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Gene Structures and Catalytic Mechanisms of Microbial Enzymes Able to Biodegrade the Synthetic Solid Polymers Nylon and Polyester Polyurethane

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Introduction

Since the middle of the 20th century, the chemical industry has generated various synthetic compounds as both industrial products and wastes material by-products. Among these synthetic compounds the water-insoluble solid polymers (with the exception of polymers synthesized specifically as biodegradable polymers, such as polylactic acid) are generally the most resistant to microbial attack, an attack which is essentially by enzyme action. An enzyme that is able to catalyze the degradation of a solid polymer must be able to access and bind to the polymer at a specific site, and to catalyze the degradation reaction extracellularly. In general, water-insoluble synthetic polymers are hydrophobic, rigid, and have a small specific surface area as compared to naturally occurring water-insoluble polymers such as cellulose. These properties make the degradation of the water-insoluble synthetic solid polymer difficult. However it has been reported that several water-insoluble synthetic solid polymers are vulnerable to microbial attack. In particular, the characteristics of the genetic sequences and catalytic mechanisms of the microbial enzymes which are able to degrade nylon and polyester polyurethane have been well studied, and this is what we will consider in this review.

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Abbreviations: PUR, polyurethane; DMP, dimethoxyphenol; SOD, superoxide dismutase; HRP, horseradish peroxidase; NMR, nuclear magnetic resonance; CBS, culture broth secreted; PAGE, polyacrylamide gel electrophoresis; ORF, open reading frame; ChE, cholinesterase; AChE, acetylcholinesterase; SCRs, structurally conserved regions; PHA, poly(hydroxyalkanoate).

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Nylon is a synthetic polymer containing an amide bond (–CONH–). Although the enzyme susceptible –CONH– bond occurs widely in natural polymers like polypeptides, nylon is believed to be relatively resistant to microbial attack. To address this, over the past twenty years, Okada and colleagues have done a considerable amount of work on two kinds of hydrolases that are able to degrade nylon oligomers (Kinoshita *et al.*, 1972; Negoro *et al.*, 1994). These two hydrolases were purified from the cultures of bacterial strains isolated from the wastewater generated in the nylon synthesis process. One hydrolase is able to degrade linear oligomers and the other can degrade cyclic oligomers. Since these enzymes are inactive against natural amide bonds, Okada and colleagues concluded that the newly acquired ability to degrade nylon oligomers had developed in the wastewater (Okada *et al.*, 1983; Ohno, 1984; Tan, 1999). The degradation activities of these enzymes depend on the water solubility of the nylon oligomer, i.e. these enzymes were not able to catalyze the degradation of water-insoluble nylon directly. More recently, it has been reported that water-insoluble nylon can be degraded by a lignin-degrading fungus and the active enzyme responsible has been purified (Deguchi *et al.*, 1997, 1998).

Polyurethanes (PURs) are the general term used for a polymer derived from the condensation of polyisocyanates and polyols creating intramolecular urethane bonds (carbamate ester bond, –NHCOO), and are classified into two types: polyether PUR and polyester PUR. Darby and Kaplam (1968) have synthesized various kinds of polyether and polyester PUR in order to examine degradability. They reported that *polyether* PUR was scarcely susceptible to microbial degradation. However, *polyester* PUR is considered to be comparatively susceptible to microbial degradation (Morton and Surman, 1994). The relative resistance of polyether PUR to microbial degradation is considered to be due to the degradation mechanism which involves exo-type depolymerization (Kawai *et al.*, 1978, 1985) whereas polyester PUR degradation involves endo-type depolymerization. Therefore, the study of microbial degradation of polyether PUR has not substantially progressed. On the other hand, polyester PUR could be degraded by several species of fungi (Darby and Kaplam, 1968; Pathirana and Seal, 1984a, b, 1985a, b). Kay and co-workers have isolated three kinds of bacteria possessing polyester PUR biodegradative activities (Kay *et al.*, 1991, 1993). One of the three bacteria has demonstrated the ability to hydrolyse ester bonds thus suggesting the presence of esterase activity. Howard and co-workers have purified six bacterial proteins containing colloidal polyester PUR degradative activity (Howard and Blake, 1998; Howard *et al.*, 1999; Allen *et al.*, 1999; Ruiz *et al.*, 1999). Five of these enzymes were shown to possess esterase activity and one cloned gene of them encodes a lipase (Stern and Howard, 2000). On the other hand, Nakajima-Kambe and co-workers have isolated a gram-negative bacterium, *Comamonas acidovorans* TB-35, which is capable of utilizing solid polyester PUR as the sole carbon source (Nakajima-Kambe *et al.*, 1995). GC–MS analysis has indicated that the degradation products are derived from the polyester segment of the PUR as a result of the hydrolytic cleavage of ester bonds (Nakajima-Kambe *et al.*, 1997). Recently, the enzyme catalyzing the hydrolytic cleavage has been purified, and both the gene encoding this enzyme and its unique structure have been revealed (Akutsu *et al.*, 1998; Nomura *et al.*, 1998).

This review describes the biodegradation of two water-insoluble synthetic solid polymers, nylon and polyurethane, by microbial enzymes.

Table 6.1. Nylon degradation by three lignin-degrading fungi (Deguchi *et al.*, 1997)

Nutritional conditions	Weight-average molecular weight of nylon-66[a]
Control[b]	84,832
Phanerochaete chrysosporium	
Nitrogen-starvation	11,482
Trametes versicolor	
Nitrogen-starvation	27,108
IZU-154	
Non-starvation	84,751
Nitrogen-starvation	5,523
Carbon-starvation	5,983

[a] The weight-average molecular weight of nylon-66 was measured by gel permeation chromatography after the incubation with each of three fungi on agar medium for 20 days.
[b] The control nylon-66 was incubated for 20 days on the nitrogen-starvation agar medium lacking fungi.

Nylon degradation

NYLON DEGRADING ENZYME

Recently, studies have revealed that three well-known species of lignin-degrading basidiomycete, *Phanerochaete chrysosporium* (ATCC 34541), *Trametes versicolor* (IFO7043) and IZU-154 are able to degrade nylon-66 polymer (*Table 6.1*) (Deguchi *et al.*, 1997). The nylon degradation activity has been investigated under various nutritional conditions, and a significant reduction in the molecular weight of nylon was observed when either the nitrogen or carbon source was limited. These results suggest that nylon degradation can be triggered by starvation of either carbon or nitrogen in the same manner as lignin degradation (Keyser *et al.*, 1978; Jeffries *et al.*, 1981). More recently, during the process of developing an enzymatic method for degrading nylon, a nylon-degrading enzyme has been purified from the nitrogen starved culture of fungus IZU-154 using four purification steps which included three different types of chromatography: anion-exchange, gel permeation, and hydrophobic chromatography. Surprisingly, the characteristics of this purified protein (molecular weight, absorption spectrum, and requirements for peroxidase activity) were identical to manganese dependent peroxidase (Mn peroxidase) which is a well-known major component of the lignin-degrading system. However, requirements for nylon degradation are significantly different from the well-known Mn peroxidase reaction.

Mn peroxidase is known to require manganese, hydrogen peroxide and α-hydroxy acid, such as lactate, which act as a manganese chelator, for its catalytic reaction. This enzyme also has manganese-binding site. The primary catalytic cycle of Mn peroxidase is similar to that of other peroxidases (*Figure 6.1*) (Mino *et al.*, 1988; Wariishi *et al.*, 1988, 1989; Farhangranzi *et al.*, 1994). Inactive Mn peroxidase is oxidized by H_2O_2 in a single two-electron step to form active Mn peroxidase compound I which is then reduced by Mn(II) to return back to the inactive enzyme in two single electron steps with the intermediate formation of Mn peroxidase compound II (Wariishi *et al.*, 1992). In each reduction step, one equivalent of Mn(III) is formed. Since Mn peroxidase was first discovered in the cultures of white rot fungi,

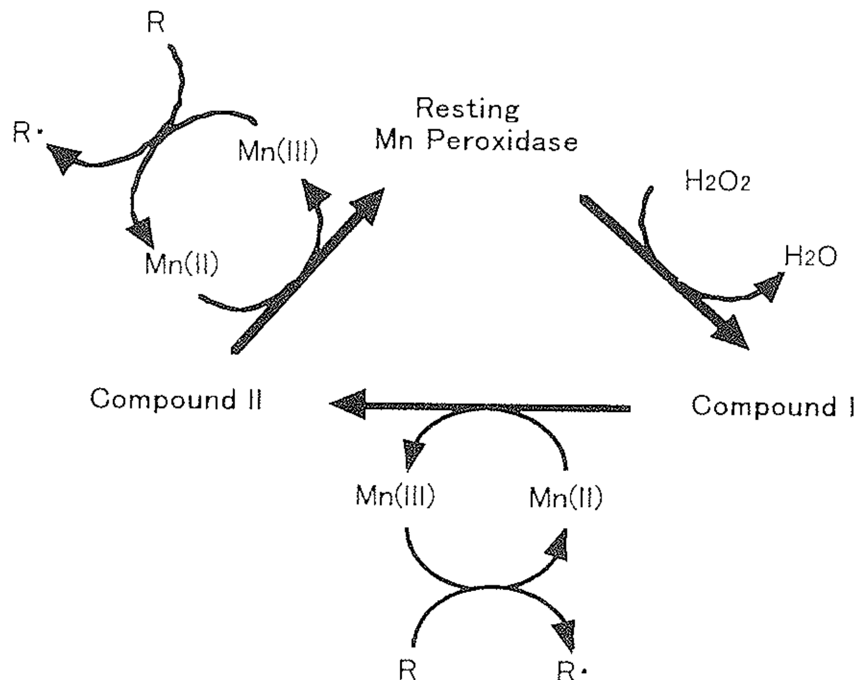

Figure 6.1. The Mn peroxidase catalytic cycle.

α-hydroxy acid has been considered to be one of the key components in the Mn peroxidase reaction system (Glenn and Gold, 1985; Wariishi *et al.*, 1989). This organic acid chelates the Mn(III) generated, both facilitating the release of Mn(III) from the enzyme–manganese complex and stabilizing this Mn(III)-chelator species in aqueous solution. Then the released Mn(III)-chelator can, in turn, oxidize various substrates (Halliwell and Rycker, 1978).

However, the requirements for nylon degradation differ significantly from the well-known Mn peroxidase reaction as shown by the 2,6-dimethoxyphenol (2,6-DMP) oxidation reaction. *Tables 6.2* and *6.3* show the requirements for 2,6-DMP oxidation and nylon degradation, respectively. Unlike 2,6-DMP oxidation, nylon degradation is apparently inhibited by lactate (α-hydroxy) and requires no exogenous H_2O_2, although both reactions were slightly inhibited by the addition of catalase. Superoxide dismutase (SOD) inhibited only nylon degrading activity. These results suggest that both H_2O_2 and superoxide anion radical are involved in nylon degradation.

Other than the peroxidase reaction, horseradish peroxidase (HRP) is also known to catalyze the peroxidase–oxidase reaction. This reaction also requires no exogenous H_2O_2 and is inhibited by both catalase and SOD (Chance, 1952). Yokota and Yamazaki have proposed a mechanism for this reaction, in which a catalytic amount of H_2O_2 is necessary for initiation and the superoxide anion radical serves as an active intermediate in the chain reaction (Yokota and Yamazaki, 1965). This mechanism may also explain the roles of H_2O_2 and the superoxide anion radical in nylon degradation.

Table 6.2. Requirements of 2,6-DMP oxidation (Deguchi *et al.*, 1998)

Reaction mixture [a]	% of activity with complete mixture
Complete mixture	100
Complete mixture – enzyme	<1
Complete mixture – Mn(II)	<1
Complete mixture – lactate	<1
Complete mixture – H_2O_2	<1
Complete mixture + catalase (650 U/ml)	4
Complete mixture + SOD (330 U/ml)	105
Complete mixture + NaN_3 (1 mM)	<1

[a] The complete reaction mixture (volume, 1 ml) contained 0.5 mM $MnSO_4$, 1 mM 2,6-DMP, 50 mM sodium lactate, 20 mM sodium acetate (pH 4.5), and enzyme. All of the reactions except the reaction with H_2O_2 were initiated with H_2O_2 (final concentration, 0.1 mM). Oxidation rates were determined by monitoring the increase in absorbance at 496 for 30 s.

Table 6.3. Requirements of nylon degradation (Deguchi *et al.*, 1998)

Reaction mixture [a]	Mol wt of nylon	
	Weight average	Number average
Complete mixture	38,206	12,477
Complete mixture – enzyme	89,845	49,423
Complete mixture – Mn(II)	90,110	50,854
Complete mixture + lactate (50 mM)	90,059	49,882
Complete mixture + catalase (650 U/ml)	75,334	33,002
Complete mixture + SOD (330 U/ml)	88,203	45,264
Complete mixture + NaN_3 (1 mM)	89,300	49,639

[a] The complete reaction mixture (volume, 1 ml) contained 1 mM $MnSO_4$, 10 mM KH_2PO_4, 20 mM sodium acetate (pH 4.5), 1 mg of nylon-66 membrane, and 17 nkat of enzyme, where a katal was defined on the basis of peroxidase activity. After 2 days of incubation at 30°C, the nylon was harvested and applied to a gel permeation chromatography column.

STRUCTURAL CHARACTERISTICS

Mn peroxidase has been purified to electrophoretic homogeneity and characterized by many researchers (Glenn and Gold, 1985; Matsubara *et al.*, 1996) with the protein purified from *P. chrysosporium* the most extensively studied to date. The enzyme contains one iron protoporphyrin IX prosthetic group, is a glycoprotein and exists as several closely related isozymes. The cDNA sequence of this enzyme has also been elucidated. The first Mn peroxidase cDNA, isolated from strain *P. chrysosporium* OGC101, was cloned using polyclonal antibodies raised against a purified Mn peroxidase isozyme (Pribnow *et al.*, 1989), and then several cDNA clones also have been characterized. *Figure 6.2* shows the deduced amino acid sequence of several Mn peroxidases. The *mnp* gene encodes a mature protein of approximately 360 amino acids preceded by a 21 or 24 amino acid leader sequence. Two histidine residues conserved among many peroxidases (His-46 and His-172/173) are essential for peroxidase activity. The distal histidine, His-46, is related to the cleavage of H_2O_2, and the proximal histidine, His-172/173, is the axial ligand of heme (Reading and Aust, 1998). There are three potential N-glycosylation sites at Asn-76, Asn-131, and Asn-217. Both PHAMP1A and PHAMNP contain all three whereas IZ-MnP1 and

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                                          ↓
IZ-MnP1    MAFTSLLSLV ALAAVARAAP AAETAVCPDG TRVSNSACCA FIPLAQDLQA  26
IZ-MnP2    MALHLSSLLS ASPRLHRAAP AAETAVCFDG TRVSNSACCA FIPLAQDLQA  26
PHAMP1A    MAFGSLLAFV ALAAITRAAP TAESAVCPDG TRVTNAACCA FIPLAQDLQE  26
PHAMNP     MAFKSLIAFV ALAAAVRAAP T---AVCPDG TRVSHAACCA FIPLAQDLQE  26
                                                   -------A-------

IZ-MnP1    TVFQNDCGED AHEVIRLIFH DAVAISRSKG PSAGGGADGS MLLFPTVEPL  76
IZ-MnP2    TVFQNDCGED AHEVIRLIFH DAWHISRSKG PSAGGGADGS MLLFPTVEPL  76
PHAMP1A    TLFQGDCGED AHEVIRLTFH DAIAISQSLG PQAGGGADGS MLHFPTIEPN  76
PHAMNP     TIFQNECGED AHEVIRLTFH DAIAISRLQG PKAGGGADGS MLLFPTVEPN  76
--      ----------B-----------                   ---B′---

IZ-MnP1    FAANNGIDDS VNNLIPFLAK HPV-SAADLV QFAGAVALSN CPGAPRLEFL  125
IZ-MnP2    FAANNGIDDS VNNLIPFLAK HPV-SAADLV QFAGAVALSN CPGAPRLEFL  125
PHAMP1A    FSANSGIDDS VNNLLPFMQK HDTISAADLV QFAGAVALSN CPGAPRLEFM  126
PHAMNP     FSANNGIDDS VNNLIPFMQK HNTISAADLV QFAGAVALSN CPGAPRLEFL  126
        ------------C---------  ------------D---------

IZ-MnP1    AGRPNHTIPA IDGLVPEPQ- DDVTTILARF EDAGGFTPFE VVSLLASHSV  174
IZ-MnP2    DGRPNHTIPA IDGLVPEPQE DDVTTILARF EDAGGFTPFE VVSLLASHSV  175
PHAMP1A    AGRPNTTIPA VEGLIPEPQ- DSVTKILQRF EDAGNFSPFE VVSLLASHTV  175
PHAMNP     AGRPNKTIAA VDGLIPEPQ- DSVTKILQRF EDAGGFTPFE VVSLLASHSV  175
                                --------E----------  -----F-------

IZ-MnP1    ARADKVDETI DAAPFDSTPF TFDTQVFLEV LLKGTGFPGL SNNTGEVASP  224
IZ-MnP2    ARADKVDETI DAAPFDSTPF TFDTQVFLEV LLKGTGFPGL SNNTGEVASP  225
PHAMP1A    ARADKVDETI DAAPFDSTPF TFDTQVFLEV LLKGTGFPGS NNNTGEVMSP  225
PHAMNP     ARADKVDQTI DAAPFDSTPF TFDTQVFLEV LLKGVGFPGS ANNTGEVASP  225
                                 ---G---
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IZ-MnP1    LPKGSGNDTG EMRLQSDFAL ARDSRTACFW QGFVNQQEFM AASFKSAVAK  274
IZ-MnP2    LPKGSGNDTG EMRLQSDFAL ARDSRTACFW QGFVNEQEFM AASFKSAVAK  275
PHAMP1A    LPLGSGSDTG EMRLQSDFAL ARDERTACFW QSFVNEQEFM AASFKAAMAK  275
PHAMNP     LPLGSGSDTG EMRLQSDFAL AHDPRTACIW QGFVNEQAFM AASFRAAMSK  275
                      ------H------ ----I----   ------------J-------

IZ-MnP1    LAVLGHNRDD LIDCSEVVPV PKPAVKKPAS FPATTSAKDL E-LNCNSQKF  323
IZ-MnP2    LAVLGHNRDD LIDCSEVVPV PKPAVNKPAS FPATTSAKDL E-LNCNSQKF  324
PHAMP1A    LAILGHSRSS LIDCSDVVPV PKPAVNKPAT FPATKGPKDL DTLTCKALKF  325
PHAMNP     LAVLGHNRNS LIDCSDVVPV PKPATGQPAM FPASTGPQDL E-LSCPSERF  324
           ---

IZ-MnP1    PTLTVDQGAT QSLIPHCSNG GQNCPAVQFD GPSQASS*               360
IZ-MnP2    PTLTVDQGAT QSLIPHCSNG GQNCPAVQFD GPSQASS*               361
PHAMP1A    PTLTSDPGAT ETLIPHCSNG GMSCPGVQFD GPA*                   358
PHAMNP     PTLTTQPGAS QSLIAHCPDG SMSCPGVQFN GPA*                   357
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Figure 6.2. Alignment of the deduced amino acid sequences of several Mn peroxidases derived from IZU-154 and *P. chrysosporium*. Amino acid sequences shown are from two IZU-154 Mn peroxidase species, IZ-MnP1 and IZ-MnP2, and from two *P. chrysosporium* Mn peroxidase species, PHAMP1A (Pease *et al.*, 1989) and PHAMNP (Pribnow *et al.*, 1989). Boxed sequences indicate residues conserved among all four MnPs; broken line shown below the sequence denotes the locations of helices in *P. chrysosporium* MnP (Sundaramoorthy, 1994); the vertical arrow at the top shows the putative N terminus of the mature MnP, which is numbered 1 (Matsubara *et al.*, 1996).

IZ-MnP2 contain Asn only at the 131 and 217 positions. Crystal structure analysis shows that, of these three residues, only carbohydrate actually binds to Asn-131 (Sundaramoorthy *et al.*, 1994).

Mn(II) is hexa-coordinated to the carboxylate oxygens of Glu35, Glu39, Asp179, a heme propionate oxygen, and two water oxygens (Harris *et al.*, 1991; Wariishi *et al.*, 1992; Banci *et al.*, 1993; Johnson *et al.*, 1994; Sundaramoorthy *et al.*, 1998). The two glutamate residues are in helix B, and Asp-179 is in the loop between helices F and G. Mn peroxidase also has two structural calcium ions, like other fungal peroxidases (Sundaramoorthy *et al.*, 1994). The calcium-binding residues, Asp-47, Gly-62, Ser-66, Asp-64, Ser-174, Asp-191, Thr-193, Thr-196, and Asp-198, are located in helix B' and in the loop between helices F and G.

DEGRADATION MECHANISM

The mechanism of nylon degradation that has been suggested is based on nuclear magnetic resonance (NMR) analysis of degraded nylon (Deguchi *et al.*, 1997, 1998). This analysis reveals the formation of four end groups, –CHO, –NHCHO, $-CH_3$ and $-CONH_2$, after the nylon is degraded, indicating that nylon is degraded oxidatively and that the C–N bond in $NH-CH_2$ and the C–C bond in CH_2-CH_2 adjacent to the nitrogen atom are cleaved. The most likely explanation for the formation of these four end groups is that the methylene groups adjacent to the nitrogen atom are attacked by the enzyme and subsequently the reaction proceeds auto-oxidatively (*Figure 6.3*).

APPROACHES FOR LARGE-SCALE PRODUCTION OF NYLON DEGRADING ENZYME

Mutants

It is well known that Mn peroxidase, which is also a nylon degrading enzyme, is one of the key components of the lignin degrading system in basidiomycetes, such as in *P. chrysosporium*. In a basidiomycete, this system is activated only in response to nitrogen (Keyser *et al.*, 1978), carbon, or sulphur (Jeffries *et al.*, 1981) starvation, which triggers secondary metabolism. The production of Mn peroxidase is regulated by nutritional conditions and occurs only under nutrient-poor conditions. Northern blot analysis demonstrates that Mn peroxidase is regulated by nutrient nitrogen at the transcription level (Pribnow *et al.*, 1989). One approach for facilitating nylon degradation could be to produce a mutant strain which is able to generate Mn peroxidase under nutrient rich conditions thus making large-scale production of the enzyme possible.

Tien *et al.* described a procedure to obtain a mutant which is able to produce Mn peroxidase under nitrogen-rich conditions. *P. chrysosporium* strains which have a lysine requirement were mutagenized and screened using lignin model compounds which were covalently attached to lysine (Tien *et al.*, 1987; Tien and Myer, 1990). Mn peroxidase activity for a mutant obtained by this procedure was shown to be 4- to 10-fold higher than that of the wild type of *P. chrysosporium* (Orth *et al.*, 1991). Miura *et al.* also reported a procedure to obtain a mutant which is able to produce Mn peroxidase under nitrogen-rich conditions (Miura *et al.*, 1997). The protoplasts of the wild type of strain were prepared, mutagenized, reproduced and screened by using an

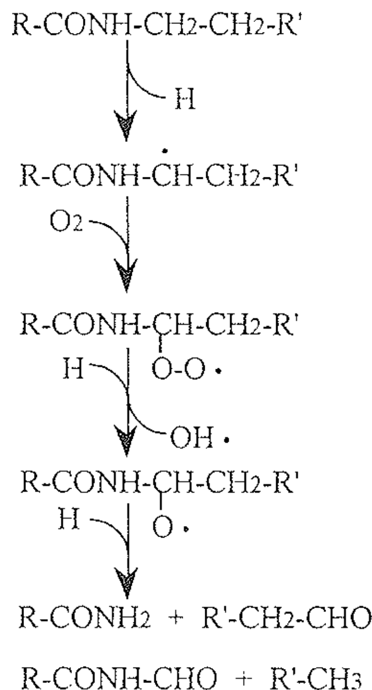

Figure 6.3. Possible mechanism for nylon degradation.

an agar including a sufficient nitrogen source and synthetic melanin. Mutants were selected for the ability to decolorize synthetic melanin under nitrogen-rich conditions, thus confirming the production of Mn peroxidase under those conditions (*Figure 6.4*).

Transformation

Another approach for producing the nylon degrading enzyme in large amounts is DNA transformation. Early reports have demonstrated the successful transformation of *P. chrysosporium* which is a well-known Mn peroxidase producing basidiomycete (Alic *et al.*, 1989, 1990). Protoplasted basidiospores of *P. chrysosporium* adenine auxotroph were transformed to prototrophy by using a plasmid containing the gene for an adenine biosynthetic enzyme derived from another basidiomycete, *Schizophyllum commune* (Munoz-Rivas *et al.*, 1986). Southern blot analysis has indicated that the transformed DNA is able to integrate into the chromosomal DNA at sites other than the resident adenine biosynthetic enzyme in multiple copies. The transformed DNA was found to be both mitotically and meiotically stable. Ogawa and co-workers have reported co-transformation of the basidiomycete *Coprinus cinereus* tryptophan auxotroph by a plasmid containing the Mn peroxidase cDNA with a plasmid containing the *C. cinereus TRP1*-gene. The Mn peroxidase cDNA came from *Pleurotus ostreatus* and was fused between the promoter and terminator in the plasmid (Ogawa *et al.*, 1998). The co-transformation was performed using protoplasted *C. cinereus* and transformants were obtained at a frequency of 5–10 transformants/μg

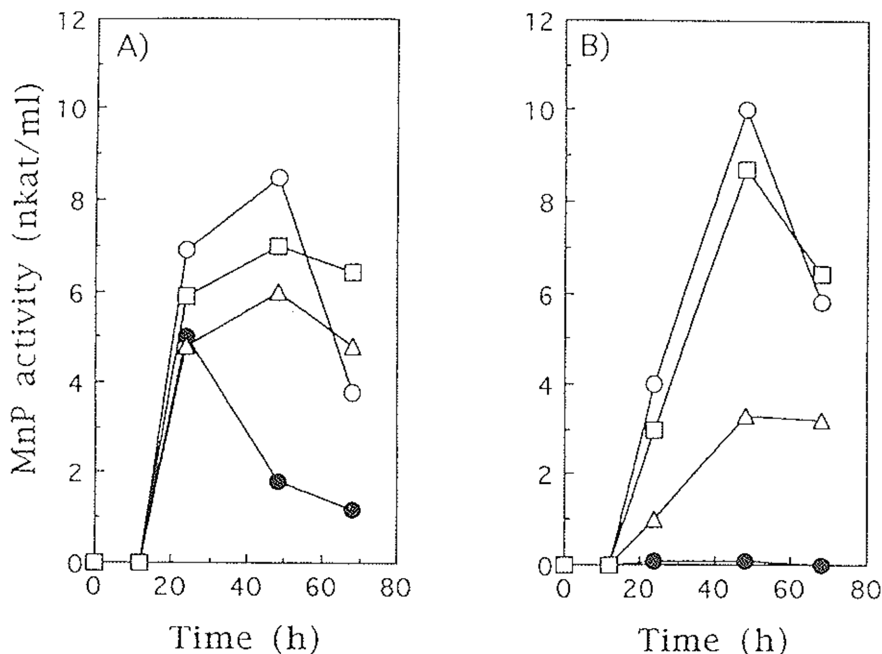

Figure 6.4. Extracellular Mn peroxidase activity for the wild-type IZU-154 and mutants, IZU-811, IZU-881 and IZU-882, under (A) low and (B) high nitrogen conditions. Each point is the mean of three replicates. Symbols: ●, IZU-154; □, IZU-811; △, IZU-881; ○, IZU-882 (Miura *et al.*, 1997).

transforming plasmid. The superior transformant carries a high number of copies of the Mn peroxidase cDNA (approx. 10) and shows about 30 times higher ligninolytic activities than the non-transformed strain.

Mn peroxidase of *P. chrysosporium* contains one protoporphyrin IX per enzyme molecule (Mino *et al.*, 1988) and is glycosylated. The cDNA of the Mn peroxidase encodes a leader sequence for secretion. This sometimes makes the heterologous expression of Mn peroxidase difficult. Pease and co-workers first reported the heterologous expression of active Mn peroxidase using the baculovirus expression system (Pease *et al.*, 1991). The recombinant Mn peroxidase was shown to have the same molecular weight as the native enzyme but a different pI, suggesting a variation in the nature of the glycosylation. The substrate profile of the recombinant Mn peroxidase is the same as the native enzyme. Another expression system was developed using *Aspergillus oryzae* (Whitwam *et al.*, 1995). This has an advantage over the baculovirus expression system for yielding high levels of expression. The recombinant protein was secreted in an active form and its physical and kinetic properties were similar to the native protein.

Recently, development of a prokaryotic expression system has been the focus of intense research since Whitwam and co-workers reported the refolding of inactive Mn peroxidase found in the inactive inclusion bodies produced in *E. coli* (Whitwam and Tien, 1996). The inclusion bodies were solubilized in 8 M urea and the reducing agent dithiothreitol, and reconstitution of activity was accomplished by diluting the urea concentration to 2 M in the presence of heme, calcium, and oxidized gulutathione.

The reconstitution of Mn peroxidase activity exhibits an absolute dependence on Ca^{2+} and heme. Maximal activity is obtained with a concentration of 50 mM $CaCl_2$ and 5 μM heme. By using this prokaryotic expression system, several researchers have attempted to add new activity to Mn peroxidase (Timofeevski *et al.*, 1999; Reading and Aust, 1998).

Polyurethane degradation

SOLID POLYURETHANE DEGRADING ENZYME

Comamonas acidovorans TB-35, which had been isolated as a solid PUR-degrading bacterium (Nakajima-Kambe *et al.*, 1995), was found to produce two kinds of esterases: one is secreted to the culture broth (CBS esterase) (Akutsu *et al.*, 1999) and the other is bound to the cell surface (PUR esterase) (Akutsu *et al.*, 1998). However, only the cell-bound esterase (PUR esterase) was shown to be able to degrade PUR. This enzyme reacts with solid polyester PUR to hydrolyse the ester bonds. PUR degradation by the PUR esterase is strongly inhibited by the addition of 0.04% deoxy-BIGCHAP, a kind of surfactant. However, deoxy-BIGCHAP did not inhibit activity when *p*-nitrophenyl acetate, a water-soluble compound, was used as a substrate. These observations have indicated that this enzyme degrades PUR in a two-step reaction: hydrophobic adsorption onto the PUR surface followed by hydrolysis of the ester bonds of PUR. Thus, PUR esterase is considered to have a hydrophobic PUR surface-binding domain and a catalytic domain, and this surface-binding domain is considered essential for PUR degradation.

The CBS esterase was also purified (Akutsu *et al.*, 1999). The CBS esterase is enzymatically active; it was shown not to be a denatured PUR esterase, because the ratio of CBS and PUR esterases produced remained constant regardless of the culture conditions and both demonstrate similar optimal conditions and thermostability. The properties of the two esterases also correspond with regard to their molecular weight and N-terminal/internal amino acid sequences. Furthermore, neither of the esterases had undergone any modification such as glycosylation, since they exhibited the same mobility on native PAGE. From these results, it can be concluded that the CBS and PUR esterases are the same polypeptide. This finding was supported by the observation that *Escherichia coli* containing the PUR esterase gene also produced two kinds of esterases. Though PUR esterase is able to degrade PUR and poly(diethylene glycol adipate) which is the soft segment of PUR, CBS esterase can only degrade poly(diethylene glycol adipate). Furthermore, the hydrophobicity of the CBS esterase was shown to be significantly lower than the PUR esterase. There is evidence that proves this PUR esterase possesses a PUR-binding domain, thus suggesting that structural change around the PUR-binding domain of the CBS esterase is responsible for its inability to degrade PUR.

STRUCTURAL CHARACTERISTICS

The structural gene (*pudA*) which encodes PUR esterase has been cloned and its primary structure has also been analysed by Nomura *et al.* (1998). The ORF consists of 1644 bp with a putative ATG initiation codon, and encodes a 548-amino

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PudA     1:CGGSDNDSSSNNQGAPAVAITVAGQVQAVDRLGMRRYFGIPFAAPPVGNLRWMPPAPPQSWAAPL-AKTQSNAPCMQ---  76
T AChE   1:-----DDHSELLVNTKSGKV-MGTRVPVLSS-HISAFLGIPFAEPPVGNMRFRRPEPKKPWSGVWNASTYPN-NCQQYVD  72

PudA    77:---TG-ATDPLRLPN-G-TEDCLYLDVHAPATGEGPFPVMVWIHGGAFSIG-GTI-TYADPSPLVSKGVIVVNIAYRMGA 148
T AChE  73:EQFPGFSGSEMWNPNREMSEDCLYLNIWVPSPRPKSTTVMVWIYGGGFYSGSSTLDVYNGKYLAYTEEVVLVSLSYRVGA 152

PudA   149:MGFLGHPSLRA-ADGTVGNYGIMDQQAALRWVQDNIAAFGGDKSNVTIFGESAGGFSV-MTHLASPLSKGLFAKAIVQSG 226
T AChE 153:FGFL---ALHGSQEAP-GNVGLLDQRMALQWVHDNIQFFGGDPKTVTIFGESAGGASVGM-HILSPGSRDLFRRAILQSG 227

                                   I                                 II
PudA   227:GYGFDRQLTQAQLEAQSTSIVNSALAAAGVSCPTV-D---AACLRG-LSAELVNNQLAT-AFTTA-NWSPVPSVDGKVLP 299
T AChE 228:SPNCPWA-----SVSVAEGRRRAVELGRNLNCNLNSDEELIHCLREKKPQELIDVEWNVLPFDSIFRFSFVPVIDGEFFP 302
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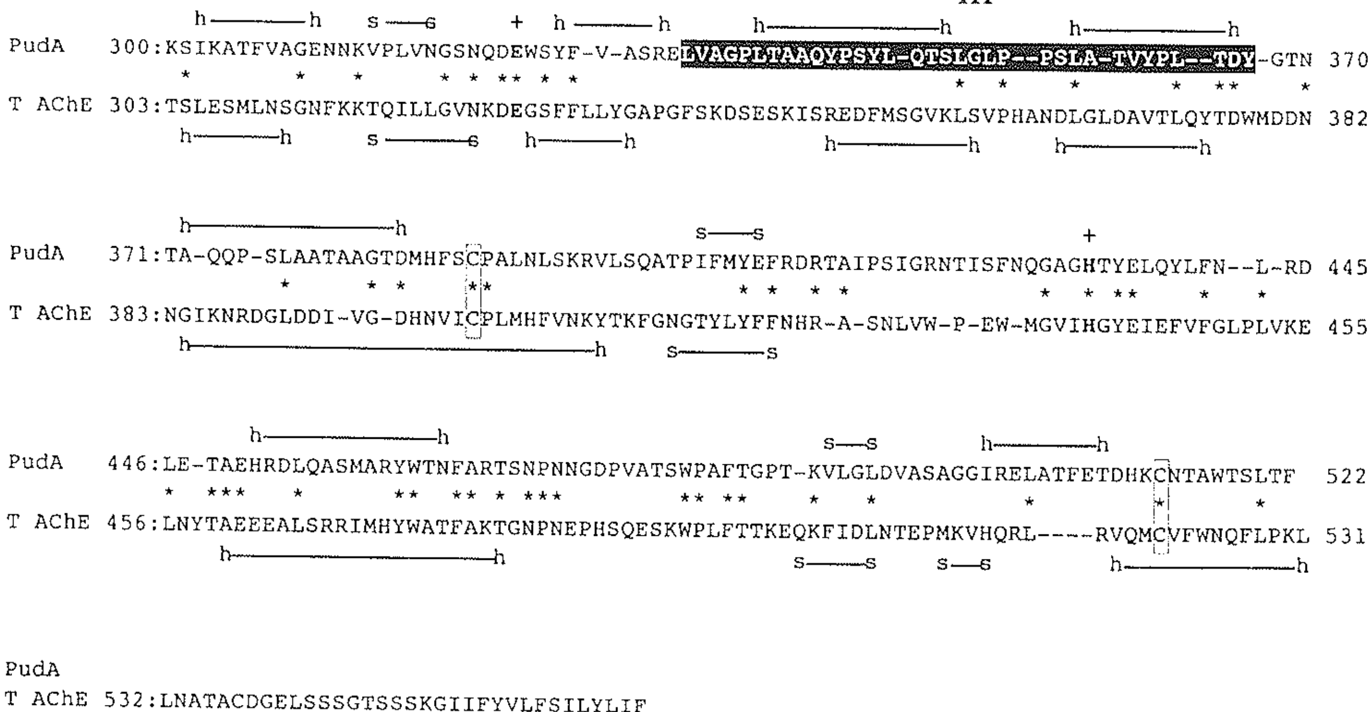


Figure 6.5. Comparison of amino acid sequences between *Comamonas acidovorans* PUR esterase (PudA) and *Torpedo californica* acetylcholinesterase (T AChE) (Sussuman *et al.*, 1991). Residues that are identical in both sequences are denoted by an asterisk (single-letter amino-acid notation). Gaps are indicated by hyphens. Regions of I, II, and III are shown in the black boxes. The secondary structure elements observed in PUR esterase and T AChE are shown above and below the sequence, respectively. α-helix is shown as h-h; β-strand is shown as s-s; +, residues from the catalytic triad; the pattern for Cys-Cys bridges are also shown in the boxes.

acid enzyme. The recombinant protein expressed in *E. coli* can degrade solid PUR. The amino acid sequence of this enzyme shows only about a 30% homology to the acetylcholinesterase from *Torpedo californica* (T AChE) (Schumacher *et al.*, 1986) and the lipase from *Geotrichum candidum* (GcL1) (Shimada *et al.*, 1990), respectively. Although alignments of PudA never showed an amino acid homology of more than 30% (T AChE), residues of similar sequences between PudA and T AChE extend throughout the entire amino acid sequence of these two proteins (*Figure 6.5*). Some of these residues were seen to occur at a high degree of conservation.

Although T AChE and GcL1 hydrolyse vastly different substrates, the determined three-dimensional (3D) structures of T AChE (Sussuman *et al.*, 1991) and GcL1 (Schrag *et al.*, 1991) have revealed a surprising degree of structural similarity that extends through the whole length of the polypeptide chain. It was also reported that the root-mean-square deviation between the 399 corresponding Cα atoms after superposition of the two molecules was 1.90 Å (Ollis *et al.*, 1992). The same topological fold, named the α/β hydrolase fold, has been identified in a number of other hydrolases with no sequence similarity to either T AChE or GcL1 or to each other. Then, based on the X-ray structures of T AChE and GcL1 and on their three-dimensional superposition, an improved alignment of 32 related amino acid sequences was generated and a collection of other esterases, lipases, and related proteins was obtained (Cygler *et al.*, 1993). The conserved residues include the active site, disulphide bridges, salt bridges, and residues in the core of the proteins. Most invariant residues are located at the edges of secondary structural elements. Greer coined the term '*structurally conserved regions*,' or *scaffold SCRs*, to describe these common regions, thus grouping proteins into families based on the superposition of their 3D structures (Greer, 1990). Then, Cygler *et al.* (1993) indicated that a clear structural basis for the preservation of many of these residues (SCRs) can be determined by comparing the two X-ray structures.

The structure of the PUR esterase (PudA) was deduced according to its amino acid sequence (Nomura *et al.*, 1998). The secondary structure of PudA was calculated by computer analyses and the result was compared with T AChE, which had already been identified by three-dimensional analysis (Sussuman *et al.*, 1991). It was shown that the number and the positions of the α-helix and β-strand regions in PudA are similar to T AChE (*Figure 6.5*). Secondary structure assignments are listed in *Table 6.4*. The amino acid sequence homology and positions of these conserved residues between PudA and T AChE/GcL1 are shown to occur in the active sites, cystein bonds, salt bonds, and other scaffold SCRs. Most of the strictly conserved residues are located in turns and loops at the edges of the β-strands or α-helices and seem to be important for maintaining the proper fold of the backbone. Based on the results of these analyses, the three-dimensional structures of PudA and T AChE must be nearly identical (*Figure 6.6*).

There are three regions (I, II and III) containing many hydrophobic amino acid residues in PudA and although the corresponding regions exist in T AChE, they are not hydrophobic (*Figure 6.5*). α-helix secondary structures exist in these three regions. The corresponding three regions in T AChE are positioned on the outer surface of the T AChE molecule (*Figure 6.6*). Interestingly, one of these hydrophobic regions in PudA exhibited significant homology to the substrate-binding domain of the PHA (poly(hydroxyalkanoate)) depolymerase (Jendrossek *et al.*, 1995a). It is

Table 6.4. Structural context of conserved residues[a]

Residue[b]	Context	Possible function
C [74, 67, 61]	C x Q	S-S bridge to C [92, 94, 105]
E [90, 92, 103]	s E D C L y	Salt bridge
G [115, 117, 130]		
G [116, 118, 131]	G G G/A F/L x x G	Oxyanion hole loop
G [121, 123, 136]		
Y [144, 148, 164]		Ring stacking
R [145, 149, 165]	Y R v g x x G F/L	Salt bridge
Q [150, 154, 170]		Packing against R [145, 149, 165]
N [166, 167, 184]	N x g l	Anchoring
N [182, 183, 200]		
F [186, 187, 204]		
G [187, 188, 205]	N i a x F G G d p	Packing
G [188, 189, 206]		
G [197, 198, 215]		Close packing
S [199, 200, 217]	V/I x I/L f G e S A G A/G	Active site
G [201, 202, 219]		Close packing
S [225, 226, 249]	i x x S G	H-bond to E [324, 327, 354]
C [258, 254, 276]		S-S bridge to C [265, 265, 288]
E [324, 327, 354]	d E g	Active site
D [385, 397, 425]	D x x V/F	Salt bridge
H [433, 440, 463]	g x x H x x E/D	Active site
F [465, 476, 488]	W/F x x F A	Packing volume

[a]One-letter code is used. In the context column a capital letter identifies a highly conserved residue, X/Y means that only residue X or Y is observed in this position, small letter indicates the type of residue with the highest abundance, and the letter x indicates a variable position.
[b]Three sequence numbers are shown: the first corresponds to the PudA numbering, and the second and third to the T AChE and GcL 1 numbering, respectively.

thought that some or all of these hydrophobic regions could be necessary for binding to the insoluble solid PUR surface.

PudA possesses a high degree of homology with the T AChE/GcL1 serine hydrolase family only in the catalytic regions which contain the Ser-His-Glu catalytic triad (*Table 6.5*). Interestingly, the glutamate residue replaces the usual aspartate residue. Comparison of the positions of each residue in the Ser-His-Glu catalytic triad reveals that the amino acid residues for PudA are similar with T AChE, GcL1 and human cholinesterase (H ChE). This infers that prokaryotic esterases possess the Ser-His-Glu catalytic triad as the active site. To confirm Glu instead of Asp as necessary for activity in prokaryotic esterase, site directed mutagenesis was performed. Results from this have demonstrated that each residue in the Ser-His-Glu catalytic triad is in fact essential for enzymatic activity (Akutsu *et al.*, unpublished results).

DEGRADATION MECHANISM

Enzymatic reactions against water-soluble substrates proceed rapidly because the enzyme molecules can easily come into contact with the substrates. However, solid substrates are thought to have extremely low contact efficacy with enzyme molecules. In order to overcome this problem, enzymes which degrade solid substrates are thought to possess some characteristics which enable them to be adsorbed onto the surfaces of the solid substrates (vanTilbeurgh *et al.*, 1986; Fukui *et al.*, 1988; Hansen, 1992).

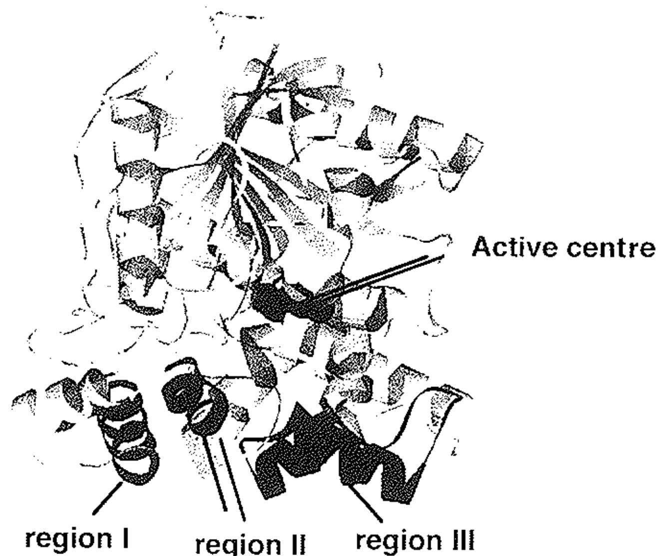


Figure 6.6. Three-dimensional structure of T AChE (PDB No. 2ACE). Putative positions for the Ser-His-Glu catalytic triad (active centre) and three regions (I, II and III) which contain many hydrophobic amino acid residues in PudA are indicated.

Table 6.5. Alignment of amino acid sequence of the PUR esterase with the sequences of four Ser-His-Glu type serine hydrolases

		Serine (S)[a]		Glutamate (E)		Histidine (H)
PudA[b]	199	FGGDKSNVTIF**GESAG**GFSV	324	GSNQD**E**WSYF	433	GAG**H**TYELQYLF
T AChE	200	FGGDPKTVTIF**GESAG**GASV	327	GVNKD**E**GSFF	440	GVI**H**GYEIEFVF
H ChE	198	FGGNPKSVTLF**GESAG**AASV	325	GVNKD**E**GTAF	438	GVM**H**GYEIEFVF
GcL 1	218	FGGDPDKVMIF**GESAG**AMSV	354	GNQED**E**GTAF	463	GTF**H**GNELIFQF
PnbA	167	FGGDPDNVTVF**GESAG**GMSI	288	GTTRD**E**GYLF	377	KAF**H**ALELPFVF
Consensus		FGGdp--Vt*FGESAG*-Sv		G---DEg*-F		g--H--E*-f-F

[a] The positions of serine, glutamate and histidine in the primary sequences of the corresponding mature proteins are indicated.
[b] PUR esterase from *C. acidovorans* TB-35 (PudA), acetylcholinesterases of *T. californica* (T AChE) (Schumacher *et al.*, 1986), cholinesterase of humans (H ChE) (Lockridge *et al.*, 1987), *Geotrichum candidum* lipase isoformI (GcL 1) (Shimada *et al.*, 1990), *p*-nitrobenzyl esterase from *Bacillus subtilis* (Zock *et al.*, 1994).

Various degradation enzymes for many kinds of solid substrates of natural origin, such as PHA, cellulose, and chitin, have been found (Jendrossek *et al.*, 1996; Ohmiya *et al.*, 1997; Felse and Panda, 1999). Almost all of these enzymes display the same modular structure as PHA depolymerases, which consist of two separate domains, namely the catalytic domain and the substrate surface-binding domain (*Figure 6.7*). These domains are linked by a flexible linker domain, which is a threonine-rich region (Jendrossek *et al.*, 1995b) or a fibronectin region (Hansen, 1992). These enzymes adhere strongly to the solid substrate before it is degraded. Results from the observations of PHA degradation demonstrate a significant detriment in the modular

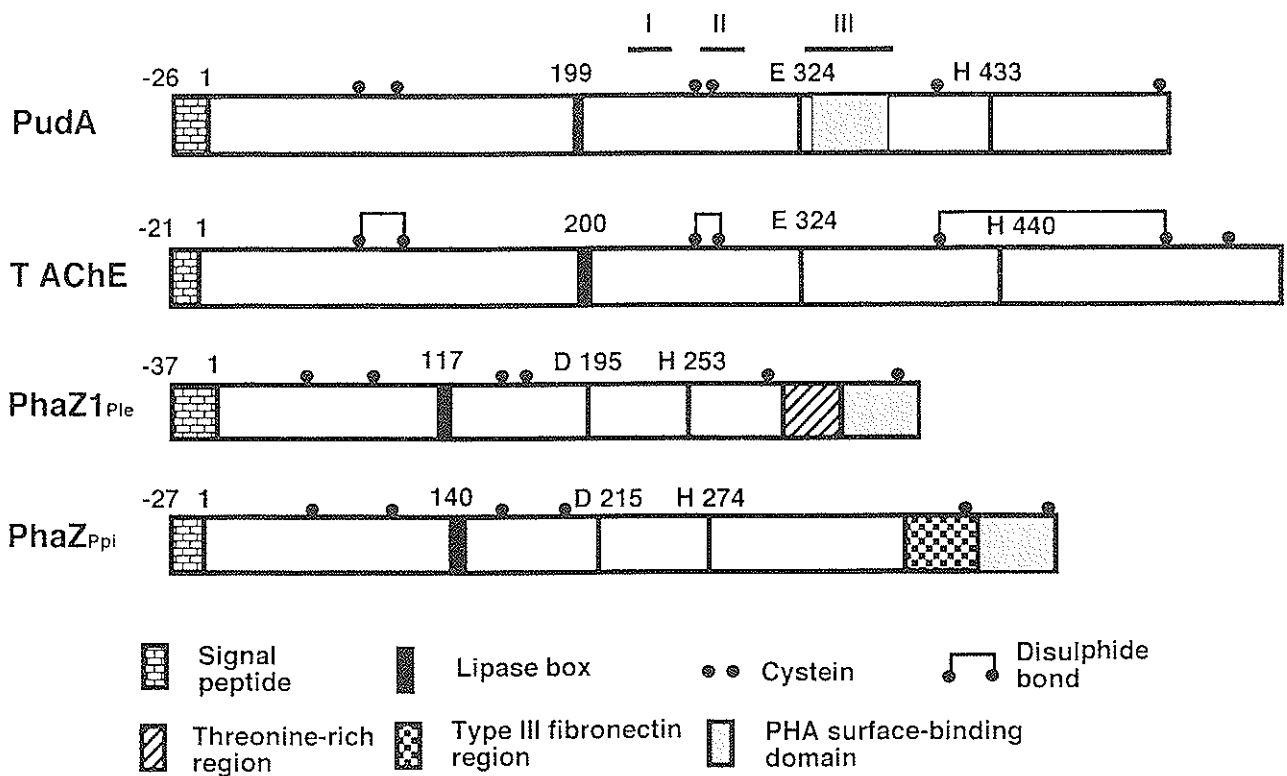


Figure 6.7. Comparison of the domain structures. The domains for *Comamonas acidovorans* PUR esterase (PudA), *Torpedo californica* acetylcholinesterase (T AChE) (Sussman *et al.*, 1991), and bacterial PHA depolymerases (PhaZ*ple* and PhaZ*ppi*) (Jendrossek *et al.*, 1995a,b) are shown.

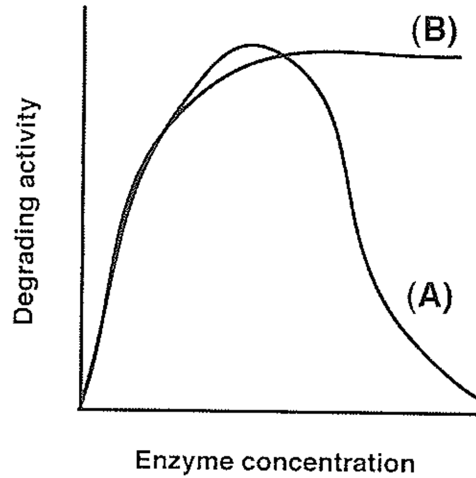
structure of the PHA depolymerase (Mukai *et al.*, 1993). The degradation activity of PHA depolymerase has been shown to decrease in the presence of excess enzyme (*Figure 6.8A*). This suggests that the PHA surface becomes saturated with only the binding domains of PHA depolymerase, so much so that the catalytic domain cannot gain access to the PHA surface.

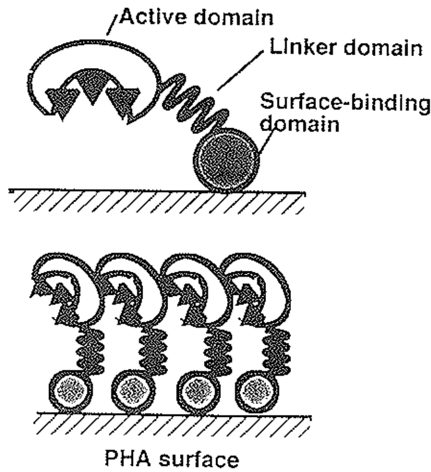
On the other hand, the PUR esterase degradation activity did not decrease but rather remained constant when an excess of the enzyme was present (*Figure 6.8B*). It is thought that since the number of adsorbable enzyme molecules per unit surface area of solid PUR is fixed, the PUR degradation activity should remain constant (*Figure 6.8B*) (Akutsu *et al.*, 1998). Based on this hypothesis, it has been inferred that the surface-binding site and the catalytic site of the PUR esterase exist in a three-dimensionally closer position when compared to the PHA depolymerase. Unlike the PHA depolymerase, the flexible linker domain is not observed in PudA (and T AChE), and three putative binding domains of which contain many hydrophobic residues are located in the centre of the enzyme's polypeptide chain (*Figure 6.7*). These observations indicate that the structure of PudA without the linker domain is significantly different compared to the PHA depolymerase which contains the modular structure with the linker domain. Therefore these observations and the remarkable structural similarities with T AChE suggest that the three putative binding domains of PudA are positioned close to the catalytic domain without the linker domain in three-dimensional space (*Figure 6.6*). These conclusions are consistent with the fact that the catalytic domain can gain access to the PUR surface even if the PUR surface is saturated with enzyme molecules (*Figure 6.8B*).

The structural properties of PudA can explain reasonably the occurrence of two esterases from *C. acidovorans* TB-35. Despite the amino acid sequence for the CBS esterase being identical to the PUR esterase, the former can only degrade poly(diethylene glycol adipate) but not PUR (Akutsu *et al.*, 1999). It is necessary for the PUR esterase to expose its hydrophobic PUR-binding domains to the enzyme exterior. The hydrophobicity of CBS esterase has been shown to be significantly lower than the PUR esterase. Since CBS esterase contains the sequence for the putative PUR-binding domain, the binding domain would appear to be buried within the body of the molecule, perhaps as a result of the folding and transportation processes. Therefore, it has been assumed that the reduced hydrophobicity of the CBS esterase resulting from the three-dimensional structure of PUR-binding domains is related to its lack of ability to degrade solid PUR.

Conclusions

Among all of the synthetic compounds which are used in our daily lives, water-insoluble non-naturally occurring synthetic polymers are the most resistant to microbial attack. However, microorganisms which can degrade these water-insoluable synthetic polymers have turned up in the environment due to environmental conditions or their ability to adapt. Currently, these microorganisms have received much attention because of the environmental problems of waste polymers, and the possible industrial use of modified polymers (Deguchi *et al*, 1998). In this review, we have considered in detail two enzymes, Mn peroxidase and PUR esterase which are able to degrade nylon and polyurethane, respectively. Both of the enzymes described in this review





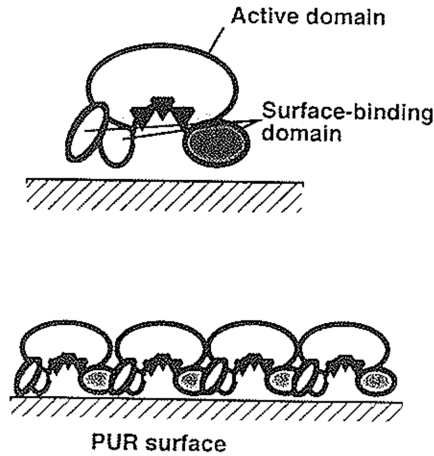


Figure 6.8. Effects of PHA depolymerase and PUR esterase concentrations on the degradation of solid substrates, and kinetic models of these enzymes. (**A**) PHA depolymerase; (**B**) PUR esterase.

have been found to have abilities to degrade synthetic polymers, but each has other biochemical roles necessary for the maintenance of the cell's life cycle. Modifications to these enzymes may generate the ability to attack and degrade other substances. Further research is needed to expand the repertoire of these enzymes and to develop new enzymes which can attack currently non-biodegradable substances which plague our modern lives.

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