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Partial Purification and Characterization of β-Ketothiolase from *Alcaligenes* sp. SH-69

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A β-ketothiolase was purified 180-fold from the cell extracts of *Alcaligenes* sp. SH-69 by a series of chromatography on DEAE-Sephadex A-50, Sephacryl S-200, and hydroxyapatite columns. The optimum pH values of the partially purified enzyme were 7.5 for condensation reaction and 8.3 for thiolysis reaction. The K_m values for acetoacetyl-CoA and free CoASH in the thiolysis reaction were estimated to be 0.12 mM and 18.7 μM, respectively. The K_m value for acetyl-CoA in the condensation reaction was 0.70 mM. The condensation reaction of the β-ketothiolase was inhibited even by low concentrations of free CoASH (K_i=30.4 μM). Pretreatment of the enzyme with NADH and NADPH markedly inhibited the thiolysis reaction of the enzyme. The potent inhibition of the enzyme by sulfhydryl reagents suggests the involvement of cysteine residue in the active site.

Key words: *Alcaligenes* sp. SH-69, condensation, inhibitors, β-ketothiolase, thiolysis

Poly-β-hydroxyalkanoates (PHAs), a family of polyesters produced by a variety of microorganisms, have been the focus of extensive research because of their potential application as biodegradable and biocompatible thermoplastics (5, 19). Particularly, a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), poly(3HB-*co*-3HV), has been of the greatest commercial interest since this polymer exibits a considerable range of thermomechanical properties that depend on its 3HV content (11). Production of poly (3HB-*co*-3HV) is usually achieved by providing bacteria with a cosubstrate such as propionate and valerate along with a main carbon source (10, 20).

Alcaligenes sp. SH-69 is an exceptional strain that produces poly (3HB-*co*-3HV) from single carbon sources such as glucose, sucrose, and sorbitol (13). This ability is potentially useful for commercial production of poly (3HB-*co*-3HV) since price reduction can be achieved not only by reducing the substrate costs but also by facilitating fermentation process (21, 22, 26). However, in this organism, the metabolic pathways for the synthesis of poly (3HB-*co*-3HV) have not been clearly elucidated. Recently, it has been reported that the molar fraction of 3HV in the copolyester produced by *Alcaligenes* sp. SH-69 can be enhanced by addition of amino acids, such as threonine, isoleucine, and valine, into glucose medium (27). These results imply that the ability to synthesize poly (3HB-*co*-3HV) from a single unrelated carbon source may be due to the efficient formation of propionyl-CoA or 3-hydroxyvaleryl-CoA. However, the possibility of different substrate specificities or distinct properties of the PHA synthesizing enzymes from Alcaligenes sp. SH-69 cannot be excluded.

In PHA biosynthesis, β-ketothiolase (EC 2.3.1.9), acetoactyl-CoA reductase (EC 1.1.1.36), and PHA synthase function as the principal enzymes. A key regulatory enzyme in PHA metabolism was reported to be β-ketothiolase which catalyzes the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA (1, 6). In this study, as an initial attempt to understand the properties of PHA synthesizing enzymes in *Alcaligenes* sp. SH-69, we purified β-ketothiolase from the cell extract by a series of column chromatographies and compared its properties with those from other PHA-synthesizing bacteria.

For enzyme preparation, batch culture of *Alcaligenes* sp. SH-69 was carried out in a 5 L jar fermentor (Korea Fermentor Co., Korea) with a working volume of 3.0 L. The culture medium and culture conditions used were the same as described previously (27). The medium was inoculated with a 10% (v/v) inoculum of an overnight culture in the same medium. After incubation for 16 h, the bacterial cells were harvested by centrifugation at 7,000 rpm for 10 min, and washed twice with 10mM phosphate buffer (pH 7.0). All purification procedures were carried out at 4°C in 10 mM phosphate buffer (pH 7.0) containing 1mM dithiothreitol. *Alcaligenes* sp. SH-69

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cells were suspended in 180 ml of the buffer containing 1 mM EDTA. The suspension was sonically disrupted and centrifuged at 10,000×*g* for 12 min. The supernatant was used as crude enzyme extract. The crude extract was fractionated by salting out with ammonium sulfate (25 to 70% saturation). The precipitate was then dissolved in 60 ml of 10 mM potassium phosphate buffer (pH 7.0) with 1 mM dithiothreitol, and dialyzed against the same buffer for 18 h with periodic changes of the buffer. The resulting solution was applied to a DEAE-Sephadex A-50 column (5×25 cm), previously equilibrated with the same buffer. The column was washed with the same buffer and then eluted with a linear gradient of 0~0.4 M KCl in the same buffer. Fractions of 8 ml were collected per 12 min. The active fractions were pooled and concentrated by ultrafiltration through a Diaflo membrane PM 10. The concentrate was loaded onto a Sephacryl S-200 column (2.5×95 cm) pre-equilibrated with 10mM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and eluted with the same buffer at a flow rate of 13 ml per hour. Active enzyme fractions were collected again and concentrated by ultrafiltration. For further purification, the concentrated enzyme solution was applied to a hydroxyapatite column (2.5×20 cm) equlibrated with 50 mM phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. After washing with the same buffer, the column was eluted with a linear gradient of 50~300 mM phosphate buffer (pH 7.0, total volume 900 ml). Active fractions were collected and concentrated, and used in this study as the source of β-ketothiolase.

β-Ketothiolase was assayed in the reaction of both forward and reverse directions. The assay mixture for the condensation reaction (total volume; 1 ml) contained 100 mM Tris-HCl (pH 8.0), 2.4 mM acetyl-CoA, 300 μM NADH, and 2.5 U of 3-hydroxyacyl-CoA dehydrogenase from porcine heart (15). After 2 min preincubation at 30°C, the reaction was initiated by addition of 10 μl enzyme, and the decrease in absorbance at 340 nm was measured at 30°C. One unit of β-ketothiolase activity was defined as the quantity of enzyme required to catalyze the formation of 1 μM of acetoacetyl-CoA per min. The thiolysis reaction was assayed by the method of Berndt and Schlegel (3) with slight modifications. The reaction mixture (total volume: 1 ml) contained 100 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 100 μM acetoacetyl-CoA, and 100 μM CoASH. After preincubation for 2 min at 30°C, the reaction was initiated by the addition of 10 μl enzyme. The decrease in acetoacetyl-CoA was then measured at 303 nm using a millimolar extinction coefficient of 12.9 (12). One unit of β-ketothiolase activity was defined as the amount of enzyme required to catalyze the cleavage of 1 μM of acetoacetyl-CoA per min. Protein concentration was determined using Bradford reagent (4) with bovine serum albumin as the standard, or by measuring absorbance at 280 nm. Analyses of dry cell weight, residual nutrients, PHA content and its composition by gas chromatography were done as described previously (18).

Activities of β-ketothiolase during the batch-fermentation of *Alcaligenes* sp. SH-69 were measured. Relatively high levels of β-ketothiolase were detected from the cells throughout the batch-fermentation despite the substantial difference in PHA content. Even though β-ketothiolase was reported to be a constitutive enzyme by other investigators (7, 8), the maximum specific activity of the enzyme was reached immediately after the exhaustion of ammonium ion in the medium. For purification of the enzyme, the cells were harvested in the early PHA accumulation phase following nitrogen exhaustion.

Table 1 summarizes the quantitative evaluation of the results from the purification procedures. The enzyme was purified approximately 180-fold with an overall recovery of 21%. However, the final preparation from a hydroxyapatite column was not homogeneous and showed a few additional bands of protein on SDS-PAGE.

The optimum pH of the partially purified β-ketothiolase was 7.7 for the condensation reaction in either phosphate or Tris-HCl buffer and 8.3 for the thiolysis reaction in Tris-HCl buffer. To assess the thermal stability of the β-ketothiolase, the en-

Table 1. Purification of β-ketothiolase from *Alcaligenes* sp. SH-69

Purification step	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Purification fold	Recovery (%)
Crude extract	1642	670.4	0.41	1	100
$(NH_4)_2SO_4$ fractionation	779	624.7	0.80	2.0	93
DEAE-Sephadex A-50	60.8	297.4	4.89	11.9	44
Sephacryl S-200	18.9	167.4	8.86	21.6	25
Hydroxyapatite	1.9	141.2	73.54	179.4	21

[a] The activity of the enzyme preparation at each step was determined by the assay for thiolysis reaction.

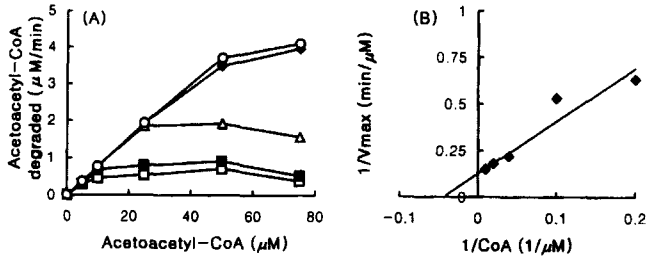

Fig. 1. Effect of concentration of acetoacetyl-CoA on the thiolysis reaction of β-ketothiolase at various concentrations of CoASH. (A) Acetoacetyl-CoA saturation curves. The following concentrations of CoASH were used: □, 5 μM; ■, 10 μM; △, 25 μM; ◆, 50 μM; ○, 75 μM. (B) Double reciprocal plot of V_{max} and concentration of CoASH.

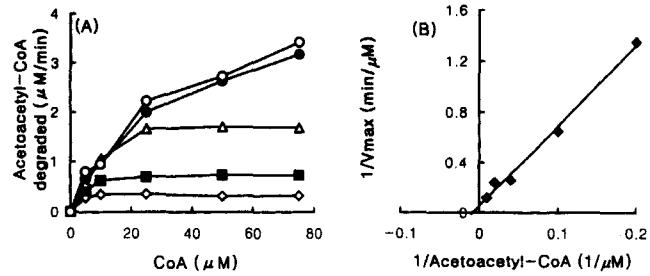

Fig. 2. Effect of concentration of free CoASH on the thiolysis reaction of β-ketothiolase at various concentrations of acetoacetyl-CoA. (A) CoASH saturation curve. The following concentrations of acetoacetyl-CoA were used: ◇, 5 μM; ■, 10 μM; △, 25 μM; ●, 50 μM; ○, 75 μM. (B) Double reciprocal plot of velocity and concentration of acetoacetyl-CoA.

zyme was preincubated for 30 min at various temperatures (20~60°C) after which the residual activity of the thiolysis reaction was measured. The enzyme retained more than 90% of the initial activity after preincubation at 40°C and 74% at 50°C, but lost all of the initial activity at 60°C.

Fig. 1A shows the dependence of initial reaction velocity on the concentration of acetoacetyl-CoA as a substrate of the thiolysis reaction at five fixed concentrations of CoASH. A slight inhibition of activity was observed at acetoacetyl-CoA concentrations greater than 50 μM when CoASH concentrations were less than 25 μM. From the double reciprocal plot of Vmax and concentration of CoASH (Fig. 1B), the apparent K_m value for CoASH was estimated to be 18.7 μM. Fig. 2A shows CoASH saturation curves at various fixed concentrations of acetoacetyl-CoA. The data (not shown) from the double reciprocal plot of velocity and concentration of CoASH were consistent with the ping-pong reaction mechanism that has been suggested for other bacterial β-ketothiolases (8, 15, 24). The K_m value for acetoacetyl-CoA and the V_{max} of the thiolysis reaction were estimated to be 0.12 mM and 19.1 μM/min, respectively, from the double reciprocal plot of V_{max} and concentration of acetoacetyl-CoA (Fig. 2B).

The effect of concentration of acetyl-CoA, as a substrate for the condensation reaction, on the initial velocity was measured at several fixed concentrations of CoASH (Fig. 3A). In the absence of CoASH, the velocity versus substrate concentration plot obeyed normal Michaelis-Menten kinetics, while the initial velocity was progressively suppressed with increasing concentrations of CoASH. The Lineweaver-Burk plot (Fig. 3B) shows that free CoASH functions as a non-competitive inhibitor for the condensation reaction. The apparent K_m value for acetyl-CoA in the absence of CoASH was estimated to be 0.7 mM and the V_{max} of condensation reaction was estimated to be 1.31 μM/min. From a replot of slope against the concentration of CoASH (Fig. 3C), the K_i value for CoASH was estimated to be 30.4 μM.

As shown in Table 2, pretreatment of the enzyme with 1.5 mM NADH and NADPH completely inhibited the thiolysis reaction of the enzyme. They were more potent inhibitors than their oxidized forms, NAD^+ and $NADP^+$. The inhibition of β-

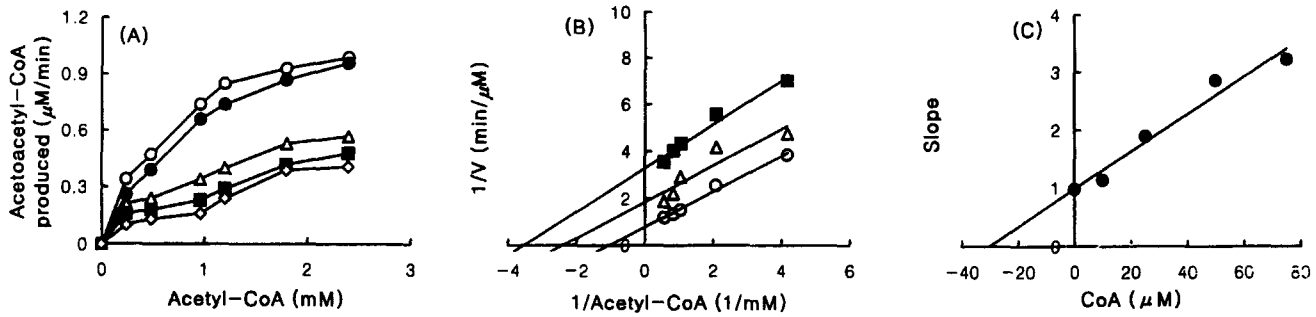

Fig. 3. Effect of the presence of free CoASH on the condensation reaction of β-ketothiolase. (A) Acetyl-CoA saturation curves. The following concentrations of CoASH were used: ○, 0 μM; ●, 10 μM; △, 25 μM; ■, 50 μM; ◇, 75 μM. (B) Double reciprocal plot of velocity and concentration of acetyl-CoA at CoASH concentrations of 0 (○), 25 (△), and 50 μM (■). (C) Replot of slope against the CoASH concentration.

Table 2. Effects of nicotinamide nucleotides and protein modification chemicals on β-ketothiolase activity[a]

Addition	Concentration (mM)	Percent inhibition
None	0	0
NADP	0.15	14
	0.3	23
	1.5	39
	3.0	41
NADPH	0.15	50
	0.3	63
	1.5	100
	3.0	100
NAD	0.15	5
	0.3	12
	1.5	41
	3.0	49
NADH	0.15	46
	0.3	70
	1.5	100
	3.0	100
Iodoacetamide	0.5	78
N-Ethylmaleimide	0.5	71
O-Nitrobenzoic acid	0.5	88
P-Chloromercuribenzoic acid	0.5	65
N-Bromosuccinate	0.5	42

[a] The enzyme was preincubated with various reagents at 30°C for 5 min prior to mixing with the standard mixture for thiolysis assay. The reaction was initiated by the addition of acetoacetyl-CoA.

ketothiolase by nicotinamide nucleotides could be overcome by increasing the concentration of CoA (data not shown). The apparent Ki value for NADPH was estimated to be 0.13 mM. The enzyme was also inhibited by sulfhydryl reagents, such as *O*-nitrobenzoic acid, iodoacetamide, *N*-ethylmaleimide, and ρ-chloromercuribenzoic acid, at a concentration of 0.5 mM. These results suggest that cysteine residue is involved in the active sites of the enzyme.

Recently we have cloned and characterized β-ketothiolase gene (*phaA*) from *Alcaligenes* sp. SH-69 (unpublished data). When the amino acid sequence was compared with other bacteria's β-ketothiolases in the GenBank database, it showed 72%, 63%, and 60% identity to those β-ketothiolases isolated from *Alcaligenes eutrophus*, *Acinetobacter* sp., and *Zooglea ramigera*, respectively. The data from the present study revealed that the enzymatic properties of β-ketothiolase from *Alcaligenes* sp. SH-69 are also similar to those from other PHA synthesizing bacteria such as *A. eutrophus* (8), *A. latus* (15), *Z. ramigera* (25), *Azotobacter beijerinckii* (23), and *Methylobacterium rhodesianum* (16). These enzymes have similar pH optima for the condensation and thiolysis reactions and fairly high Km values for acetyl-CoA (*A. beijerinckii*, 0.9 mM; *M. rhodesianum*, 0.5 mM; *A. eutrophus*, 1.1 mM). In addition, the condensation reaction of the enzymes was inhibited by low concentrations of CoASH and the thiolysis reaction by high concentrations of acetoacetyl-CoA. Moreover, these enzymes are characterized by a Bi Bi ping-pong reaction mechanism for the thiolysis reaction. The presence of cysteine residue (Cys^{88} in the case of *Alcaligenes* sp. SH-69 β-ketothiolase, unpublished data) in their active sites is also a characteristic property which is conserved among many different microbial β-ketothiolases.

It is interesting to note that not only $NAD(P)^+$ but also NAD(P)H had inhibitory effects on the thiolysis reaction. The β-ketothiolases from *M. rhodesianum* (16) and *Bradyrhizobium japonicum* (24) are also inhibited by $NAD(P)^+$ and NAD(P)H. However, those from *A. eutrophus* (8), *A. beijerinckii* (23), and *Hydrogenomonas eutropha* (now *A.* eutrophus) H-16 (17) are not inhibited by NADH and NADPH. It is likely that the inhibitory effect of nicotinamide nucleotides could be attributed to structural similarity between CoASH and nicotinamide nucleotides. The relief of this inhibition by addition of CoASH support this possibility. It has been known that NADH and NADPH inhibit citrate synthase and isocitrate dehydrogenase in the TCA cycle while high levels of NADH and NADPH facilitate the shift of metabolic flux of the acetyl-CoA to PHA synthetic pathway (9, 14, 16). Although the stimulating effects of nicotinamide nucleotides on the condensation reaction could not be determined as this assay was a coupled reaction employing the conversion of NADH to NAD^+, the results in this study suggest that both reduced and oxidized forms of nicotinamide nucleotides, as well as CoASH, are the important factors that are involved in regulation of PHA biosynthesis (condensation) and degradation (thiolysis) at the level of β-ketothiolase.

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