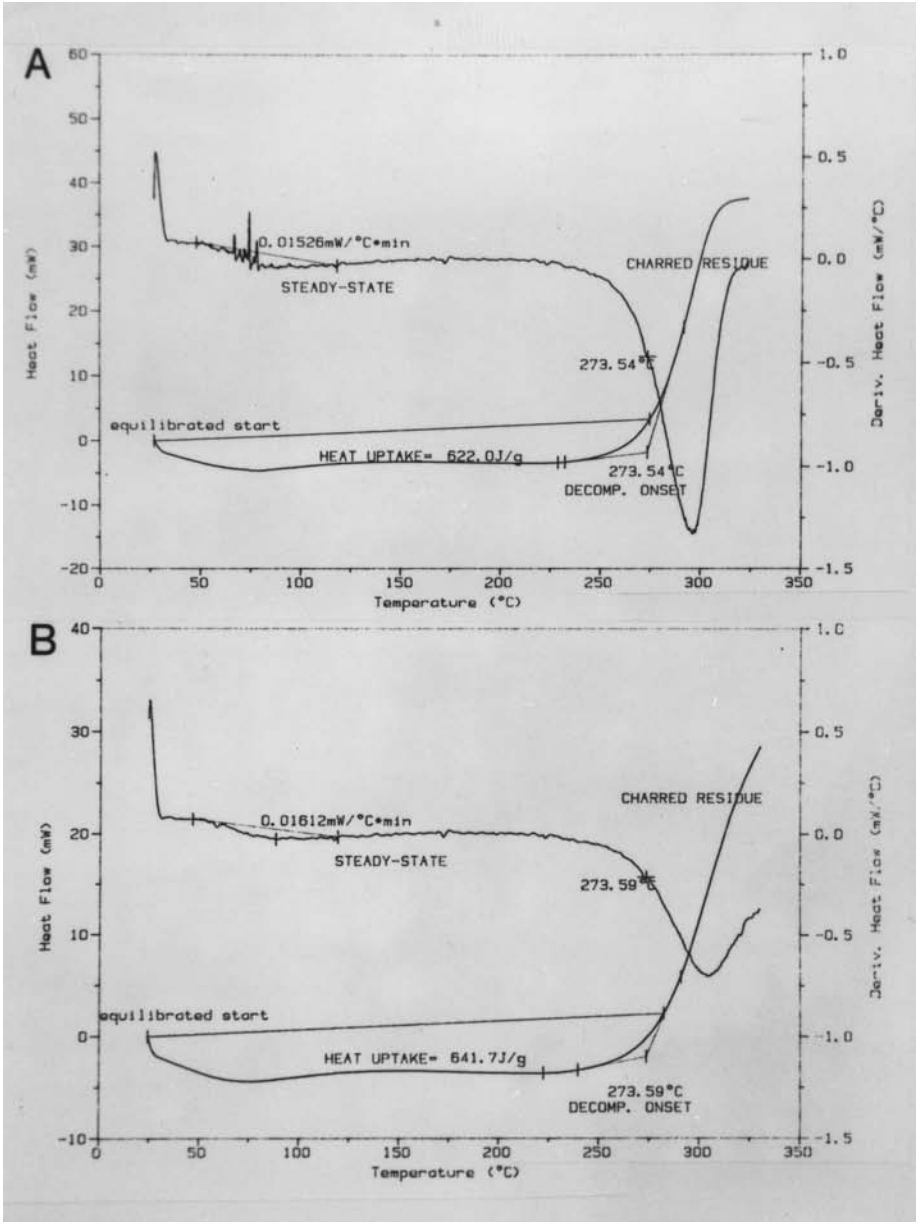

Fig. 1. Heat uptake measurements of control and transgenic cotton by DSC.

studied, indicating that the melt of PHB in a mechanical mixture influences the onset of decomposition of the matrix.

X-ray diffraction and high resolution NMR spectroscopy studies showed that the PHB granules within bacterial cells were amorphous in nature [18,19], whereas PHB when extracted from the cells showed a 60–70% crystallinity [20]. It has been postulated that intracellular water acts as a plasticizer of PHB [19]. Thus, we assume the PHB within fiber lumen to be in

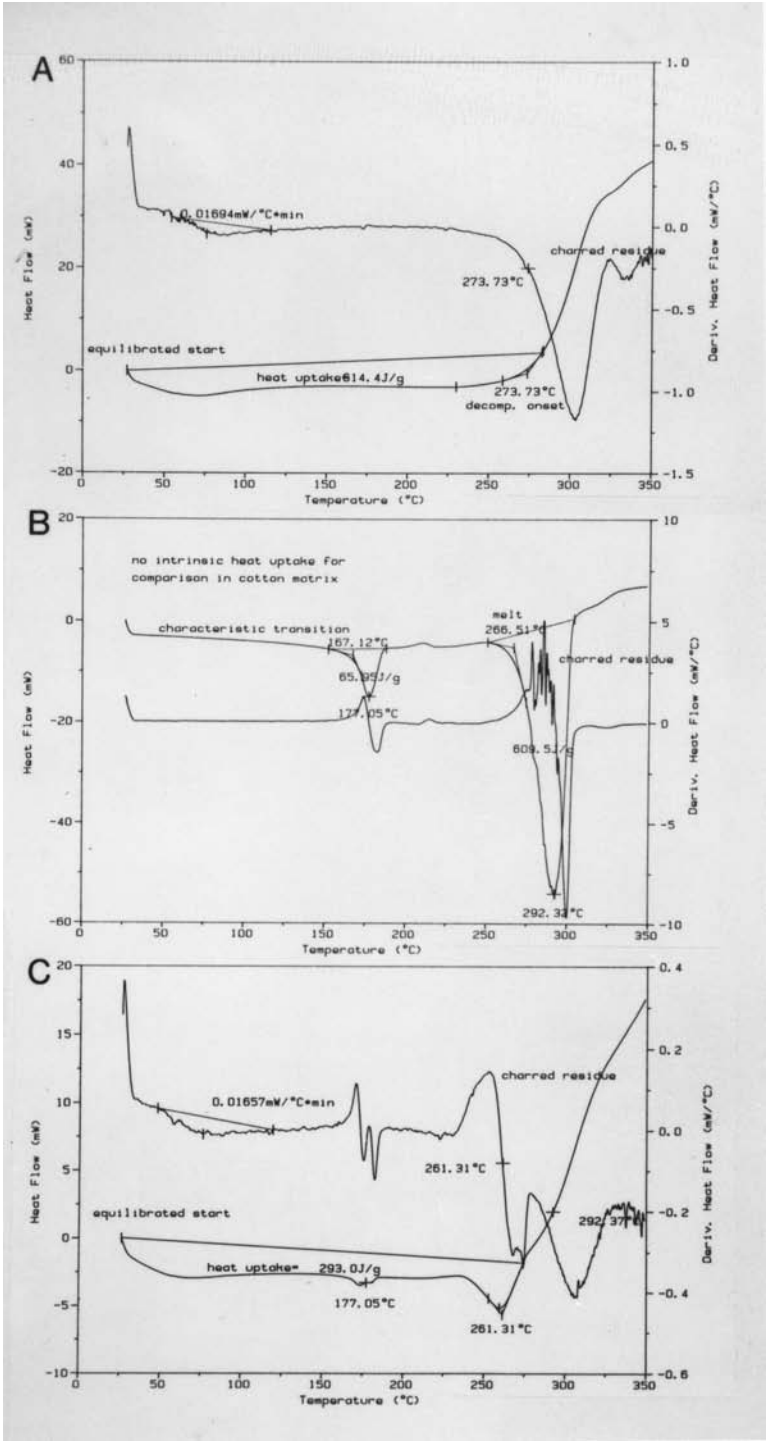

Fig. 2. DSC profiles of cotton, PHB and PHB/cotton fiber granules mixture.

the amorphous state, probably plasticized by water. Extracted bacterial PHB that was mixed with ground cotton, on the other hand, may be crystalline in nature. Thus, one of the factors affecting the decreased heat uptake upon mixing PHB with ground cotton may be the different states of the PHBs. Therefore, the precise role of PHB in a mixture cannot be calculated. From these we conclude that the quantitative role of PHB in transgenic cotton heat uptake cannot be measured by in-vitro physical mixtures, but only by measurement of intact biological samples. We further speculate that the interaction of PHB, cellulose and the water molecules in transgenic fiber collectively influenced its thermal properties. The precise relationship of PHB to increased thermal properties in fiber can be established as fibers with greater PHB content are developed.

PHB also depressed the heat uptake values of finely powdered (200 μm) cellulose. Cellulose powder alone had significantly lower heat uptake (360.95 J/g) than cotton fiber. The change in crystallinity may account for this decrease. When cellulose powder was mixed with PHB (1 : 1 w/w), the heat uptake further decreased (351.1 vs. 360.9 J/g).

We also investigated the role of moisture on the heat uptake of transgenic fibers containing PHB. Transgenic sample #D-5 and control SG-125 were conditioned at 12, 50 and 90% RH and heat uptake was measured. These results are presented in Fig. 3. Increase in moisture content resulted in increased heat uptake. The increase in relative heat uptake was similar for both control and transgenic fibers (∼3% at 90% RH). The increased heat uptake may be explained by the additional heat required for the vaporization of moisture and a discrepancy in sample weight due to moisture. These studies show that transgenic fibers do not have any additional advantage over conventional fibers at various humidity levels.

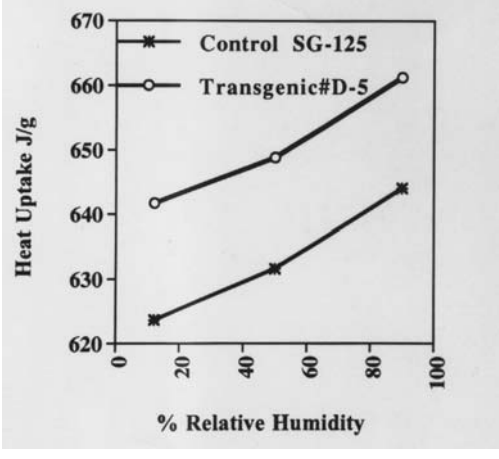

Fig. 3. Heat uptake (J/g) for samples exposed to different RH. Control SG-125 and transgenic #7148-5 were conditioned at 12, 50 and 90%RH.

Table 2
Comparison of thermal conductivity values at 36°C and specific heat values at 36°C and 60°C for control and transgenic cotton

Sample	Thermal conductivity/ (W/m K)	Specific heat/ 36°C (J/g°C)	Specific heat/ 60°C (J/g°C)
DP-50	0.283	1.862	2.692
#7148-D	0.264	2.022	3.889

The difference in the onset of decomposition observed in DSC measurements was confirmed by TG analysis. TG analysis showed the onset of decomposition to be somewhat higher for regular cotton than for transgenic cotton. Weight loss at all stages was higher and faster for transgenic cotton except at the last stage, which is governed by mass action law. These are shown in Fig. 4. The indication by DSC of initial moisture release, centered at about the same temperature for both samples, is supported by the TG results. The rate of release is slower for the transgenic sample, suggesting tighter moisture bonding for transgenic cotton.

The relative heat transmission properties were determined by thermal conductivity measurements. Typical values are given in Table 2 and details are shown in Fig. 5. These values indicate that the transgenic sample cools down more slowly than regular cotton and is therefore more insulating. The same conclusion was derived from the specific heat values given in Table 2 and plotted in Fig. 6.

Any product application of transgenic fibers containing PHB depends on its degree of improvements in the thermal attributes. Therefore, it is necessary to

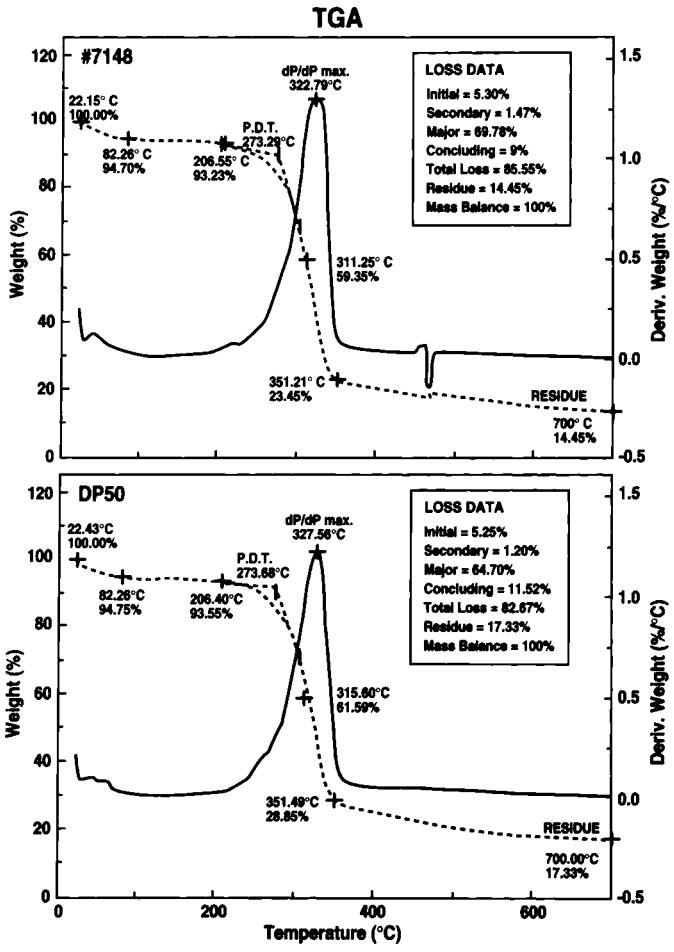

Fig. 4. Onset of decomposition and weight loss determined by TG. Sample size was 17.070 mg for DP-50 and 16.973 mg for #7148-D.

measure the thermal properties of fabrics made from transgenic fibers. Heat uptake values for spun fabrics from each type of cotton, given in Table 1, match those obtained for fibers, suggesting no loss in thermal property when cotton is made into fabric. This result is significant in terms of product applications. In order to compare the insulation value of synthetic and transgenic materials, thermal conductance measurements were carried out. Commercial Thinsulate™ products are characterized by their high thermal resistance (R). The measured values include both, loft and compressible insulation for constructed materials. We compared the loft thermal resistance of commercial Thinsulate™ with those of regular cotton and transgenic cotton. The method involved measur-

Table 3
Comparative thermal resistance (R) values for Thinsulate™, control cotton and transgenic cotton

Sample	R-value/ (h.ft^2·°F)/BTU
Thinsulate™ UDS70	1.01
DP-50	0.047
7148	0.051

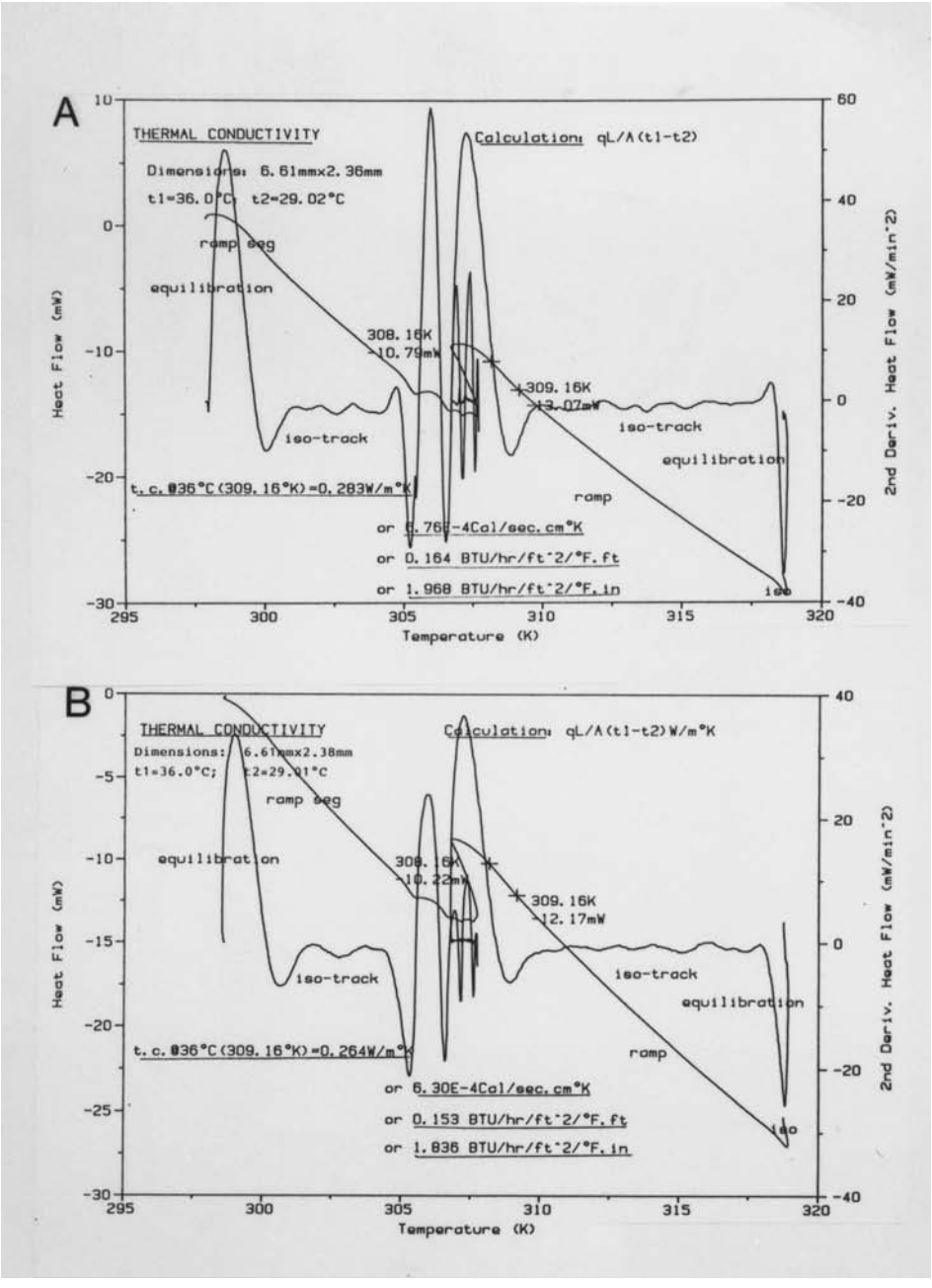

Fig. 5. Details of thermal conductivity measurements by DSC.

ing the conductance per ASTM C-177 and taking the reciprocal for *R*-value calculation. The calculated values are given in Table 3. Although cotton does not have the high insulation of Thinsulate™, transgenic cotton has greater insulating capacity than regular cotton.

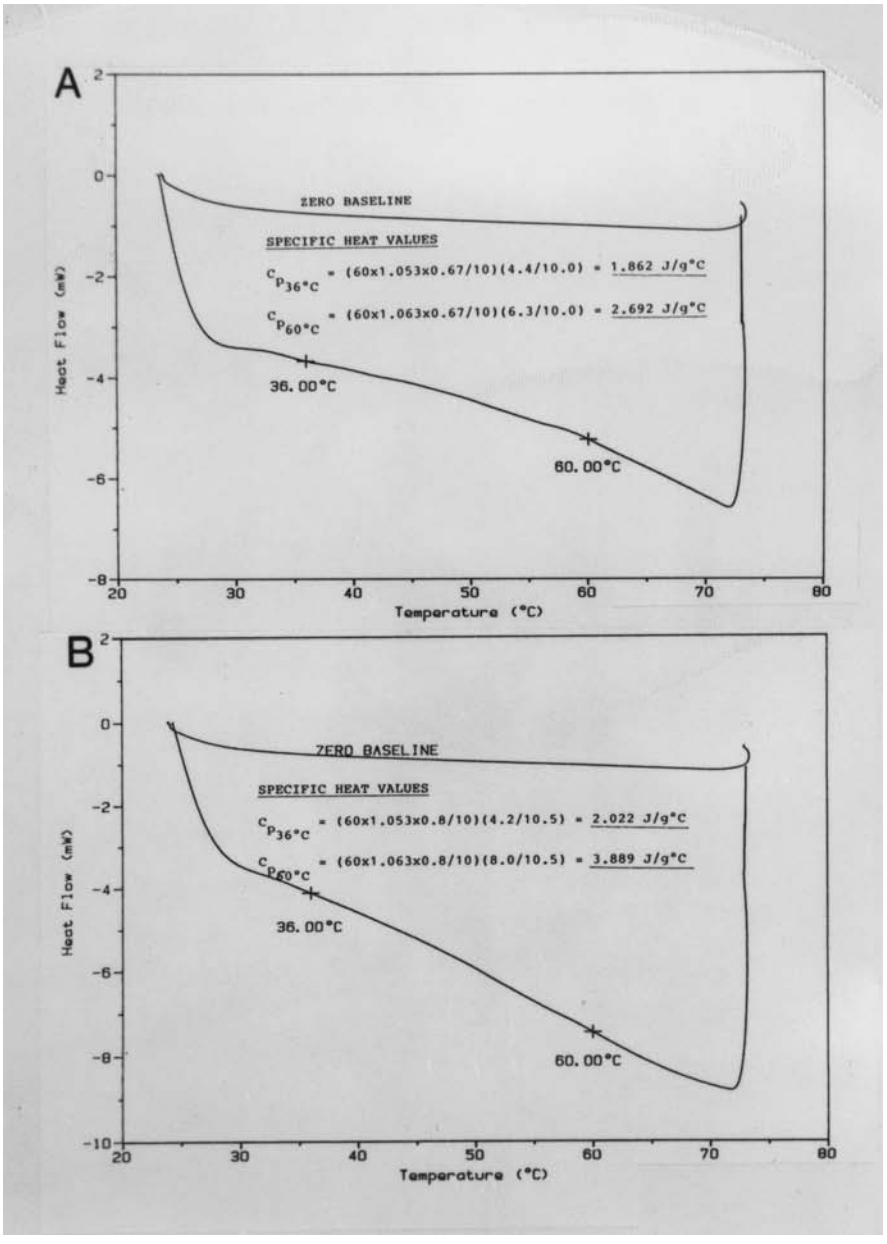

Fig. 6. Specific heat measurement by DSC.

4. Conclusion

Thermal evaluation of transgenic cotton produced through genetic engineering shows distinct differences with those of controls, particularly with regard to insulation characteristics. The changes in overall thermal properties are, however, relatively small, a fact that can be expected from the small amount of PHB in fibers (maximum 0.34% fiber weight). It is likely that a several-fold increase in PHB systhesis is required for product applications. Nevertheless, the positive change in fiber quality demonstrated here indicate

the potential of this technology. Thus, the initial studies demonstrate that thermal parameters are influenced by the net interaction of PHB and cellulose. The interaction of water molecules with those of PHB may also influence the heat absorption properties. Thus, the present study demonstrates that the addition of new biopolymers into cotton fiber influences its chemical and physical properties.

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