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CELLULAR AND MOLECULAR APPROACHES TO POLYMER SYNTHESIS BY BACTERIA

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OBJECTIVE: The objective of this project was a multi-disciplinary study of the biosynthesis, regulation and materials properties of microbial poly-ß-hydroxyalkanoates (PHAs).

APPROACH: Three principal areas were investigated, including: (1) the production of new poly-ß-hydroxyalkanoates, PHAs, (2) the structure and function of proteins associated with the PHA inclusion bodies in the cells, and (3) the enzymatic degradation of PHAs.

In our overall studies on the production of new PHAs, we investigated the growth rates, polymer yields and polymer compositions obtained with two anaerobic, phototrophic bacteria, *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*, and one aerobic bacterium, *Pseudomonas oleovorans*. The first two produced PHAs with short chain ß-alkyl substituents (primarily methyl and ethyl) while the last produced PHAs with long alkyl chains (C_4 - C_8) at the ß-position. *P. oleovorans* was also much more versatile in the types of polyesters it could produce, especially in its ability to produce PHAs containing ß-substituents with terminal functional groups, including ester, phenyl, cyano and vinyl groups and bromine atoms. However, *R. sphaeroides* showed a capacity of accumulating PHA in high quantities, and deuterated PHAs were formed by this bacterium when it was grown on d_3-acetate. Similarly, *R. rubrum* was able to incorporate units with varying amounts of the methyl- and ethyl-containing units, and the production of copolymers appeared to be the rule rather than the exception for this bacterium.

When *P. oleovorans* was grown on *n*-alkanoic acids, the formation of intracellular PHAs was observed only for hexanoate and the higher *n*-alkanoic acids. The maximum isolated polymer yields were approximately 40% of the cellular dry weight when grown on either octanoate or nonanoate. In most cases, the major repeating unit in the polymer had the same chain length as the *n*-alkanoic acid used for growth, but units with two carbon atoms less or more than the acid used as carbon source were also generally present in the polyesters formed. Indeed, copolymers containing as many as six different types of ß-hydroxyalkanoate units could be formed. The weight average molecular weights of the PHA copolymers produced by *P. oleovorans* ranged from 90,000 to 370,000.

P. oleovorans could be grown on a wide variety of functionalized organic compounds as either a sole carbon source or in mixtures with traditional carbon sources to produce functionalized PHAs. Among these substrates were methyl-branched alkanoic acids, ω-bromoalkanoic acids, ω-

cyanoalkanoic acids, ω-phenylalkanoic acids and terminally unsaturated alkanoic acids. For example, three different ω-bromoacids were evaluated as substrates separately, including 6-bromohexanoic acid (6-BH), 8-bromooctanoic acid (8-BO) and 11-bromoundecanoic acid (11-BU). Growth was much faster with 11-BU than with 8-BO, and there was no growth with 6-BH. These results showed that cell growth was favored only when the distance between the functional substituent (Br) and the bioreactive group (COOH) was sufficiently long. However, no PHA granules were observed in cells grown on any of these bromoalkanoic acids, so the three bromoalkanoic acids were combined with equimolar amounts of *n*-nonanoic acid and fed to *P. oleovorans*, which had been grown initially on *n*-nonanoic acid. PHA granules were observed in the cells grown on all of the 1:1 molar mixtures, and the amount of ω-bromo-ß-hydroxyalkanoate units was as high as approximately 38 mole % when a mixture of 1:1 moles of nonanoic acid to 11-BO was used as the carbon source.

Very similar results were obtained with the methyl-branched substrates. That is, *P. oleovorans* was grown on a series of branched alkanoic acids, including 6-methylnonanoic acid (6-MNA), 7-methylnonanoic acid (7-MNA), 8-methylnonanoic acid (8-MNA), 9-methyldecanoic acid (9-MDA), 7-methyldecanoic acid (7-MDA) and 2,6-di-methylhept-5-enoic acid (2,6-DMHA). Polyesters were obtained from growth on pure 9-MDA, 8-MNA, 7-MNA, 6-MNA, but not from 7-MDA and 2,6-DMHA. Mixtures of n-nonanoic acid (n-NA) and either 7-MDA or 2,6-DMHA gave almost pure nonanoic acid homopolymers, but the other branched substrates produced polymers which contained the type of methyl branching expected from the starting alkanoic acid. The melting points, T_m, of most polymers were similar to that of the nonanoic acid polymer except for that of the polymer from 6-MNA the T_m was considerably higher, and this polymer also crystallized much faster than the others even though the 6-methylnonanoate units in the polymer included equal amounts of the two chiral isomers.

Our studies on the microbial polymer cellular inclusions were focused primarily on the structural and functional role of the protein components. Antibodies to each of the polypeptides were prepared and immunoassay techniques were established for the rapid identification of the polymerase and depolymerase enzymes. This technology (i.e., having Elisa assays available) will accelerate the determination of the primary and secondary structures of these two enzyme systems. Amino acid composition and sequencing studies were carried out. The isolation and analysis of a non-enzymatic protein, which may play a role in the assembly and organization of the granule, was also accomplished.

In our studies on the enzymatic degradation of PHAs we followed the course of the degradation by scanning electron microscopy, SEM. The SEM studies of biodegraded PHB/V films indicated that amorphous regions biodegraded faster than the crystalline regions. This conclusion was implied by micrographs of originally smooth surfaces which, when degraded, revealed what appeared to be crystallites surrounded by roughened, degraded areas. Also, biodegradation may have started at the hydroxyl group terminus and proceeded in a linear fashion along the length of the chain. This mechanism was indicated by the retardation of degradation when the chain ends were blocked by heptafluorobutyrl chloride labeling.

SIGNIFICANCE: The information gained from these studies will be of great importance for the scale-up and development of new PHAs which may find practical applications in the plastics industry and in medicine.

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