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Catalytic Triad of Intracellular Poly(3-Hydroxybutyrate) Depolymerase (PhaZ1) in *Ralstonia eutropha* H16

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The amino acid sequence of an intracellular poly[D(−)-3-hydroxybutyrate] (PHB) depolymerase (PhaZ1) from *Ralstonia eutropha* H16 was compared with the sequences of various proteins using the BLAST search. It showed a number of matches including with intracellular PHB depolymerases, conserved hypothetical proteins, and PHB synthases. From an alignment of these proteins, we constructed nine mutants: C87A, S118A, H120Q, C183A, C183S, D355A, D356A, C370A, and H388Q. The C183A, D355A, and H388Q mutants lost their activities, but C183S and the other mutants did not. C183, D355, and H388 in PhaZ1 were positioned similarly to the amino acids of the catalytic triad of PHB synthase. These results indicated that C183, D355, and H388 make up the catalytic triad of PhaZ1.

[**Key words:** *Ralstonia eutropha* H16, poly(3-hydroxybutyrate) (PHB), intracellular PHB depolymerase, catalytic triad]

Poly(D-3-hydroxybutyrate) (PHB), a homopolymer of D(−)-3-hydroxybutyrate (3HB), is a storage material produced by many bacteria under certain conditions (1, 2). In the past few decades, the application of this biopolymer to biodegradable polymers/plastics has been studied extensively (3). In these studies, several genes involved in the biosynthesis of PHB have been cloned and characterized in detail. Although the degradation of crystalline PHB by extracellular PHB (ePHB) depolymerases has been examined extensively (4, 5), intracellular degradation of PHB is not well understood, and the biochemical and genetical clarification of the intracellular degradation system is awaited (6–11). The intracellular PHB degradation system in *Rhodospirillum rubrum* was first reported in 1964, and consisted of a thermostable activator and a thermolabile esterase (6). This system is still not well understood in spite of a recent reinvestigation (7). The molecular cloning of an intracellular PHB (iPHB) depolymerase from *Ralstonia eutropha* H16 has been reported (8). This enzyme ($PhaZ1_{Reu}$), which localizes solely on PHB inclusion bodies, degraded artificial amorphous PHB granules, but not crystalline PHB. Recently, a protein homologous to $PhaZ1_{Reu}$ was identified as an iPHB depolymerase ($PhaZ1_{Pde}$) from *Paracoccus denitrificans* (12) and two new candidates of PHB depolymerases homologous to $PhaZ1_{Reu}$ were cloned from *R. eutropha* H16 (13). All ePHB depolymerases examined so far contain a typical lipase box sequence (Gly-X_1-Ser-X_2-Gly) and are regarded as serine-dependent hydrolases (4, 5). However, PhaZ1 does not contain such a sequence, and the amino acid residues essential to the catalysis are unknown. To understand the PHB metabolism, it is important to know how intracellular PHB depolymerases degrade the PHB granules and which amino acid residues of PHB depolymerase are involved in catalysis.

In this report, the amino acid residues in $PhaZ1_{Reu}$ that play a role in catalysis were identified by site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids *Escherichia coli* BLR(DE3)pLysS (F⁻*ompT hsd*S_B($r_B^-m_B^-$) *gal dcm* Δ(*srl-recA*)*306*::Tn*10* (Tc^r) DE3 pLysS (Cm^r); Novagen, Madison, WI, USA) which was used as the host cell for the recombinant plasmids carrying the wild-type and mutated iPHB depolymerase genes (*phaZ1*) was grown in Luria–Bertani (LB) medium supplemented with ampicillin (50 μg/ml), tetracycline (12.5 μg/ml), and chloramphenicol (34 μg/ml). *E. coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]; Takara Shuzo, Kyoto) which was used for plasmid manipulation was grown in LB medium at 37°C.

Site-directed mutagenesis Site-directed mutations were introduced by a two-step PCR approach into the His-tagged wild-type gene (8) in an expression vector (pET23b; Novagen). DNA fragments of the N-terminal side and C-terminal side of the cloned wild-type gene were amplified separately in two independent PCRs. Each reaction mixture contained a complementary mutagenic primer. Primers used in this study are listed in Table 1. Each primer contained one to three base changes, which create serine, alanine, and glutamine codons. The flanking primers used were T7 promoter primer #69348-1 and T7 terminator primer #69337-1 (Novagen). Both fragments were mixed and amplified by PCR using T7 primers. The product was cleaved with *Nde*I and *Xho*I, and ligated into the corresponding sites of pET23b. The ligated DNA was introduced into *E. coli* JM109 cells. The changes in the nu-

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TABLE 1. Primers used in this study

Mutation	Strain	Sequence (5′→3′)
C87A	C87A-1	CACCAGCTTGGCGAACGGCTTTTCAAGCACGGTCTGCTC
	C87A-2	TTGAAAAGCCGTTCGCCAAGCTGGTGCGCTTCAAGCGC
S118A	S118A-1	ATGGTGGCCCGCCAGCGGCGCGGCCACCAGCAC
	S118A-2	CGCGCCGCTGGCGGGCCACCATGCCACGCTGC
H120Q	H120Q-1	CAGCGTGGCATGTTGGCCCGACAGCGGCGCGGC
	H120Q-2	GCTGTCGGGCCAACATGCCACGCTGCTGCGCGAC
C183A	C183A-1	CGGTGGGCTGGGCTACCGAGATCACATGCAGGTTCTCGGCGCC
	C183A-2	CATGTGATCTCGGTAGCCCAGCCCACCGTGCCGGTGCTG
C183S	C183S-1	CGGTGGGCTGGGATACCGAGATCACATGCAGGTTCTCGGCGCC
	C183S-2	CATGTGATCTCGGTATCCCAGCCCACCGTGCCGGTGCTG
D355A	D355A-1	CCGAGATGTCGGCCAGTTCGCCCTCGACGGTCATC
	D355A-2	GGGCGAACTGGCCCGACATCTCGGGCGCGGGCC
D356A	D356A-1	CGCCCGAGATGGCGTCCAGTTCGCCCTCGACGG
	D356A-2	CGAACTGGACGCCATCTCGGGCGCGGGCCAGAC
C370A	C370A-1	GGATGCCGGCGGCCAGGTCGTGCGCCGCGGC
	C370A-2	GCACGACCTGGCCCGCCGGCATCCCGAAAATCCGC
H388Q	H388Q-1	AGAAGATGCCGTATTGGCCGCAGTGTGCCGCGTTC
	H388Q-2	CACTGCGGCCAATACGGCATCTTCTCGGGCCGGC

Mutated codons are underlined.

cleotide sequence of the plasmids obtained were verified by nucleotide sequencing.

Expression and purification of recombinant enzyme *E. coli* BLR(DE3)pLysS was transformed with pET171H and the mutant plasmids. The transformed cells were grown in LB medium at 27°C with vigorous shaking. At an OD 600 nm of 0.6, the culture temperature was lowered to 22°C, and the gene expression was induced by the addition of isopropyl-β-D-thiogalactoside at a final concentration of 10 μM. The cultures were incubated overnight. All subsequent procedures were carried out at 4°C or below. Bacteria were harvested by centrifugation, and the cells were suspended in 20 mM Tris–HCl (pH 8.0). The resuspended cells were sonicated on ice for 4 min (20 kHz, 30 W), and centrifuged at 10,000×*g* for 30 min. The supernatant was mixed with glycerol (20% v/v, final concentration) and used as the crude extract for purification. The crude extract was applied to a Co-chelating column (1 ml, HiTrap; Amersham Bioscience, Tokyo), and the column was washed with 20 mM sodium phosphate (pH 7.4) containing 20% glycerol and 0.5 M NaCl. The enzyme was eluted with a linear gradient of imidazole (total volume 15 ml, 0–0.5 M) and the activity was found at about 350 mM of imidazole. The purified enzyme was dialyzed against 10 mM Tris–HCl (pH 8.0) containing 50% glycerol overnight.

Enzyme assays For PHB depolymerase activity, the released 3HB was routinely assayed by the enzymatic method using D(−)-3HB dehydrogenase and hydrazine hydrate (14). The reaction mixture (100 μl) was composed of 100 mM Tris–HCl (pH 8.5), artificial amorphous PHB granules (0.5 mg/ml as a solid), and enzyme. The reaction was started by the addition of substrate at 30°C. The reaction was stopped by the addition of 6 M HCl to pH 2 and vortexed for 5 min at room temperature. The reaction mixture was centrifuged at 15,000×*g* for 10 min. The supernatant fraction was used for the quantification of 3HB. The 3HB-oligomers in the supernatant fraction were completely hydrolyzed by 0.05 unit of extracellular 3HB-oligomer hydrolase from *Ralstonia pickettii* (formerly *Alcaligenes faecalis*) T1 (15). For 3HB-oligomer hydrolase activity, 3HB-oligomers (10 mM) were used as substrates instead of artificial amorphous PHB granules. The linear 3HB-oligomers were prepared as described previously (16). Artificial PHB granules and 3HB-oligomers used were stable at pH 8.5 for at least 30 min, and these esters were also stable at pH 2 during the assay. Artificial PHB granules were prepared according to a previous report (8) except that sodium deoxycholate was used instead of sodium oleate. To investigate the effect of inhibitors, each concentration of inhibitors was added in the reaction mixture and preincubated for 10 min at room temperature. The reaction was started by addition of a substrate.

Protease K proteolysis Purified wild-type and mutant proteins were subjected to limited protease K proteolysis as follows. Protease K was used to digest a 1000-fold-molar excess of the target protein in a buffer containing 25 mM Tris–HCl (pH 8.0) and 50 mM NaCl. Digestions were performed at 30°C and were stopped at 5-min intervals by adding phenylmethylsulfonyl fluoride (1 mM final concentration). A portion of the mixture was withdrawn and analyzed by SDS–PAGE.

Other methods Protein concentrations were measured by the method of Lowry *et al*. (17) with bovine serum albumin as the standard. The purified proteins were examined for purity and size by SDS–PAGE as described by Laemmli (18). Proteins on the gel were stained with Coomassie Brilliant Blue R250.

RESULTS

Comparison of amino acid sequences and construction of mutated PhaZ1 A BLAST search for amino acid sequences similar to that of $PhaZ1_{Reu}$ from *R. eutropha* yielded a number of matches, and we selected 10 proteins for sequence alignment using the Clustal W program. The proteins used in this analysis were PhaZ1 from *Ralstonia eutropha* (O87189) and *Paracoccus denitrificans* (Q9WX79), conserved hypothetical proteins (probably PhaZ1) from *Ralstonia solanacearum* (Q8Y0N5), *Xanthomonas axonopodis* (Q8PMM6), *Agrobacterium tumefaciens* (Q8UJB3), *Rhodospirillum rubrum* (AA062349), *Sinorhizobium meliloti* (Q92TD3), *Methylobacterium extorquens* (Q9AP15), and *Rickettsia prowazekii* (Q9ZCP2), and PHB synthases from *Bacillus megaterium* (Q9ZF92), and *Chromatium vinosum* (P45370) (identity at the amino acid level for $PhaZ1_{Reu}$: 42%, 86%, 66%, 50%, 47%, 47%, 47%, 44%, 21%, and 25%, respectively). The result showed several conserved amino acid residues and regions. Particularly, cysteine 183, aspartate 355, and histidine 388 were completely conserved in PhaZ1s and two synthases (Fig. 1). Since serine, histidine,

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R. eutropha     ----------MLYQLHEFQRSILHPLTAWAQATAKTFTNPLSPLSLVPGAPRLAAGYELL
    A                                                                    :
C. vinosum      -----------MFPID------IRPD-------KLTQ--------------------EML
    B

                                                        87
R. eutropha     YR-LGKEYEKPAFDIKSVRSNGRDIPIVEQTVLEKPFCKLVRFKRYADDPETIKLLKDEP
    A            *   :   ** : :  .   ..   :    :   **  :  * :
C. vinosum      DY--SRKLGQGMENLLNAEA-IDTGVSPKQAVYSEDKLVLYRYDRPEGAPEAQ----PVP
    B                :   :    :  .           :       :  :

                     118 120
R. eutropha     VVLVAAPLSGHHAT---LLRDTVRTLL-QDHKVYVTDWIDARMVP-VEEGAFHLSDYIY-
    A            :*: **::**::*   *** ** ::*     :::*** :*. :*    * * :  *
C. vinosum      LLIVYALVNRPYMTDIQEDRSTIKGLLATGQDVYLIDWG----YPDQADRALTLDDYING
    B            ::: * :   :          .:  :*     ::: **      *      : :  *

                                                183
R. eutropha     YI---QEFIR-HIGAENLHVISVCQPTVPVLAAISLMASAGEKTPRT-MTMMGGPIDARK
    A           *:   :  :   :*    : :.**** .* ::: :*:       * ::  ::***:*.
C. vinosum      YIDRCVDYLREAHGVDKVNLLGICQG--G---AFSLMYSALHPDKVRNLVTMVTPVDFK-
    B           *:      :    .      :. *        :::            :  :  *.*

R. eutropha     SPTAVNSLATNKSFEWFEN-NVIYTVPANYPGHGRRVYPGFLQHAGFVAMNPDRHLSSHY
    A            .* *. :.      :        *      .  * ****: * :.*:::*   *  :.
C. vinosum      TP--DNLLSA-----WVQNVDIDLAVD-T---MG-NI-PG--ELLNWTFLSL-KPFSLTG
    B                :        .        *          : *   :  .:

R. eutropha     DFYLSLVEGDADDAEAHVRFYDEYNA-VLDMA--A-EYYLDTIREVFQEFRLANGTWAID
    A           : :     .: . .:    ***** :  :*:.  * *:**.*:  :* :  :...
C. vinosum      QKYVNMV--DLLDDPDKVKNFLRMEKWIFDSPDQAGETFRQFIKDFYQNNGFLNGGVVLG
    B             :      :          :       * .  * * : . :  .: :  : ..

                                  355 356          370                 388
R. eutropha     GNPVRPQDIKSTALMTVEGEHDDISGAGQTAAAHDICAGIPKIRKQHLNAAHCGHVGIFS
    A            . :   *    :: :**  ***:. **  *.  :  .:     :          ****:*
C. vinosum      GQEVDLKDI-TCPVLNIFALQDHLVPPDASRALKGLTSS-PD--YTELAFP-GQHIGIYV
    B            . :   *    :: : .  *.:       *                        ** .:

R. eutropha     GRRWREEIYPQLRDFIRKYHQASATR
    A           *  ::  : * :  *
C. vinosum      SGKAQKEVTPAIGKWLNER-------
    B           .       * :  :
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FIG. 1. Results of multiple alignment for 11 amino acid sequences using the Clustal W program. PhaZ1 from *Ralstonia eutropha* was compared with 10 proteins as follows: iPHB depolymerases from *Paracoccus denitrificans*, conserved hypothetical proteins from *Ralstonia solanacearum*, *Xanthomonas axonopodis*, *Agrobacterium tumefaciens*, *Rhodospirillum rubrum*, *Sinorhizobium meliloti*, *Methylobacterium extorquens*, and *Rickettsia prowazekii*, and PHA synthase from *Bacillus megaterium*, and *Chromatium vinosum. R. eutropha*, Amino acid sequence of PhaZ1 from *R. eutropha*. Mutational positions are indicated by reversed characters. *C. vinosum*, Amino acid sequence of PHB synthase from *C. vinosum*. The catalytic triad were indicated by boxes and the modified lipase box is underlined. A, Result with Clustal W for iPHB depolymerases and conserved hypothetical proteins; B, result with Clustal W for all proteins. Asterisk (*), Conserved in all proteins; double dots (:), substitution of the amino acid residue with one of high similarity; dot (.), substitution of the amino acid residue with one of slightly lower similarity.

and aspartate residues are involved in the catalytic triad in many hydrolases and a cysteine residue is involved in the active center of PHB synthases, we thought these amino acid residues may be important for activity. Serine 118, histidine 120, and aspartate 356 were shown to be highly conserved in PhaZ1s using Clustal W program. Although cysteine 87 or 370 was not shown to have similarity using the Clustal W program, it was highly conserved in PhaZ1s: cysteine 87 was conserved in seven proteins, and cysteine 370 was conserved in eight proteins. From these results, we constructed nine mutants: C87A, S118A, H120Q, C183A, C183S, D355A, D356A, C370A, and H388Q (*e.g.*, C87A indicates that cysteine 87 was changed to alanine).

Purification of the wild-type and mutant enzymes $PhaZ1_{Reu}$ and its mutants were constructed as His-tagged proteins at the C-terminus, and were purified from *E. coli* BLR(DE3)pLysS harboring plasmids with those genes. All enzymes were applied to a Co-chelating column and eluted in the same manner with 0–400 mM of imidazole. The final preparations showed apparent homogeneity on SDS–PAGE with a molecular mass of about 48 kDa (Fig. 2). The chemically determined amino acid sequence at the N-terminus of

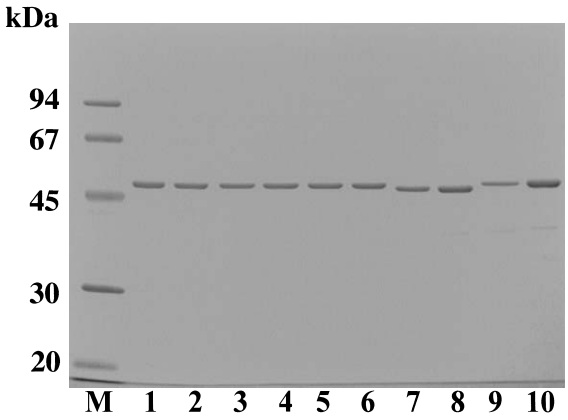

FIG. 2. SDS–PAGE of the purified wild-type and mutants. Lane M, Molecular mass markers; 1, wild-type; 2, C87A; 3, S118A; 4, H120Q; 5, C183A; 6, C183S; 7, D355A; 8, D356A; 9, C370A; 10, H388Q. One microgram of purified protein was used for analysis.

the purified enzyme corresponded to that deduced from the nucleotide sequence (8) (data not shown). D355A and D356A showed a slightly lower molecular mass on SDS–PAGE, but judging from the nucleotide sequence, N-terminal amino acid sequences, and the presence of the His-tag at the C-terminus, these mutants also had the same primary structure as the wild-type except for one position. It is possible that a slight lower molecular mass on SDS–PAGE observed in D355A and D356A is due to partial resistance of two mutants to denaturation with SDS.

Enzyme activity Table 2 shows the degradation activity of all enzymes for artificial amorphous PHB granules and 3HB-oligomers. C183A, D355A, and H388Q did not degrade either substrate. C87A showed a slightly lower activity toward PHB granules than the wild-type. S118A degraded the PHB granules at a significantly lower rate. C370A degraded PHB granules more effectively than the wild-type when the substrate was used at 1 mg/ml, although it had similar degradative activity toward 3HB-oligomers. C87A, S118A, and C183S showed a slightly lower 3HB-oliogmer hydrolase activity. H120Q degraded PHB granules at a similar rate to the wild-type, but 3HB-oligomer hydrolase activity was very low. The mutants with activity hydrolyzed the 3HB-tetramer, pentamer, and PHB granules, but did not hydrolyze the dimer and trimer in the same way as the wild-type (Table 2).

Structural properties Mutants and the wild-type were treated with protease K, and the degradation patterns were analyzed by SDS–PAGE. Figure 3 shows the patterns of the wild-type, C183A, D355A, D356A, and H388Q. All mutants, including mutants not shown in Fig. 3, showed similar patterns except for D355A and D356A. Although the degradation products of D355A and D356A had a slightly lower molecular mass than other proteins at initial proteolysis, it seems that the degradation pattern is essentially similar in all proteins. At least, the degradation patterns of these two proteins were similar to each other. Since D356A retained the enzyme activity, it seems that D355 did not loose its activity due to incorrect folding of the protein. These results indicate that the mutants were not inactivated by structural factors such as incorrect folding or low stability of the en-

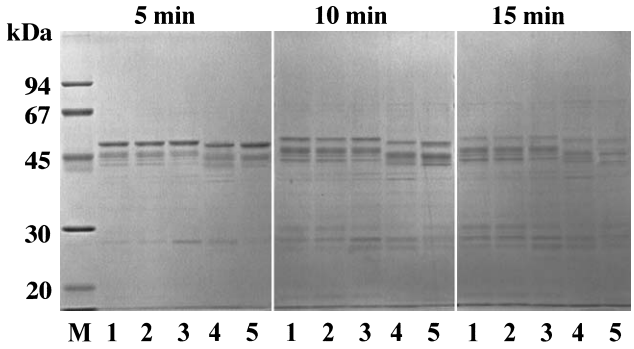

FIG. 3. Protease K degradation of wild-type, C183A, D355A, D356A, and H388Q. Purified enzymes were subjected to limited protease K digestion as described in Materials and Methods. Samples (2 μg each) were removed at 5-min intervals and analyzed by SDS–PAGE. Lane M, Molecular mass markers; 1, wild-type; 2, C183A; 3, H388Q; 4, D355A; 5, D356A.

zyme.

Effect of various compounds Table 3 shows the effect of various compounds on enzyme activity. When amorphous PHB granules were used as a substrate, 0.1% Triton X-100 fully inhibited the activity. The 3HB-tetramer-hydrolyzing activity of the wild-type and mutants was significantly inhibited by 0.1% Triton X-100 (2–22%, residual activity) except for C183S, which showed increased activity toward the 3HB-tetramer in the presence of the detergent. Dithiothreitol (DTT) did not affect the activity for degrading PHB granules, but for degrading 3HB-tetramer, wild-type, C87A, S118A, and D356A revealed a slightly increased activity. Twenty μM *p*-chloromercuribenzoic acid (PCMB) completely inhibited all the enzymes except for C370A, which was not inhibited by 20 μM PCMB with either substrate. Interestingly, the activity of the C183S mutant was strongly inhibited by diisopropylfluorophosphate (DFP), although the wild-type and other mutants were only slightly inhibited.

DISCUSSION

In this report, we constructed nine PhaZ1 mutants and examined their properties. A database search uncovered several proteins similar to $PhaZ1_{Reu}$. They include PhaZ1 from *P. denitrificans*, putative iPHB depolymerases, and PHB synthases, but ePHB depolymerases were not included among them. As far as we know, all bacteria whose genome analysis has finished have *phaZ1* or a similar gene whenever they contain a PHB synthase gene. It is possible that the primordial bacteria which gained the ability to synthe-

TABLE 2. Degradation activity of wild-type and mutants against PHB granules and 3HB-oligomers

Enzyme	PHB-degrading activity[a] (μmol/min/mg)		Oligomer hydrolase activity[b] (μmol/min/mg)			
	0.5 mg/ml	1 mg/ml	2 mer	3 mer	4 mer	5 mer
Wild-type	710	460	ND	ND	1100	1200
C87A	460	310	ND	ND	790	950
S118A	50	34	ND	ND	400	640
H120Q	900	830	ND	ND	50	90
C183A	ND	ND	ND	ND	ND	ND
C183S	800	460	ND	ND	730	730
D355A	ND	ND	ND	ND	ND	ND
D356A	690	290	ND	ND	1200	1300
C370A	970	1400	ND	ND	1300	1300
H388Q	ND	ND	ND	ND	ND	ND

ND, Not detected.
[a] PHB-degrading activity was measured using 0.5 or 1 mg/ml of artificial amorphous PHB granules.
[b] Oligomer hydrolase activity was measured using 10 mM of 2–5 mer of 3HB.

TABLE 3. Effect of various compounds

Enzyme	Substrate	Relative activity (%)							
		DFP (mM)		PCMB (μM)		DTT (mM)		Triton X-100 (%, w/v)	
		1	10	20	100	10	100	0.01	0.1
Wild-type	PHB-granules	97	64	0	0	97	97	89	0
	3HB-tetramer		66	0			150		12
C87A	PHB-granules	72	36	0	0	110	90	76	0
	3HB-tetramer		64	0			150		10
S118A	PHB-granules	73	62	0	0	120	100	74	0
	3HB-tetramer		55	0			150		6
H120Q	PHB-granules	91	74	0	0	100	90	92	0
	3HB-tetramer		32	0			74		12
C183S	PHB-granules	18	1	1	0	99	92	120	2
	3HB-tetramer		0	0			92		130
D356A	PHB-granules	95	76	1	0	100	95	72	0
	3HB-tetramer		70	0			120		15
C370A	PHB-granules	110	100	90	0	110	100	110	0
	3HB-tetramer		89	59			100		22

size PHB acquired or developed both PHB synthase and PhaZ1 simultaneously.

In the amino acids sequence alignment, PhaZ1 have some conserved regions. They are very similar to each other and none of the phaZ1s examined had a typical lipase box, -Gly-X_1-Ser-X_2-Gly-, which all the ePHB depolymerases contain (4, 5). The only conserved serine residue in PhaZ1 is Ser^{118}, but the S118A mutant did not loose the activity (Table 2). Recently, the second structure of components of the catalytic triad in the PHB synthase of *C. vinosum* was predicted by comparison of that of lipases whose three-dimensional protein structures were elucidated by X-ray crystallography (19). *C. vinosum* PHB synthase has a modified lipase box, -Gly-Ile-Cys-Glu-Gly-, whose cysteine was identified as essential for catalysis by site-directed mutagenesis. Interestingly, the cysteine, aspartate and histidine residues that make up the catalytic triad in PHB synthase (19) were well conserved in PhaZ1 in similar positions (Fig. 1). PhaZ1 and PHA synthase may be homologous. Actually, three mutants, C183A, D356A, and H388Q, lost the activity. Analysis of the C183S mutant indicated that serine could replace cysteine at position 183 for catalysis and the serine was modified by DFP. We therefore conclude that cysteine 183 is an amino acid residue making up the catalytic triad of this enzyme. When this cysteine was changed to serine in the PHA synthase from *C. vinosum*, the activity was almost completely lost (reduced to about 1/1000 of the original level). Although C183S did not have cysteine 183, it was inhibited by 20 μM of PCMB in a similar manner to the wild-type. It seems that cysteine 370 is very sensitive to PCMB compared with cysteine 87 and 183, because C370A was only slightly inhibited in the presence of 20 μM of PCMB. Additionally, C370A showed high activity when 1 mg/ml of PHB granules were used as a substrate (Table 2). It is difficult to explain the catalytic role of cysteine 370 in $PhaZ1_{Reu}$.

Gao *et al*. reported that crude extract containing PhaZ1 from *P. denitrificans* expressed in *E. coli* was inhibited by 1 mM DFP. Therefore, they suggested that the enzyme is a serine esterase (12). In our hands, 1 mM DFP did not inhibit $PhaZ1_{Reu}$ (Table 3). We do not know the reason for this discrepancy. The purified $PhaZ1_{Reu}$ was not completely inhibited even by 10 mM DFP, but all the ePHB depolymerases (4, 20) and the soluble type iPHB depolymerase of *Zoogloea ramigera* (9) and *R. eutropha* (10) were inhibited by a low concentration of DFP or phenylmethylsulfonyl fluoride. Therefore, the $PhaZ1_{Reu}$ purified in this study completely differs from ePHB depolymerase and the soluble iPHB depolymerase.

Triton X-100 (0.1%) inhibited the 3HB-tetramer-degrading activity of the wild-type and all the mutants except for C183S (Table 3). These results indicate that Triton X-100 may interact with the substrate binding region or a region which exists by whose structure is influenced by cysteine 183. The substrate binding regions for PHB granules and 3HB-oligomers may overlap in $PhaZ1_{Reu}$. On the contrary, ePHB depolymerases have distinct domains, catalytic domains and PHB (substrate)-binding domain, and Triton X-100 or a similar detergent strongly inhibits PHB-binding activity, but not the hydrolyzing activity of water-soluble 3HB-oligomers (21). These results indicate clearly the difference in structure between PhaZ1 and ePHB depolymerase.

None of the wild-type and mutants were inhibited by DTT, which indicates that PhaZ1 has no disulfide bond(s) that affects its enzyme activity. Furthermore, S118A showed weak degradation activity only for PHB granules, whereas H120Q showed weak degradation activity only for 3HB-tetramer. It is interesting that two activities may be separable. It suggests that serine 118 and histidine 120 are also activity-affective residues.

In this study, we determined catalytic triad residues, cysteine 183, aspartate 355, and histidine 388, and found some activity-affective residues, serine 118, histidine 120, and cysteine 370. However, the precise roles of serine 118, histidine 120, and cysteine 370 in catalysis are not clear. Certainly further investigation is required.

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