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REVIEW

Biosynthesis, Modification, and Biodegradation of Bacterial Medium-Chain-Length Polyhydroxyalkanoates

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Medium-chain-length polyhydroxyalkanoates (MCL-PHAs), which have constituents with a typical chain length of C_6-C_{14}, are polyesters that are synthesized and accumulated in a wide variety of Gram-negative bacteria, mainly pseudomonads. These biopolyesters are promising materials for various applications because they have useful mechanical properties and are biodegradable and biocompatible. The versatile metabolic capacity of some *Pseudomonas* spp. enables them to synthesize MCL-PHAs that contain various functional substituents; these MCL-PHAs are of great interest because these functional groups can improve the physical properties of the polymers, allowing the creation of tailor-made products. Moreover, some functional substituents can be modified by chemical reactions to obtain more useful groups that can extend the potential applications of MCL-PHAs as environmentally friendly polymers and functional biomaterials for use in biomedical fields. Although MCL-PHAs are water-insoluble, hydrophobic polymers, they can be degraded by microorganisms that produce extracellular MCL-PHA depolymerase. MCL-PHA-degraders are relatively uncommon in natural environments and, to date, only a limited number of MCL-PHA depolymerases have been investigated at the molecular level. All known MCL-PHA depolymerases share a highly significant similarity in amino acid sequences, as well as several enzymatic characteristics. This paper reviews recent advances in our knowledge of MCL-PHAs, with particular emphasis on the findings by our research group.

Keywords: polyhydroxyalkanoate, medium-chain-length polyhydroxyalkanoates, biopolyester, modification, biodegradation, MCL-PHA depolymerase

Introduction to medium-chain-length polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are versatile polyesters produced by many bacterial species as intracellular storage compounds for carbon and energy under nutrient-limited conditions in the presence of excess carbon source. Since the discovery of polyhydroxybutyrate (PHB) in *Bacillus megaterium* in 1926, a wide variety of PHAs with different properties have been found in more than 90 genera of bacteria. As constituents of biosynthetic PHAs, a great number of hydroxyalkanoates have been detected using different carbon substrates, culture conditions and microorganisms. Based on the carbon-chain length of the constituents, PHAs are generally divided into two groups, short-chain-length (SCL) PHAs and medium-chain-length (MCL) PHAs. SCL-PHAs consist of (*R*)-hydroxyalkanoates of C_3-C_5, while MCL-PHAs are comprised of aliphatic and/or aromatic (*R*)-hydroxyalkanoates of C_6-C_{14}. Although the great majority of PHA-synthesizing bacteria accumulate either SCL-PHAs or MCL-PHAs, microbial copolyesters consisting of SCL- and MCL-hydroxyalkanoates have been reported in some bacterial strains (Steinbüchel and Hein, 2001).

SCL-PHAs are thermoplastics with a high degree of crystallinity, while MCL-PHAs are elastic or tacky materials with a low degree of crystallinity and a low melting temperature. MCL-PHAs may be much more suitable biomaterials for biomedical applications in view of their physical properties (Zinn *et al.*, 2001). The most attractive feature of MCL-PHAs is that various MCL-PHAs bearing different functional groups in the side chains can be synthesized by some organisms, including *Pseudomonas oleovorans* and *P. putida,* when they are grown with substrates containing the corresponding chemical structures (Kim *et al.*, 2000a). MCL-PHAs with functional groups are of great interest, because the functional groups can improve the physical properties of the polymers. Moreover, some functional groups can be modified by chemical reactions to obtain more useful polymers and extend the potential application of MCL-PHAs as environmentally biodegradable polymers and functional biomaterials for biomedical uses (Hazer and Steinbüchel, 2007).

MCL-PHA biosynthesis in bacteria is closely linked to three different metabolic routes that generate MCL-PHA precursor molecules (Fig. 1): (i) *de novo* fatty acid biosynthesis pathway, which produces (*R*)-3-hydroxyacyl-CoA precursors from non-related carbon sources such as glucose and gluconate; (ii) fatty acid degradation by β-oxidation, which is the main metabolic route of fatty acids; (iii) chain

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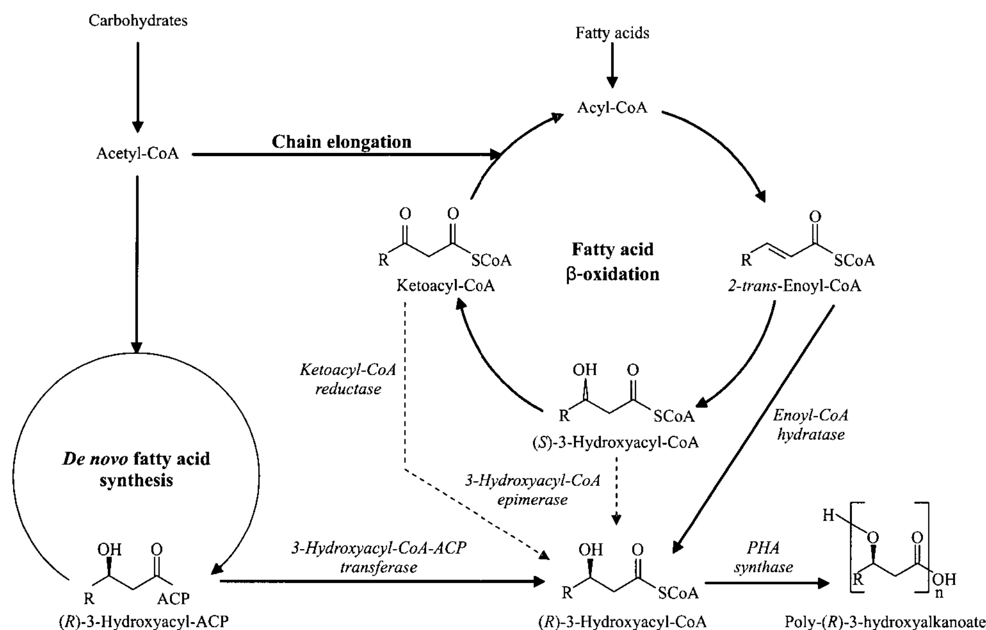

Fig 1. Metabolic pathways for MCL-PHA biosynthesis.

elongation, in which acyl-CoA is extended with acetyl-CoA (Witholt and Kessler, 1999). These metabolic pathways produce various intermediate precursors of MCL-PHA, such as (*R*)-3-hydroxyacyl-acyl carrier protein (ACP), 2-*trans*-enoyl-CoA, (*S*)-3-hydroxyacyl-CoA, and 3-ketoacyl-CoA.

The current supposition is that the (*S*)-3-hydroxyacyl-CoA and 3-ketoacyl-CoA molecules are subsequently converted to (*R*)-3-hydroxyacyl-CoA molecules by 3-hydroxyacyl-CoA epimerase and 3-ketoacyl-ACP reductase, respectively (Steinbüchel and Hein, 2001). In addition, (*R*)-specific enoyl-CoA hydratase (PhaJ), which catalyzes the (*R*)-specific hydration of β-oxidation intermediate, 2-*trans*-enoyl-CoA, to (*R*)-3-hydroxyacyl-CoA, plays a critical role in supplying monomer units from β-oxidation to PHA synthesis (Fiedler *et al.*, 2002; Tsuge *et al.*, 2003). Indeed, this enzyme actually functions as a supplier of (*R*)-3-hydroxyacyl-CoA monomer for PHA production in recombinant bacteria when fatty acids or plant oils are used as carbon sources (Fukui *et al.*, 1999). (*R*)-3-hydroxyacyl-ACP-CoA transferase (PhaG), which has been identified in *P. putida* and *P. aeruginosa*, plays an important role in the metabolic connection of *de novo* fatty acid biosynthesis with MCL-PHA biosynthesis (Hoffmann *et al.*, 2000). PhaG catalyzes the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA and contributes to the MCL-PHA biosynthesis from gluconate or other non-related carbon compounds.

In the final step of MCL-PHA biosynthesis, MCL-PHA synthases (PhaC) catalyze the conversion of (*R*)-3-hydroxyacyl-CoA molecules into MCL-PHAs with the concomitant release of CoA. To date, two MCL-PHA synthase genes, *phaC1* and *phaC2,* have been identified and characterized in *Pseudomonas* spp. Attempts have been made to analyze the functional differences between PhaC1 and PhaC2 in *Pseudomonas* spp. Although some results showed that both exhibited very similar properties (Qi *et al.*, 1997), recent studies have demonstrated that PhaC1 and PhaC2 from the same *Pseudomonas* strain had obvious differences in substrate specificity (Hein *et al.*, 2002; Chen *et al.*, 2004, 2006). In *P. stutzeri*, PhaC2 had a broader substrate specificity, ranging from 3-hydroxybutyrate to MCL 3-hydroxyalkanoates, while PhaC1 could only polymerize MCL 3-hydroxyalkanoates to PHA products (Chen *et al.*, 2004, 2006).

Diversity of biosynthetic MCL-PHAs

Many fluorescent *Pseudomonas* strains that belong to rRNA homology group I produce MCL-PHAs as copolymers that contain at least two types of 3-hydroxyalkanoate units when grown with alkanes and alkanoic acids longer than pentane and pentanoic acid (Kim and Lenz, 2001). With only a few exceptions, MCL-PHAs synthesized by different microorganisms grown with the same organic substrate have very similar monomer compositions. Generally the main monomeric unit in MCL-PHAs synthesized from a substrate with an even number of carbon atoms is 3-hydroxyoctanoate, while that from a substrate with an odd number of carbon atoms is 3-hydroxynonanoate. Also, non-alkyl-based organic substrates can be used for MCL-PHA synthesis. For example, *P. putida* and *P. aeruginosa* can synthesize MCL-PHA copolymers that contain 3-hydroxydecanoate as a major constituent from glucose and gluconate (Timm and Steinbüchel, 1990; Huijberts *et al.,* 1992). A recent report showed that a number of *Pseudomonas* species can accumulate MCL-PHAs bearing phenyl groups from a variety of aromatic hydrocarbons (Tobin *et al.*, 2005). In addition, several types of SCL/MCL hybrid PHA copolymers have been isolated from *Aeromonas* and *Pseudomonas* cells grown on appropriate carbon sources (Doi *et al.,* 1995; Kang *et al.,* 2001). However, compared to the frequent occurrence of SCL- or MCL-PHAs in nature,

the prevalence of SCL/MCL hybrid PHA copolyesters in microorganisms is relatively rare. The ability to biosynthesize these hybrid PHAs is considered to be closely related to the extraordinarily broad substrate specificity of PHA synthases in these organisms (Steinbüchel and Lütke-Eversloh, 2003). Hybrid PHA copolyesters are expected to have a wide

Table 1. Classification of carbon substrates containing functional groups based on their ability to support growth and PHA synthesis by *P. oleovorans* and *P. putida*

Group	Carbon substrates used in *P. oleovorans*	Carbon substrates used in *P. putida*
A	7-methyloctanoic acid, 5-phenylvaleric acid, 6-phenylhexanoic acid, 7-phenylheptanoic acid, 9-phenylnonanoic acid, 11-phenylundecanoic acid, 9-*p*-tolylnonanoic acid, 4-cyclohexylbutyric acid, 5-cyclohexylvaleric acid, 6-octenoic acid, 7-octenoic acid, oleic acid, γ-linolenic acid, 10-undecenoic acid, 10-undecynoic acid, methyl octanoate, methyl heptanoate, methyl decanoate, ethyl heptanoate, propyl heptanoate, 1-heptene, 1-octene, 1-decene, 11-methoxyundecanoic acid, 11-ethoxyundecanoic acid, 8-*p*-methylphenoxyoctanoic acid, 6-phenoxyhexanoic acid, 8-phenoxyoctanoic acid, 11-phenoxyundecanoic acid, 9-*p*-tolylnonanoic acid, 9-*p*-styrylnonanoic acid	10-undecenoic acid, 10-undecynoic acid, 6-phenylhexanoic acid, 7-phenylheptanoic acid, 9-phenylnonanoic acid, 11-phenylundecanoic acid, 6-*p*-methylphenoxyhexanoic acid, 8-*p*-methylphenoxyoctanoic acid, 8-*m*-methylphenoxyoctanoic acid, 8-*o*-methylphenoxyoctanoic acid, 11-thiophenoxyundecanoic acid, 11-phenoxyundecanoic acid
B	6-bromohexanoic acid, 8-bromooctanoic acid, 11-bromoundecanoic acid, 5-methyloctanoic acid, 6-methyloctanoic acid, 11-cyanoundecanoic acid, 8-hydroxyoctanoic acid, 12-hydroxydodecanoic acid, 10-epoxyundecanoic acid, 1,12-dodecanediol, 6-methoxyhexanoic acid, 6-ethoxyhexanoic acid, 8-methoxyoctanoic acid, 8-methoxyethoxyoctanoic acid, 8-*p*-methoxyphenoxyoctanoic acid, 8-*p*-ethoxyphenoxyoctanoic acid	5-chlorovaleric acid, 8-bromooctanoic acid, 11-bromoundecanoic acid, 8-methoxyoctanoic acid, 5-phenylvaleric acid, 11-methoxyundecanoic acid, 11-ethoxyundecanoic acid, 11-cyanoundecanoic acid, 3-phenylpropionic acid, 16-hydroxyhexadecanoic acid, 8-methoxyethoxyoctanoic acid, 8-*p*-methoxyphenoxyoctanoic acid, 8-*p*-ethoxyphenoxyoctanoic acid, 11-(4-methylthiophenoxy)undecanoic acid, 5-thiophenoxypentanoic acid
C	4-phenylbutyric acid, 6-aminohexanoic acid, 8-aminooctanoic acid, 11-aminoundecanoic acid, 1,6-hexanediol, 1,8-octanediol, octanethiol, octanedioic acid, 4-octylbenzenesulfonic acid, 4-heptylbenzoic acid, 6-heptynoic acid, 8-*p*-cyanophenoxyoctanoic acid, 11-*p*-methylphenoxyundecanoic acid, nonanethiol, nonylphenol	6-bromohexanoic acid, 6-methoxyhexanoic acid, 6-ethoxyhexanoic acid, 8-hydroxyoctanoic acid, 6-aminohexanoic acid, 11-aminoundecanoic acid, 4-pentenoic acid, 6-heptynoic acid, nonanethiol, hexanedioic acid to decanedioic acid, dodecanedioic acid, nonylphenol, 4-cyclohexylbutyric acid, 6-phenoxyhexanoic acid, 8-*p*-cyanophenoxyoctanoic acid, 11-*p*-methylphenoxyundecanoic acid

variety of mechanical properties ranging from hard crystalline to elastic, depending on the mole percentage of their constituents (Chen *et al.*, 2006).

To date, more than 140 hydroxyalkanoates have been detected as constituents in MCL-PHAs; these constituents are produced by microorganisms grown on carbon substrates containing different types of chemical structures (Steinbüchel and Valentin, 1995; Witholt and Kessler, 1999). The diversity of monomeric units is principally attributed to the low substrate specificity of MCL-PHA synthases, which play a key role in MCL-PHA biosynthesis. The metabolic capabilities of *Pseudomonas* strains that utilize functionalized carbon substrates for cell growth and/or MCL-PHA production are also responsible for the constituents' variability (Kim *et al.*, 2000a).

Of the microorganisms capable of producing MCL-PHAs, *P. oleovorans* and *P. putida* have been investigated most extensively. Many reports have shown that these bacteria can produce PHAs with the corresponding functional groups from carbon substrates with certain functional groups only in the presence of a good polymer-producing substrate, such as octanoic acid or nonanoic acid (Lenz *et al.*, 1992; Kim *et al.*, 2000a). These unusual phenomena of PHA biosynthesis were explained by Lenz *et al.* (1992) as a co-metabolic effect. They divided the various carbon substrates for PHA biosynthesis into three groups according to their individual abilities to support either cell growth or PHA production, or both. Group A corresponds to alkanoic acids that support both cell growth and PHA production. Group B represents alkanoic acids that support cell growth, but not PHA production, and group C contains alkanoic acids that do not support cell growth. Obviously, in *P. oleovorans* and *P. putida*, co-metabolism is the only metabolic route to produce PHAs with chemical structures corresponding to carbon substrates belonging to groups B and C. Table 1 shows the various carbon substrates used for biosynthesis of functional MCL-PHAs, classified into three groups according to the description suggested by Lenz *et al.* (1992).

Aliphatic MCL-PHAs containing functional moieties: Over the two past decades, a great deal of effort has been spent on preparing microbial PHAs with novel properties. As a result, various MCL-PHAs with diverse functional groups in their side chains have been synthesized and their properties characterized. Many aliphatic MCL-PHAs containing substituted groups, such as bromine (Kim *et al.*, 1992), chlorine (Doi and Abe, 1990), fluorine (Kim *et al.*, 1996a), branched alkyl (Lenz *et al.*, 1992), cyano (Lenz *et al.*, 1992), hydroxyl (Lenz *et al.*, 1992), alkoxy (Kim *et al.*, 2003a), acetoxy (Jung *et al.*, 2000), epoxy (Bear *et al.*, 1997), cyclohexyl (Kim *et al.*, 2001a), propylthio (Ewering *et al.*, 2002), and alkyl esters (Scholz *et al.*, 1994) have been produced microbially. Moreover, several reports have shown that *P. oleovorans* and *P. putida* can biosynthesize unsaturated MCL-PHAs that contain carbon-carbon double bonds (Fritzche *et al.*, 1990a) and carbon-carbon triple bonds (Kim *et al.*, 1998, 2002a) in the terminal reactive groups. Functional MCL-PHAs are generally produced by growing microorganisms on carbon substrates with the respective chemical structures given as either the sole carbon source or as a mixture with octanoic or nonanoic acid. However, *P. stutzeri* and *P. cichorii* were also reported to synthesize MCL-PHAs bearing epoxy groups from non-related carbon sources such as soybean oil and 1-alkenes of C_7-C_{12} (He *et al.*, 1998; Imamura *et al.*, 2001).

Generally, the introduction of hydrophilic moieties, such as hydroxyl, amine, alkoxy, and carboxyl groups, into polymers results in the formation of polymers with enhanced hydrophilicity. These hydrophilic polymers are expected to have a wide range of applicability as biocompatible materials in a variety of biomedical fields. However, microbial production of MCL-PHAs containing hydrophilic groups is quite difficult, but not impossible. Recently, microbial MCL-PHAs containing substantial amounts of methoxy or ethoxy groups were successfully prepared from *P. oleovorans* cells grown on mixtures of methoxy- or ethoxyalkanoic acids with nonanoic acid (Kim *et al.*, 2003a). In this case, MCL-PHA copolymer with more than 60 mol% 3-hydroxyalkoxyalkanoates exhibited high solubility, even in a methanol/water (70:30) solution, which indicates that the inherent hydrophobic nature of MCL-PHAs was greatly altered by the introduction of hydrophilic moieties into the side chain.

MCL-PHAs that contain reactive groups, such as chlorine, epoxy, and unsaturated groups, have attracted much attention because they can be chemically modified to yield novel biomaterials with improved properties (Hazer and Steinbüchel, 2007). The unsaturated MCL-PHAs are also useful as cross-linking centers in photochemical and thermal radical reactions (Kim *et al.*, 2001b). Several studies have shown that epoxidized polymers, which are chemically more reactive than unsaturated PHAs, might be very useful in further cross-linking reactions to improve their mechanical (elastomeric) properties (Park *et al.*, 1998; Ashby *et al.*, 2000).

Aromatic MCL-PHAs containing bulky side groups: Aromatic polyesters are expected to have improved mechanical properties compared to aliphatic polyesters. Currently, synthetic polyesters with aromatic groups in the backbone such as poly (ethylene terephthalate) and poly(butylene terephthalate) are produced chemically and are used extensively in the plastic industry. Some PHAs that contain aromatic substituents have been biosynthesized by *P. oleovorans* and *P. putida* (Kim and Lenz, 2001). Of the microbially produced aromatic polyesters, poly(3-hydroxy-5-phenylvalerate) with phenyl groups in the side chain, which was first produced by *P. oleovorans* from 5-phenylvaleric acid in 1990, has been studied most intensively (Fritzche *et al.*, 1990b; Kim *et al.*, 1999; Abraham *et al.*, 2001). Wholly aromatic PHAs consisting of 3-hydroxy-ω-phenylalkanoates synthesized from ω-phenylalkanoic acids of C_5-C_8 are non-crystalline (Kim *et al.*, 1999; Abraham *et al.*, 2001). *P. oleovorans* and *P. putida* usually produce a physical mixture of polymers with significantly different compositions when grown on various molar mixtures of 5-phenylvaleric acid and nonanoic acid; this mixture can be separated by *n*-hexane to poly (3-hydroxy-5-phenylvalerate)-rich fractions (*n*-hexane insoluble) and poly(3-hydroynonanoate)-rich fractions (*n*-hexane soluble) (Kim *et al.*, 1999). The formation of a polymer mixture of MCL-PHAs bearing nitrophenyl (Aróstegui *et al.*, 1999) or *p*-methylphenyl (Curley *et al.*, 1996) groups has also been observed in *P. oleovorans*. However, random copolyesters with a pendant phenyl group have been biosynthesized by *P. putida* cultivated either on a mixture of 5-phenylvaleric acid and 8-*p*-methylphenoxyoctanoic acid (Kim *et al.*, 1999) or on a

mixture of 6-phenylhexanoic acid and 7-phenylheptanoic acid (Abraham *et al.,* 2001).

When cultivated on a mixture of 11-phenoxyundecanoic acid and octanoic acid, *P. putida* has been reported to simultaneously consume the two carbon substrates, depending on the cultivation time, and to produce a random copolyester consisting of 3-hydroxyalkanoates and 3-hydroxy-ω-phenoxyalkanoates (Song and Yoon, 1996). Another study showed that *P. oleovorans* could synthesize aromatic MCL-PHAs with a phenoxy substituent using various ω-phenoxyalkanoic acids as the sole carbon source (Kim *et al.,* 1996b). In fact, PHAs bearing phenoxy substituents are completely amorphous, like poly(3-hydroxy-5-phenylvalerate), but the glass transition temperatures (~20°C) of the polymers (Kim *et al.,* 1996b) are slightly higher than that (13°C) of poly(3-hydroxy-5-phenylvalerate) (Fritzche *et al.,* 1990b). The introduction of thiophenoxy group into PHA has recently been reported (Takagi *et al.,* 1999). PHA synthesized by *P. putida* from 11-thiophenoxyundecanoic acid contained 3-hydroxy-5-thiophenoxyvalerate as the main constituent, as well as 3-hydroxy-7-thiophenoxyheptanoate. The biopolyester with a thiophenoxy side group is an elastic amorphous polymer and is the first example of a sulfur-containing MCL-PHA.

Aromatic MCL-PHAs bearing *p*-methylphenoxy and *m*-methylphenoxy substituents have been prepared from 8-*p*-methylphenoxyoctanoic acid and 8-*m*-methylphenoxyoctanoic acid, respectively (Kim *et al.,* 1999). MCL-PHA that contains a *p*-methylphenoxy group is a highly crystalline polymer with a T_g of 14°C, a T_m of 97°C, and a melting enthalpy of 33.5 J/g, while the PHA containing a *m*-methylphenoxy group is almost amorphous. A crystalline aromatic MCL-PHA with a *p*-methylphenyl substituent is also produced by *P. oleovorans* from 5-(4'-tolyl)valeric acid (Curley *et al.,* 1996). Meanwhile, *p*-cyano- and *p*-nitrophenoxy groups have also been incorporated into MCL-PHA, although the extent of incorporation of these functional groups into PHA was significantly lower than that of other aromatic groups (Kim *et al.,* 1995a). However, *P. putida* and *P. oleovorans* were able to synthesize MCL-PHAs with the corresponding functional groups from 6-*p*-cyanophenoxyhexanoic and 6-*p*-nitrophenoxyhexanoic acids, respectively, but only in the presence of more than 35 mol% octanoic acid in the carbon substrate mixture.

Modification of MCL-PHAs for biomedical applications

PHAs are promising materials for various applications, because they have useful mechanical properties and are biodegradable and biocompatible. Therefore, PHAs have recently attracted interest in the biomedical field (Williams *et al.,* 1999; Zinn *et al.*, 2001). Even though a number of *in vivo* and *in vitro* studies have demonstrated the biocompatible properties of SCL-PHAs (Deng *et al.*, 2002; Volova *et al.*, 2003), SCL-PHAs are too rigid and brittle and thus may lack the superior mechanical properties required for biomedical applications such as heart valves and other vascular applications or controlled drug delivery systems. In contrast, elastomeric MCL-PHAs are better suited for biomedical applications due to their physical properties. But, until now, these applications have been limited by low physical properties and high hydrophobicities of these polymers.

The elastomeric feature of MCL-PHAs can be maintained over a narrow temperature range because of their low T_m (40-60°C). At temperatures above or close to T_m, MCL- PHAs are entirely amorphous and sticky (Witholt and Kessler, 1999); thus, the biopolymers tend to soften at ambient temperatures, which may seriously limit their potential applications. Moreover, in order for MCL-PHAs to serve as the material of choice in the biomedical field, their hydrophilicity must be tailored to suit specific applications. Therefore, attempts to modify the properties of MCL-PHAs by chemical and physical methods, such as blending, crosslinking, and graft copolymerization, have attracted a great deal of interest.

Blending of MCL-PHAs with other polymers: For applications of MCL-PHAs in drug delivery systems and tissue engineering, improved hydrolytic degradation and mechanical properties are usually required. One approach is to blend MCL-PHA with other polymers to achieve new properties for various requirements. In combination with racemic poly(*R,S*-lactic acid) (PLA) oligomers, which is a well-known hydrolysable polymer, the hydrolytic degradation of polyhydroxyoctanoate (PHO), a representative MCL-PHA, was enhanced when PLA was present in the polymeric blend (Mallardé *et al.*, 1998). In contrast, Renard *et al.* (2004) reported that the hydrolytic degradation (weight loss) of PHO was unaffected by blending with PLA or poly(ethylene glycol) (PEG), due to the incompatibility of PLA and PEG with PHO. They observed that the introduction of polar carboxylic groups in side chains increased the PHO degradation rate, and further suggested that adequate modification of the macromolecular chain arrangement to enhance the compatibility of both components in PHO/PEG blend would promote water penetration into the polymer and accelerate the hydrolytic degradation of the polymer. A recent study on the mechanical properties and the degradation profiles of semi-interpenetrating networks (semi-IPNs) of polyhydroxyundecenoate (PHU) and poly(lactide-co-glycolide) (PLGA) revealed that mechanical strength was enhanced as PLGA content in the semi-IPNs was increased and that the hydrolytic degradation of the semi-IPNs was much faster than that of cross-linked PHU (Kim *et al.*, 2005c).

Crosslinking of unsaturated MCL-PHAs: MCL-PHAs that contain high levels of unsaturated repeating units are of great interest because polymers with different properties can easily be obtained by chemical modification of these MCL-PHAs. Unsaturated MCL-PHAs, such as PHU, have very low crystallinity and cross-link when irradiated with UV light, even in the absence of photosensitizer or photoinitiator. Cross-linking of these MCL-PHAs is expected to yield useful biomaterials, such as biodegradable rubbers. In addition, since cross-linked PHAs are less susceptible to PHA depolymerase than natural PHAs, these polymers can be useful as photosensitive materials for microlithography that are also environmentally friendly (Kim *et al.*, 2001b). Because many unsaturated MCL-PHAs are sticky, these polymers may find use as biomedical adhesives. Moreover, some chiral (*R*)-3-hydroxycarboxylic acids obtained from unsaturated MCL-PHAs may also be used as starting materials for new biomaterials. However, unsaturated MCL-PHAs have not been studied as biocompatible materials for biomedical applications due to their shortcomings, i.e., their stickiness and hydrophobicity. So far, several attempts to improve the mechanical properties of unsaturated MCL-PHAs have been published. Cross-linking can be achieved

Table 2. Typical changes in properties resulting from the modified of MCL-PHAs

Modification methods	Product	Molecular weights		Thermal properties		Mechanical properties		Reference
		$M_n \times 10^{-4}$	M_w/M_n	T_g (°C)	T_m (°C)	Tensile strength (MPa)	Elongation at break (%)	
Blending	PHO/PLA50 (80/20, w/w)	8.0/0.1		-39.0/-10.0	49.0			Mallardé *et al.,* 1998
	Semi-IPN PHU/PLGA (80/20, w/w)	5.0/5.0				1.1	205.0	Kim *et al.*, 2005c
Crosslinking	Radiation treated PHA-tal	4.4~7.3	3.6~4.9	-41.7~-42.3	43.1~43.3	4.0~5.1	195.0~235.0	Ashby *et al.,* 1998
	Gamma-ray treated PHOU	1.1~8.2		-35.0~-40.0	49.0~59.0			Dufresne *et al.,* 2001
Chemical reaction	Chlorinated PHAs	1.0~2.0	1.3~1.7	2.0~58.0	104.0~134.0			Arkin *et al.,* 2000
	Epoxidized PHAs	9.3~18.4	1.4~2.5	-28.0~-34.0	41~54			Park *et al.,* 1998
Grafting	PEG-grafted PHO	4.8~10.7	2.8~3.3	-34.5~-37.5	48.9~53.0	9.5~10.5	417.0~647.0	Kim *et al.,* 2005b
	PMMA-grafted PHN	5.5~47.3	2.5~5.8			37.3~52.1	5.7~12.7	Eroğlu *et al.,* 1998
	PEG-grafted PHU					0.2~0.4	379.0~621.0	Chung *et al.,* 2003

by using chemicals such as peroxides (Gagnon *et al.*, 1994), benzoyl peroxide, benzophenone, and ethylene glycol dimethacrylate in combination with thermal treatment and UV irradiation (Hazer *et al.*, 2001; Chung, 2005). Although these chemical methods are effective for cross-linking, they also introduce unwanted substances into the system. In contrast, irradiation of polymeric materials generally results in three-dimensional network structures with improved tensile strength, without the addition or formation of contaminants in the matrix. Cross-linking of unsaturated MCL-PHAs by gamma-irradiation has been studied (Ashby *et al.*, 1998; Dufresne *et al.*, 2001).

Chemical substitution of unsaturated MCL-PHAs: To achieve unusual physical properties, unsaturated groups in the side chains of MCL-PHAs can be converted into other functional groups, such as epoxy (Bear *et al.*, 1997; Park *et al.*, 1998), carboxyl (Bear *et al.*, 2001), hydroxyl (Lee *et al.*, 2000; Eroğlu *et al.*, 2005), or chlorine groups (Arkin *et al.*, 2000). The epoxidated-MCL-PHAs have increased tensile strength and Young's modulus (Ashby *et al.,* 2000). Poly(3-hydroxyoctanoate-*co*-3-hydroxyundecenoate) that contain 25% of repeating units pendant carboxylic acid groups (Bear *et al.*, 2001; Renard *et al.*, 2004) and 40-60% hydroxylated groups (Lee *et al.*, 2000) are perfectly soluble in polar solvents, such as methanol, dimethylsulfoxide or an acetone/water mixture, indicating that the hydrophilicity of these modified PHAs is considerably enhanced. Chlorination of the sticky MCL-PHAs with a double bond produces hard, brittle, and crystalline physical properties, depending on the chlorine content (Arkin *et al.*, 2000).

Graft copolymerization of MCL-PHAs: Grafting of vinyl monomers onto MCL-PHAs often changes their chemical and physical properties. Grafting reactions can be induced chemically, by plasma discharge, or by radiation. Plasma-induced graft copolymerization is an effective method for modifying the surface of polymeric materials. One of the great advantages of this method is that it can modify materials on the surface layer only, without harming the bulk mechanical properties. When a PHO film was treated with ammonia gas plasma, the wettability of the surface increased (Williams *et al.*, 1999). In addition, graft copolymerization of acrylamide onto PHO films was achieved by plasma treatment to induce surface hydrophilicity for cell-compatible biomedical applications (Kim *et al.*, 2002c). PEG-grafted PHO (PEG-g-PHO) (Kim *et al.*, 2005b) and PEG-g-PHU (Chung *et al.*, 2003) can be prepared by irradiating homogeneous solutions of PHO and PHU with monoacrylate-PEG, respectively. In this way, the surface and bulk of the graft copolymer can be made more hydrophilic as the PEG grafting density in the polymer chains increases. These graft copolymers could potentially be used as blood-contacting devices in a broad range of biomedical applications because of their excellent blood compatibilities. In addition, many grafting reactions have been reported to improve the thermal and mechanical properties of MCL-PHAs. Hazer (1996) studied grafting reactions of polyhydroxynonanoate (PHN) with polystyrene and PMMA peroxidic initiators. Gamma-irradiation of a mixture of PHN and methylmethacrylate also yielded PHN-g-PMMA graft copolymers (Eroğlu *et al.*, 1998). Double bonds in unsaturated MCL-PHA participate in the free-radical polymerization of methylmethacrylate initiated by benzoyl peroxide that produces PHA-soya-g-PMMA (Ilter *et al.*, 2001). Table 2 summarizes typical changes in properties that result from modification of MCL-PHAs by blending, graft copolymerization, crosslinking and chemical substitution.

Microbial degradation of MCL-PHAs

The ability to degrade PHAs depends on the secretion of specific extracellular PHA depolymerases that hydrolyze the polymer to water-soluble products. Extracellular PHA depolymerases are also divided into two groups, SCL-PHA depolymerases and MCL-PHA depolymerases, which differ with respect to substrate specificity for SCL-PHAs or MCL-PHAs. Although a few bacteria have been reported to degrade both SCL-PHAs and MCL-PHAs by producing two types of depolymerases (Klingbeil *et al.*, 1996; Kim *et al.*, 2003b),

Table 3. Biochemical characteristics of various MCL-PHA depolymerases

Characteristics	PhaZ$_{Pfl\mathrm{GK13}}$	PhaZ$_{Psp\mathrm{RY\text{-}1}}$	PhaZ$_{Xsp\mathrm{JS02}}$	PhaZ$_{Pal\mathrm{LB19}}$	PhaZ$_{Ssp\mathrm{KJ\text{-}72}}$	PhaZ$_{Pin\mathrm{K2}}$	PhaZ$_{Pal\mathrm{M4\text{-}7}}$	PhaZ$_{Plu\mathrm{M13\text{-}4}}$	PhaZ$_{Req\mathrm{P2}}$
Quaternary structure	dimer	tetramer	monomer	monomer	monomer	ND	ND	monomer	ND
M_r (SDS-PAGE) (kDa)	25.0	28.0	41.7	27.6	27.1	28.0	29.0	28.0	26.0
p*I*	5.7 [a]	5.9	ND [b]	5.7	4.7	ND	5.9 [a]	6.0	ND
Carbohydrate content	–	ND	ND	ND	–	ND	ND	ND	ND
Optimum pH	8.5	8.5	8.5	9.0	8.7	8.5	9.0	10.0	9.0
Optimum temp. (°C)	45	35	60	45	50	35	35	40	50
Sensitivity to									
DTT	–	–	ND	–	–	–	–	–	–
DFP	ND	ND	ND	+	+	+	+	–	ND
PMSF	–	–	ND	+	–	+	+	+	+
PHMB	–	ND	ND	+	–	+	+	–	ND
Sodium azide	–	–	ND	+	–	+	–	–	–
Iodoacetamide	ND	ND	ND	+	–	ND	–	–	–
NEM	ND	ND	ND	+	ND	ND	+	–	ND
NBSI	ND	ND	ND	+	+	ND	+	+	ND
Acetic anhydride	ND	ND	ND	+	+	ND	+	+	+
EDTA	–	+	ND	+	–	+	+	+	+
Triton X-100	+	+	ND	+	+	+	+	+	ND
Tween 80	+	+	ND	+	+	+	+	–	+
Main hydrolysis product of MCL-PHA	dimer	ND	ND	monomer	dimer	ND	ND	ND	ND
Reference	Schirmer *et al.*, 1993	Kim *et al.*, 2000c	Kim *et al.*, 2000b	Kim *et al.*, 2002b	Kim *et al.*, 2003b	Elbanna *et al.*, 2004	Kim *et al.*, 2005a	Rhee *et al.*, 2006	Lim, 2006

[a] Theoretical value calculated from amino acid sequences. [b] Not determined.
DTT, dithiothreitol; DFP, diisopropylfluoro phosphate; PMSF, phenylmethylsulfonyl fluoride; PHMB, *p*-hydroxymercuribenzoic acid; NEM, *N*-ethylmaleimide; NBSI, *N*-bromosuccinimide.

the great majority of PHA-degrading microorganisms produce only one type of PHA depolymerase that acts upon either SCL-PHAs or MCL-PHAs.

A wide range of PHA-degrading microorganisms have been isolated and most are SCL-PHA degraders. SCL-PHA-degrading microorganisms of many taxa are widely distributed in various ecosystems such as soil, sewage sludge, compost, and marine water (Mergaert and Swings, 1996; Jendrossek, 2001; Kim and Rhee, 2003). The percentages of SCL-PHA-degrading bacteria in soil environment have been estimated to be 0.8 to 11.0% of the total colonies by Nishida and Tokiwa (1993) and 2 to 18% by Suyama *et al.* (1998). However, few reports concerning the abundance and diversity of MCL-PHA-degrading microorganisms in natural environments have been documented, most likely due to the limited availability of MCL-PHAs on the market.

Recently, Nam *et al.* (2002) described a list of taxa (60 different species belonging to 13 genera) of MCL-PHA-degrading bacteria isolated from various soil samples and demonstrated that Gram-negative bacteria belonging to the genera *Stenotrophomonas* and *Pseudomonas* are the predominant MCL-PHA degraders in soil. Our recent attempts to examine the abundance and diversity of MCL-PHA-degrading microorganisms in marine environments showed that MCL-PHA degraders in seawater samples were less than 1.4% of all the heterotrophic bacteria and that *Pseudomonas* and *Stenotrophomonas* strains accounted for approximately 65% and 23%, respectively, of the total MCL-PHA degraders isolated (our unpublished data). These results suggest that biodegradation of MCL-PHA in soil and marine environments may be predominantly performed by *Pseudomonas* and *Stenotrophomonas* species. Among Gram-positive bacteria, the presence of MCL-PHA depolymerase activity has been found only in *Streptomyces* spp. (Klingbeil *et al.*, 1996; Kim *et al.*, 2003b) and *Rhodococcus equi* (Lim, 2006). Particularly, members of the genus *Rhodococcus* have attracted great interest due to their capabilities to degrade various hydrophobic substances such as petroleum hydrocarbon, benzene, polychlorinated biphenyls, and SCL-PHAs (Jang *et al.*, 2005; Taki *et al.*, 2007; Valappil *et al.*, 2007). The ability of *Rhodococcus* to degrade MCL-PHAs suggests that *Rhodococcus* may play a considerable role in decomposing various materials in natural environments. To date, no MCL-PHA-degrading fungi have been reported. It is well known that SCL-PHAs are synthesized by a wide range of bacteria, while MCL-PHAs are synthesized primarily by some *Pseudomonas* strains that belong to rRNA homology group I. Therefore, the smaller proportion of MCL-PHA-degrading microorganisms could be

attributed to the less common occurrence of MCL-PHAs in natural environments than that of SCL-PHAs.

Until now, more than 80 extracellular SCL-PHA depolymerases have been purified and characterized from prokaryotic and eukaryotic microorganisms (Jendrossek, 2001). In contrast to SCL-PHA depolymerases, which have been intensively investigated, only a limited number of reports concerning MCL-PHA depolymerases have been documented. Extracellular PHO depolymerase from *P. fluorescens* GK13 was the first MCL-PHA depolymerase to be studied in detail at the molecular level (Schirmer *et al.*, 1993; Schirmer and Jendrossek, 1994). After reports on this enzyme, molecular cloning of MCL-PHA depolymerase genes and the biochemical characteristics of the corresponding gene products were reported from some pseudomonads, such as *P. alcaligenes* LB19 (Kim *et al.*, 2002b; Kim *et al.*, 2005a), *P. alcaligenes* M4-7 (Kim *et al.*, 2005a), and *P. luteola* M13-4 (Park *et al.*, 2006; Rhee *et al.*, 2006), as well as *R. equi* P2 (Lim, 2006). In addition, poly(3-hydroxy-5-phenylvalerate) depolymerase from *Xanthomonas* sp. JS02 (Kim *et al.,* 2000b) and the MCL-PHA depolymerases from *Pseudomonas* sp. RY-1 (Kim *et al.,* 2000c), *P. indica* K2, (Elbanna *et al.,* 2004), and *Streptomyces* sp. KJ-72 (Kim *et al.,* 2003b) were also purified and characterized, but these depolymerase genes were not cloned.

Extracellular MCL-PHA depolymerases share several properties (Table 3). The following descriptions indicate the common characteristics among the bacterial MCL-PHA depolymerases: (1) M_r (25.0-30.0 kDa) of most MCL-PHA depolymerases is smaller than that (40.0-50.0 kDa) of SCL-PHA depolymerases, although the M_r of MCL-PHA depolymerase from *Xanthomonas* sp. JS02 (Kim *et al.*, 2000b) was 41.7 kDa; (2) *p*I value is in the acidic range; (3) these enzymes are most active at alkaline pH (8.5-10.0); (4) in contrast to SCL-PHA depolymerases, MCL-PHA depolymerases are insensitive to dithiothreitol, which reduces disulfide bonds; (5) the enzymes are highly susceptible to *N*-bromosuccinimide and acetic anhydride, indicating that tryptophan and lysine residues play an essential role in the catalytic domain of MCL-PHA depolymerases; and (6) they are inactivated by serine protease inhibitors, such as phenylmethylsulfonyl fluoride or diisopropylfluoro phosphate, as well as by non-ionic detergents, such as Tween 80 or Triton X-100.

Nucleotide sequence analyses of all available MCL-PHA depolymerase genes deposited in the GenBank database show that the open reading frame of the genes consist of 834-849 bp encoding 277-282 amino acids. The nucleotide sequence identities among MCL-PHA depolymerase genes are very high, ranging from 70-87%. It is worthwhile to note that the genes exhibit an extreme bias for guanine and cytosine at the third codon position (>69%), with cytosine as the preferred nucleotide (>47%). Moreover, the high content of amino acids with aromatic and uncharged aliphatic side chains in the deduced amino acid sequences of mature MCL-PHA depolymerases suggest that the enzymes are strongly hydrophobic. The amino acid sequence similarity among MCL-PHA depolymerases ranges from 87 to 98%. However, the amino acid sequence of *P. luteola* M13-4 (Park *et al.*, 2006) shares 69 to 78% similarity with those of other MCL-PHA depolymerases.

Comparison of the primary amino acid sequences of MCL-PHA depolymerases reveals that the enzymes consist of three domains, in sequential order: a signal peptide, an *N*-terminal substrate binding domain, and a *C*-terminal catalytic domain. This sequential order is comparable to the general domain structures of SCL-PHA depolymerases, in which the substrate-binding domain and the catalytic domain are located in the *C*-terminus and *N*-terminus, respectively (Jendrossek, 2001). In addition, the substrate-binding domain and the catalytic domain of SCL-PHA depolymerases are connected to each other by a linking domain such as a fibronectin type Ⅲ domain or a threonine-rich region (Jendrossek, 2001), whereas no linking domain has been found in the MCL-PHA depolymerases. However, like extracellular SCL-PHA depolymerases, MCL-PHA depolymerases contain strictly conserved amino acids (Ser, Asp, His) that comprise a catalytic triad in the active center (Table 4). The three catalytic amino acids in the active site are also commonly found in lipases and esterases. Additionally, the catalytic domain of all MCL-PHA depolymerases contains a lipase consensus sequence, G-I-S-S-G. Serine hydrolases, such as lipases, esterases, and PHA depolymerases, are known to share the lipase box pentapeptide sequence (G-X_1-S-X_2-G). In SCL-PHA depolymerases, X_1 is generally Leu or Val, and X_2 is Ala, Trp or Ser (Jendrossek, 2001). However, in all MCL-PHA depolymerases analyzed thus far, X_1 is an Ile, and X_2 is a Ser.

In addition, all PHA depolymerases have an oxyanion hole amino acid that is known to participate in stabilizing the transition state of the hydrolysis reaction by allowing the formation of a hydrogen bond to the negatively charged oxygen atom of the active site Ser (Jaeger *et al.,* 1994). The oxyanion hole amino acid of the MCL-PHA depolymerases in *P. alcaligenes* LB19 (Kim *et al.*, 2005a), *P. luteola* M13-7 (Park *et al.*, 2006), and *R. equi* P2 (Lim, 2006) is an Asn. His and Ser were also found as other oxyanion hole amino acids in the MCL-PHA depolymerase of *P. fluorescens*

Table 4. Alignment of the catalytic active amino acids of extracellular MCL-PHA depolymerases

Peptide	Pos.	Serine	Pos.	Aspartate	Pos.	Histidine	Pos.	Oxyanion hole	Reference
PhaZ$_{Pfl\mathrm{GK13}}$	172	LNAQRQYAT[GI**S**SG]GYNT	228	FLHGFV**D**AVV	260	LGG**H**EWFAASP	111	QNLLD**H**GYAVIAP	Schirmer and Jendrossek, 1994
PhaZ$_{Pal\mathrm{LB19}}$	172	LNAQRQYAT[GI**S**SG]GYNT	228	FLHGFV**D**AVV	260	LGG**H**EWFAASP	111	QALLD**N**GYAVIAP	Kim *et al.*, 2005a
PhaZ$_{Pal\mathrm{M4\text{-}7}}$	171	LNSQRKYAT[GI**S**SG]GYNT	227	FLHGFV**D**LTV	259	LGG**H**EWFAASP	110	RALLD**S**GYAVIAP	Kim *et al.*, 2005a
PhaZ$_{Plu\mathrm{M13\text{-}4}}$	176	LNGNRKYAT[GI**S**SG]GYNT	232	FLHGFV**D**LTV	264	TGG**H**EWFPASP	115	KTLLE**N**GYAVIAP	Park *et al.*, 2006
PhaZ$_{Req\mathrm{P2}}$	172	LNAQRQYAT[GI**S**SG]GYNT	228	FLHGFV**D**AVV	260	LGG**H**EWFAASP	111	QALLD**N**GYAVIAP	Lim, 2006

Amino acids of the catalytic triad and the conserved putative oxyanion are shown in *bold letters.* The lipase consensus sequence is boxed.

GK13 (Schirmer and Jendrossek, 1994) and *P. alcaligenes* M4-7 (Kim *et al.*, 2005a), respectively. The diversity of oxyanion hole amino acids found in the MCL-PHA depolymerases is dissimilar to SCL-PHA depolymerases, in which only His acts as the oxyanion hole amino acid. Asn, Ser and His residues have been described as good hydrogen-bond donors in transition-state stabilization of hydrolytic reactions (Roberts *et al.*, 1972). Overall, the highly significant homology of the deduced amino acid sequences among the known MCL-PHA depolymerases and several common characteristics among these enzymes strongly suggest horizontal transfer of the MCL-PHA depolymerase gene in bacterial strains. Prior to widespread application of MCL-PHAs, further ecological studies on MCL-PHA-degraders and further advances in biochemistry and molecular biology concerning MCL-PHA depolymerases are necessary to gain a better understanding of MCL-PHA degradation.

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