[SI-3]

Purification and Characterization of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase from *Comamonas* sp.

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Abstract

A genomic library of biphenyl-degrading strain *Comamonas* sp. SMN4 was constructed by using the cosmid vector pWE15 and introduced into *Escherichia coli*. Of 1,000 recombinant clones tested, two clones that expressed 2,3-dihydroxybiphenyl 1,2-dioxygenase activity were found (named pNB1 and pNB2). From pNB1 clone, subclone pNA210 , demonstrated 2,3-dihydroxybiphenyl 1,2-dioxygenase activity, is isolated. 2,3-Dihydroxybiphenyl 1,2-dioxygenase (23DBDO, BphC) is an extradiol-type dioxygenase that involved in third step of biphenyl degradation pathway. The nucleotide sequence of the *Comamonas* sp. SMN4 gene *bphC*, which encodes 23DBDO, was cloned into a plasmid pQE30. The His-tagged 23DBDO produced by a recombinant *Escherichia coli*, SG13009 (pREP4)(pNPC), and purified with a Ni-nitrilotriacetic acid resin affinity column using the His-bind Qiagen system. The His-tagged 23DBDO construction was active. SDS-PAGE analysis of the purified active 23DBDO gave a single band of 32 kDa; this is in agreement with the size of the *bphC* coding region. The 23DBDO exhibited maximum activity at pH 9.0. The CD data for the pHs showed that this enzyme had a typical α-helical folding structures at neutral pHs ranged from pH 4.5 to pH 9.0. This structure maintained up to pH 10.5. However, this high stable folding strucure was converted to unfolded structure in acidic region (pH 2.5) or in high pH (pH 12.0). The result of CD spectra observed with pH effects on 23DBDO activity, suggested that charge transition by pH change have affected change of conformational structure for 23DBDO catalytic reaction. The K_m for 2,3-dihydroxybiphenyl, 3-metylcatechol, 4-methylcatechol and catechol was 11.7 μM, 24 μM, 50 mM and 625 μM.

Introduction

Nearly all pathways responsible for the bacterial degradation of aromatic compounds such as toluene, PCBs, naphthalene, and phenanthrene is to transform the initial substrates into intermediates that carry two or more adjacent hydroxyl groups on the aromatic ring. The cleavage of these intermediates in the biodegradation pathway is mediated by extradiol or intradiol dioxygenase. Intradiol dioxygenase cleaves the aromatic ring between two hydroxylated carbons in an intradiol fashion, whereas extradiol dioxygenase cleaves between the hydroxylated carbon and adjacent nonhydroxylated carbon in the aromatic ring (Nozaki *et al.*, 1970; Wallis *et al.*, 1990).

Extradiol dioxygenases play a key role in determined pathway for the degradation of toluene and presumably other aromatic compounds (Asturias *et al.*, 1994). A number of extradiol ring cleavage enzymes have been characterized for a variety of aromatic compounds including PCBs/biphenyl

(Kimbara *et al.*, 1989; Furukawa *et al.*, 1987; Hayase *et al.*, 1990; Hofer *et al.*, 1993; Taira *et al.*, 1988), toluene (Zylstra *et al.*, 1993), naphthalene (Denome *et al.*, 1993), and phenanthrene (Takizawa *et al.*, 1994). These extradiol dioxygenases have highly specificities for the intermediates (Asturias *et al.*, 1994; Furukawa *et al.*, 1993; Taira *et al.*, 1992). Especially, the homology between the 2,3-dihydroxybiphenyl dioxygenase (BphC, Zylstra and Gibson, 1989) and 3-methylcatechol 2,3-dioxygenase (TodE) of toluene degrader *P. putida* F1 (Gibson *et al.*, 1970a,b) reported to be even higher than that between two 2,3-dihydroxybiphenyl dioxygenase.

Despite the discrete substrate specificities, the amino acid sequences of these enzymes were found to be homologous to each other (Harayama *et al.*, 1992; van der Meer *et al.*, 1992; Fukuda, 1993). Moreover there are lines of evidence indicating that all of these extradiol type dioxygenases have a ferrous (Fe(II)) iron in their active sites (Howard *et al.*, 1991; Nozaki, 1979). Among these, 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DHDO), encoded by the *bphC* gene, is a component of aerobic biphenyl degradation pathways of microorganisms and *meta* cleaves 2,3- dihydroxybiphenyl yielding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) with the insertion of two atoms of oxygen. These enzymes possess many features in common such as molecular weight (ca. 270,000), subunit structure ($[\alpha Fe(II)]_8$), and substrate specificity (Taira *et al.*, 1988). They are specific for only 2,3-dihydroxybiphenyl; thus, its isomer, 3,4-dihydroxybiphenyl, is resistant to the enzymatic degradation. Bearing the importance of these enzymes in degradation of biphenyl is required to understanding the basis of substrate specificity to improve the strains capacity for mineralizing biphenyl.

In this report, purification and characterization of 2,3-dihydroxybiphenyl 1,2-dioxygenase which was overexpressed in *E. coli* were carried out in terms of pH and thermal stability, temperature and pH effect, and conformation.

Results

1. Cloning of *bphCD* gene from *Comamonas* sp. SMN4

To understand the biodegradation pathway in *Comamonas* sp. SMN4 at molecular level, the cloning and characterization of the corresponding genes have been carried out. A cosmid library of *Comamonas* sp. SMN4 was constructed with partially *Bgl* II-digested total genomic DNA and the cosmid pWE15. Each cosmid clone should contain approximately 35-40 kb of the *Comamonas* sp. SMN4 genome. The cosmid library (1,000 clones) was screened for the formation of the yellow compound 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid from 2,3-dihydroxybiphenyl through the action of 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO) by being sprayed with an ethanol solution of 2,3-dihydroxybiphenyl. Two cosmid clones that had 23DBDO activity were isolated and purified. The recombinant plasmids conferring this activity were designated as pNB1 and pNB2.

A variety of derivative plasmids were constructed with the restriction fragments from the 35 kb DNA inserted in pNB1 by using plasmid vectors for *E. coli*. The *Apa* I fragments of pNB1 DNA were cloned into pGEM5Zf(+) with selection for ampicillin resistance. The resultant clone, pNA210 with 4.9 kb in size, demonstrated 23DBDO activity. According to a restriction map of the pNA210 as shown in Figure 1, subclones of pNA210 were constructed and 23DBDO activities was detected to

know where *bphC* gene expressed or not. Subcloning experiments showed that the *bphC* gene is located between the *Pst* I and *Stu* I of pNA210 (Figure 1). Subclone pNAS6 (containing 2.6 kb *Sal* I -to-*Apa* I fragment) and pNPX9 (containing 1.9 kb *Pst* I -to-*Xba* I fragment) had 23DBDO activities Subclone pNAS6-1 (containing 2.6 kb *Sal* I -to-*Apa* I fragment) which was subcloned reverse direction to pNSA6 also expressed *bphC* gene by showing 23DBDO activity. Same was observed in pNPX9-1 (containing 1.9 kb *Pst* I -to-*Xba* I fragment). A subclone containing 1.2 kb *Sal* I -to-*Cla* I fragment (pNSC5) did not show 23DBDO activity.

2. Characterization of *bphC* gene products

For the comprehensive analysis of 2,3-dihydroxybiphenyl dioxygenase activity with strains that have pNA210, pNAS6, pNAS6-1, pNPX9, pNPX9-1, and pNCP plasmid, 23DBDO activities were assayed with various dihydroxylated substrates. Same tests were conducted with *Comamonas* sp. SMN4 for comparison. Strains harboring 23DBDO gene show maximal activity against 2,3-dihydroxybiphenyl and are able to cleave 3-methylcatechol with three-fifth rate of 2,3-dihydroxybiphenyl. They have the cleavage activity in substantial for catechol and 4-methylcatechol. Although *bphC* gene was expressed in oppositely oriented subclones (pNAS6-1 and pNPX9-1), the activity of pNAS6-1 and pNPX9-1 was very poor than that of pNAS6 and pNPX9 against 2,3-dihydroxybiphenyl and other dihydroxylated compounds. In the case of pNAS6 and pNPX9 (with *bphC* and *bphD*), pNPX9 has shorter fragment than pNAS6. However, activity of pNPX9 was 1.5-fold higher than that of pNAS6 for each substrate. Higher 23DBDO activity was detected in pNCP strain harboring only 23DBDO gene (*bphC*). In order to conform location of *bphC* gene, the nucleotide sequences of the 1.9 kb *Pst* I -to-*Xba* I fragment pNPX9 were determined and the accession number for nucleotide sequences of the 1.9 kb *Pst* I -to-*Xba* I fragment pNPX9 in GenBank database was AY028943. Analysis of the nucleotide sequence of the 1.9-kb *Pst* I -to-*Xba* I fragment pNPX9 revealed two complete ORFs (open reading frames) corresponding to *bphC* and *bphD*. The length of the ORF corresponding to *bphC* was 879 bp. The polypeptide sequence deduced from *bphC* consists of 293 residues with a calculated molecular mass of 32,717 Da.

3. Purification and molecular weight determination of 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO)

His-tagged *Comamonas* sp. SMN4 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO) was expressed in *E. coli* SG13009 (pNCP) and was purified by affinity chromatography. The purification protocol, using the imidazole gradient system, was efficient enough to eliminate most of the unwanted proteins at 20 mM imidazole. The enzyme of interest was eluted at 250 mM imidazole. No contaminating bands were detected on SDS-PAGE gels (Figure 2A). The molecular weight of the native His-tagged protein was estimated to be 270,000 by gel filtration chromatography (Figure 2C). The observed molecular weight denatured subunit was 32,000 for the purified enzyme determined by SDS-PAGE (Figure 2B). The calculated molecular weight and pI by the ExPASy (Expert Protein Analysis System) molecular biology server (www.expasy.ch/tools /pi_tool.html) were estimated to be 32717 and 6.34.

4. Characterization of 23DBDO

1) pH effects

The 23DBDO exhibited maximum activity at pH 9.0 and the activity declined in basic and acidic regions. Only 5% of enzyme activity was retained even at neutral pH 6.5 (Figure 3A). CD spectra of 23DBDO for the various pHs which was determined by spectropolarimeter were shown in Figure 3. The enzyme showed a typical α-helical folding structures at neutral pHs ranged from pH 4.5 to pH 9.0. They have peak minima around 210 nm and 222 nm which was represented in typical α-helix structure (Fasman, 1996). This structure maintained by pH 10.5. However, this high stable folding structure was converted to unfolded structure (Figure 3). The conformational structure changes were also observed in terms of pH by determining the ellipticity at 222 nm. Since 222 nm of spectra maximum ellipticity change was detected between folding and unfolding structure (Kwon and Kim, 1994), residual ellipticity at 222 nm was represented for comparing the structure and activity of 23DBDO (Figure 3B). The CD data for the pHs showed that below pH 4.0 the structure of enzyme was unfolded like as in random coil, the structure was stabilized as increase of pH up to pH 6.0. After pH 6.0, the data of θ_{222} showed to level off plateau even after pH 10.5 (Figure 3B), in CD data of θ_{222}, structure of enzyme look like stable after pH 10.5. The enzyme stability which was measured enzyme activity at pH 9.0 after incubating 23DBDO for 24 h at 4℃ for different the pHs, showed that enzyme was stable at neutral pHs although catalytic activity was very poor such as pH 7.5 at these pHs. In pH 9.0, which 23DBDO showed high activity, the enzyme was very unstable for long time incubation (Figure 3A and B). For storage of enzyme, pH 7.5 buffer was recommended.

2) Temperature effects

The temperature effects on 23DBDO activity and thermal stability were also determined at pH 9.0 by changing temperatures from 4℃ to 100℃ The activity increased as increase of reaction temperature up to 30℃ and 40% of activity was reduced at 40℃(Figure 4). The enzyme showed the maximal activity at 30℃. The activation energy from Arrhenius plot of this Figure was 3.78 kcal/mol·K°. Up to 40℃, the enzyme maintains its activity when the activity was checked at standard conditions (30℃ and pH 9.0) after incubating the enzyme for 24 h at given temperatures (Figure 4A). Figure 4C showed the spectra of 23DBDO at various temperatures. Up to 40℃ CD spectra were almost same. After 60℃, CD spectra showed similar unfolding conformation which was shown at high pH conditions (Figure 3). This is consistent with the stability data of 23DBDO as mentioned above. In case of temperature effect study, the wavelength where maximum CD spectra changed between folding and unfolding structures was 208 nm not 222 nm (for pH effect, θ_{222} nm was chosen). Here, the change θ_{208} was for checking the temperature effect on CD.

5. Kinetic calculations

Initial velocity (v_0) vs substrate concentration of 23DBDO of *Comamonas* sp. SMN4 expressed in *E. coli* for 4 substrate analogues; 2,3-dihydroxybiphenyl, catechol, 3-methylcatechol, and 4-methylcatechol, showed that typical Michaelis-Menten curves. Kinetic parameters represented by

Michaelis-Menten constants such as K_m and V_{max} values for each substrate were determined by Lineweaver-Burk plots. The K_m and V_{max} for 2,3-dihydroxybiphenyl were estimated to be 11.7 μM and 2,700U, respectively. The enzyme also bound to catalyze the cleavage of catechol, 3-methylcatechol, 4-methylcatechol with the affinities expressed by the K_m values as 625 μM, 24 μM and 50 mM. No cleavage activity for 3-chlorocatechol, 2,3-dihydroxybenzoate, and 1,2-dihydroxynaphtalene was detected. Cleavage activity of 4-chlorocatechol was observed by the enzyme. However, the activity was determined. The catalytic activity for 23DBDO is most powerful as 2,700 U of V_{max}. V_{max} values of the enzymes for 3-methylcatechol and 4-methylcatechol showed not so big difference, only 3-methylcatechol had 2.5 times greater activity than 4-methylcatechol.

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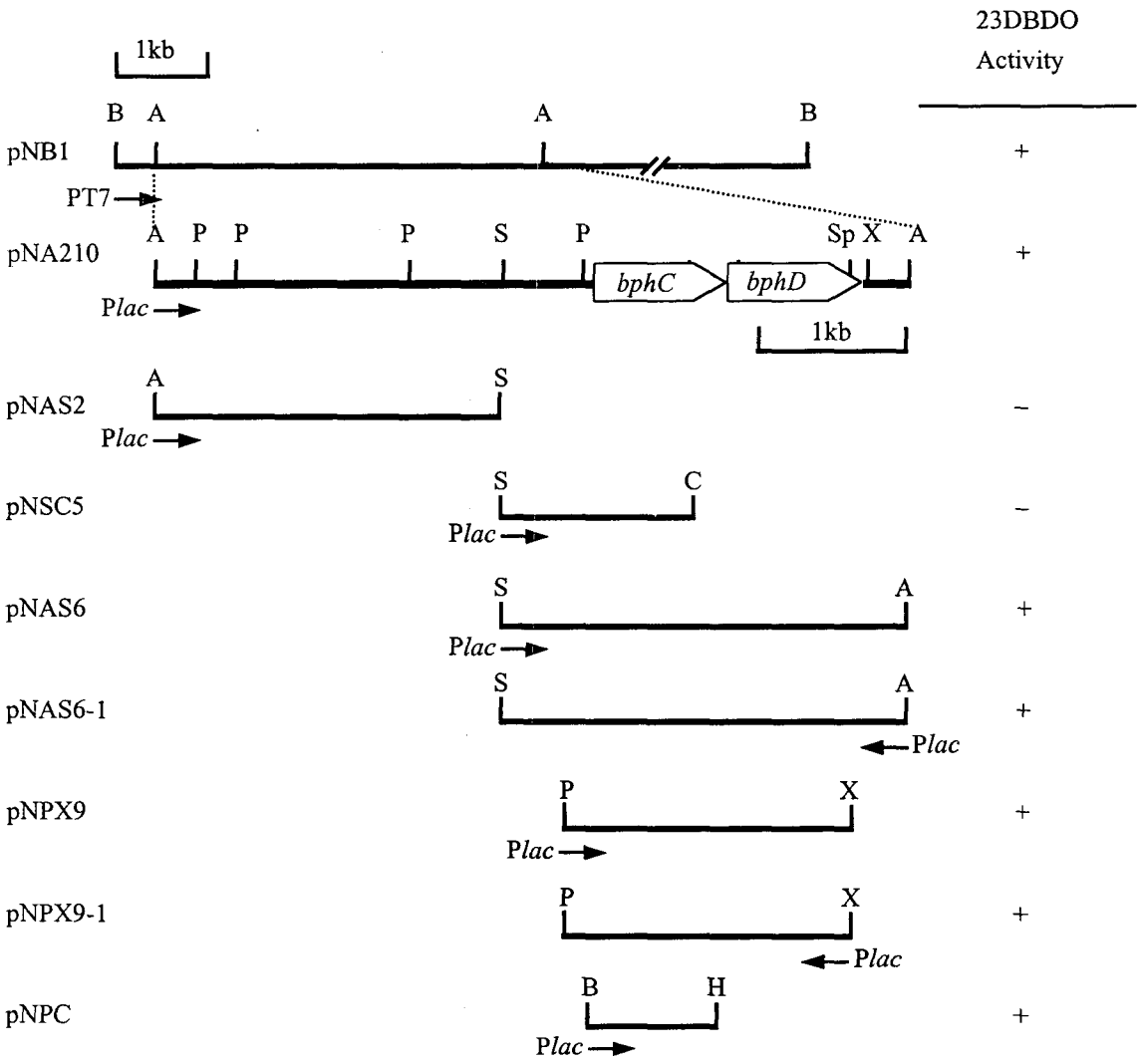

Figure 1. Restriction map of the cloned region in pNA210 and localization of the 2,3-dihydroxybiphenyl 1,2-dioxygenase gene. The arrowed boxes indicated the deduced positions of *bphC* and *bphD*. The activity column designates whether the clone having 23DBDO activity was expressed (+) or not (–). The direction of the transcription is indicated by arrow. The pNB1 is recombinant plasmid which is cloned into pWE15 cosmid vector. The restriction enzyme sites of *Bam*H I and *Hin*dIII in pNPC are inserted site for cloning into expression vector pQE30. Abbreviations: A, *Apa* I ; B, *Bam*H I ; C, *Cla* I ; H, *Hin*dIII; P, *Pst* I ; S, *Sal* I ; Sp, *Sph* I ; T, *Stu* I ; X, *Xba* I .

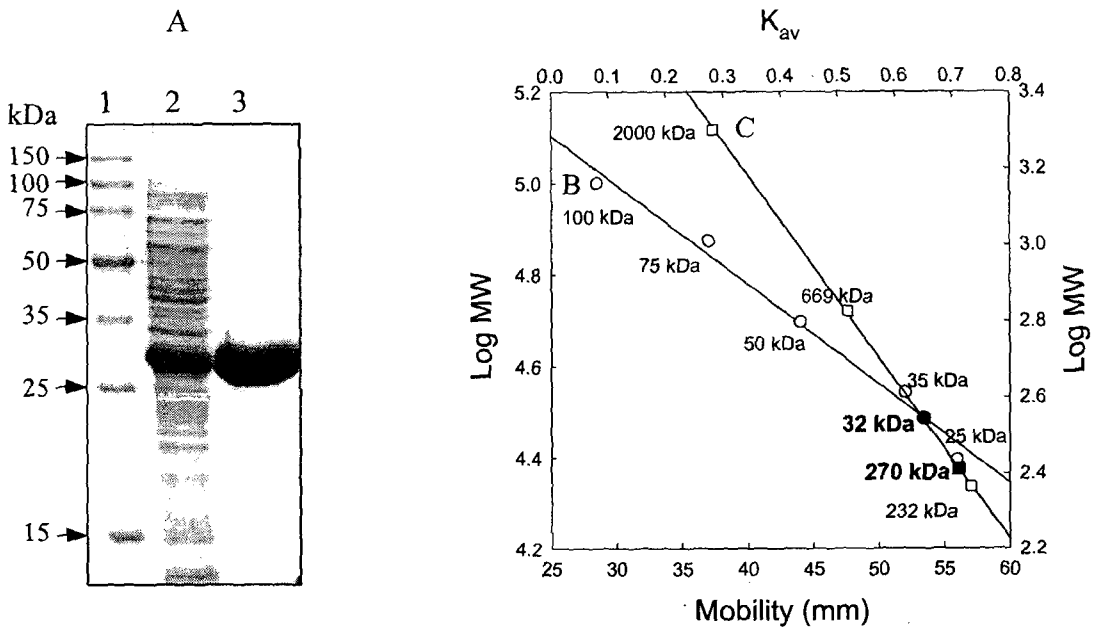

Figure 2. Determination of molecular mass for 23DBDO. (A) SDS-PAGE of His-tagged 23DBDO of *Comamonas* sp. SMN4. Lane 1, molecular weight standards; lane 2, crude preparation; lane 3, purified fraction obtained after elution of Ni-nitrilotriacetic acid resin. Estimation of molecular mass for monomeric 23DBDO from SDS-PAGE gel (B) and native 23DBDO by gel filtration (C). The molecular mass of marker proteins is designed beside the open symbols. The size marker proteins were : blue dextran (2000 kDa), thyroglobulin (669 kDa), catalase (232 kDa). $K_{av}= (V_e-V_0)/(V_t-V_0)$, where V_e, V_0, and V_t are the elution volume, void volume, and the total column volume, respectively.

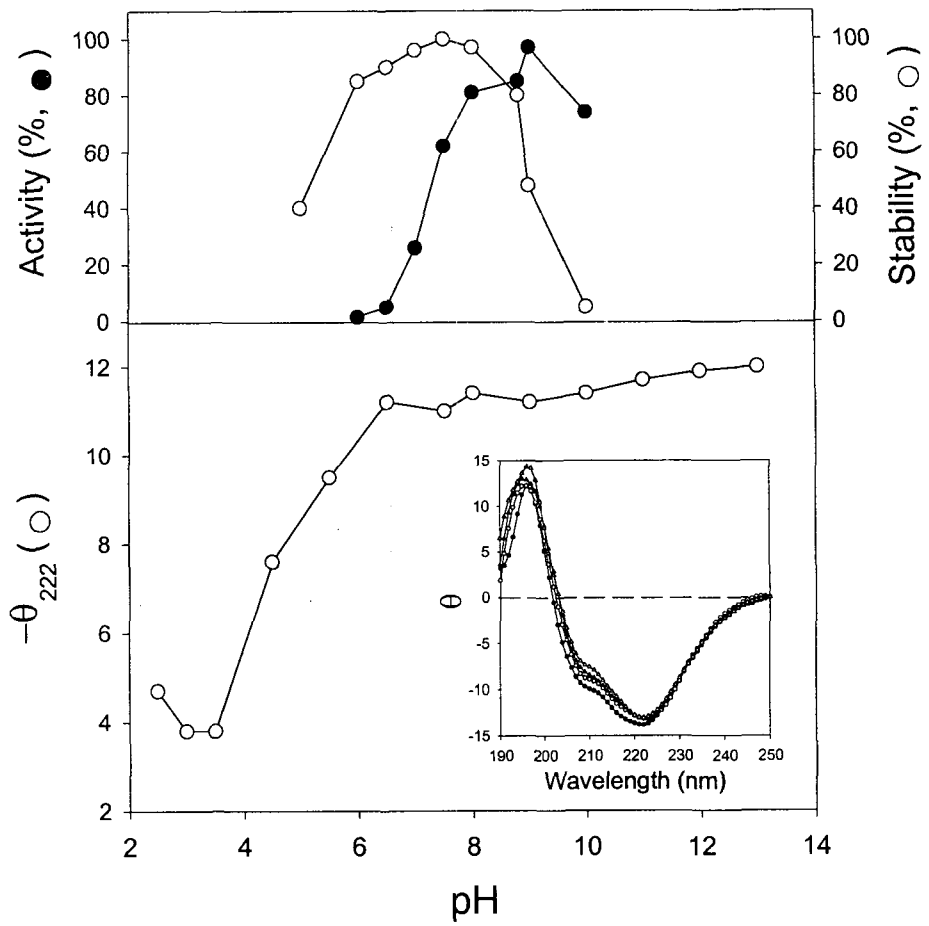

Figure 3. Effects of pH on activity and stability (A) of 23DBDO and CD spectra (B). Enzyme activities were determined by the initial velocity (V_0) under the standard conditions. For the conformational stability of enzyme (C), CD spectra of 23DBDO for conformational of 23DBDO for 24h incubation at pH 9.0 was shown as an inserted Figure. Symbols: ●, 0 h; ○, 8 h; ▲, 16 h; △, 24 h.

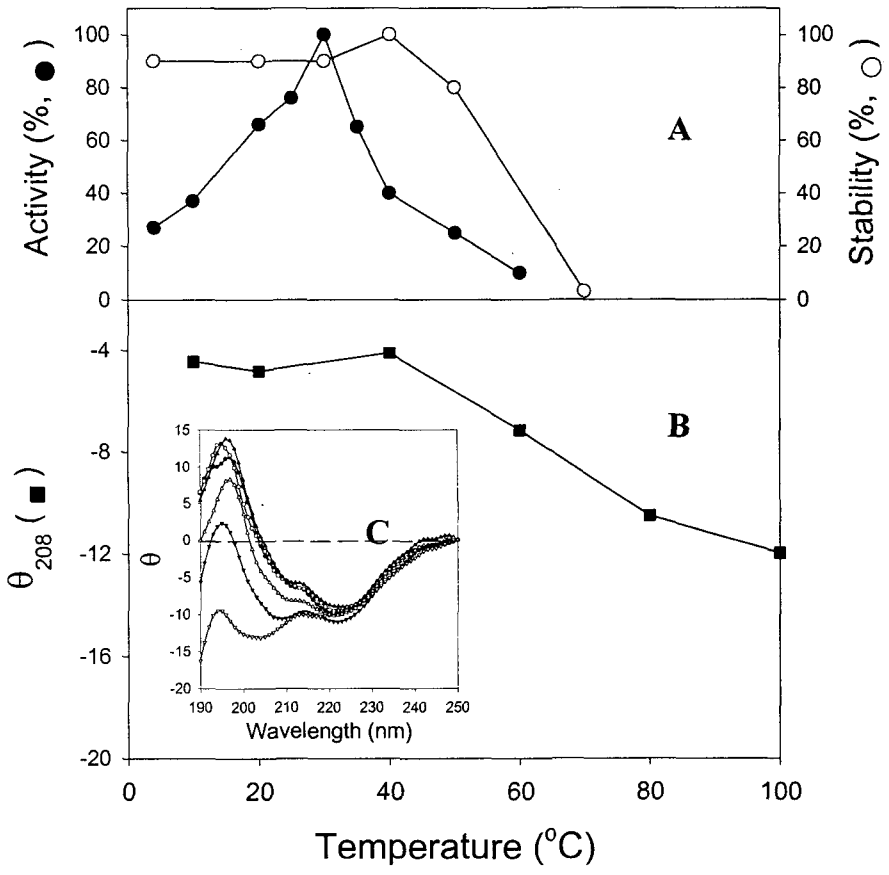

Figure 4. Effects of temperature on the 23DBDO activity and thermal stability (A) and CD spectra of the enzyme (B and C). 23DBDO activities were measured by determining the initial velocity (v_0) at given temperatures and pH 9.0. CD spectra (C) was observed by changing the temperature after 2min equilibrium. The concentration for CD work was the same as approximately 5 mM. Temperature stabilities were determined by observing the residual activities after incubating the enzyme for 24h at each temperature in 0.1M glycine buffer, pH 9.0. Residual activities were measured under the standard condition at pH 9.0 and 25℃. Symbols in C : ●, 10℃; ○, 20℃; ▲, 40℃; △, 60℃; ▼, 80℃; ▽, 100℃.