Residues Around the Catalytic Pocket of DdsA Are Important for Isoprenoids Chain Elongation

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Abstract: Nineteen mutants of decaprenyl diphosphate synthase from *Gluconobacter suboxydans* were generated and their production of ubiquinones (UQs) was compared to that of the wild type protein. The accessible surface area and the polarity of amino acid around the catalytic pocket wield remarkable influences on the isoprenoid chain elongation of UQs.

Keywords: Decaprenyl diphosphate synthase, ubiquinone, site-directed mutagenesis.

INTRODUCTION

The synthesis of isoprenoids, which provide the side chain of ubiquinone (UQ), is catalyzed by isoprenyl diphosphate synthase via the consecutive condensation of isopentenyl pyrophosphate (IPP) with allylic substrates. This enzyme family is classified by the length of the final product and the stereochemistry of the newly formed double bonds, *E* (*cis*) or *Z* (*trans*) [1]. Comparison of the primary amino acid sequences of *E*-type enzymes suggests that they have evolved from a common ancestor and these enzymes have seven conserved regions (I—VII) including two DDxxD motifs (II and VI), which form a catalytic pocket [2-6]. Several *E*-type isoprenyl diphosphate synthases from bacteria and yeasts have been purified and characterized [7-13]. A number of site-directed mutagenesis experiments have been carried out to evaluate the roles of amino acid residues in *E*-type isoprenyl diphosphate synthases [4,5,14,15]. Those results revealed the importance of the six Asp residues in the consensus $D^1D^2xxD^3$ (region II) and $D^4D^5xxD^6$ (region VI) motifs: D^2, D^3, and D^4 are critical and essential for enzyme activity. However, D^1 can be replaced by Glu but not by Ala and D^6 even tolerates the substitution of Ala. To data, most studies on *E*-type isoprenyl diphosphate synthase focused on the substitution of amino acid residues in conserved regions. Few studies have been performed on the substitution of the residues upstream of the first DDxxD motif in this enzyme, and these have revealed several mutants capable of synthesizing end products with longer chains [16]. Up to now, a systematic study on the relationship of amino acid residues inside or around the predicted catalytic pocket and the isoprenoid chain elongation has not been undertaken. Herein we report the systematic mutagenesis studies of an *E*-type decaprenyl diphosphate synthase (DdsA) from *Gluconobacter suboxydans* and the corresponding influences on the production of long-chain-length UQs. The expression of the wild type DdsA in *E. coli* makes the host produce UQ-9 and UQ-10 other than the endogenous UQ-8 (Fig. **1**). Thus, we generated nineteen DdsA mutants by site-directed mutagenesis and evaluated their abilities to produce UQs with longer chains by Long Isoprenoids Chain (LIC) value, which is designated by $(Mol_{UQ9}+Mol_{UQ10})/(Mol_{UQ8}+Mol_{UQ9}+Mol_{UQ10})$.

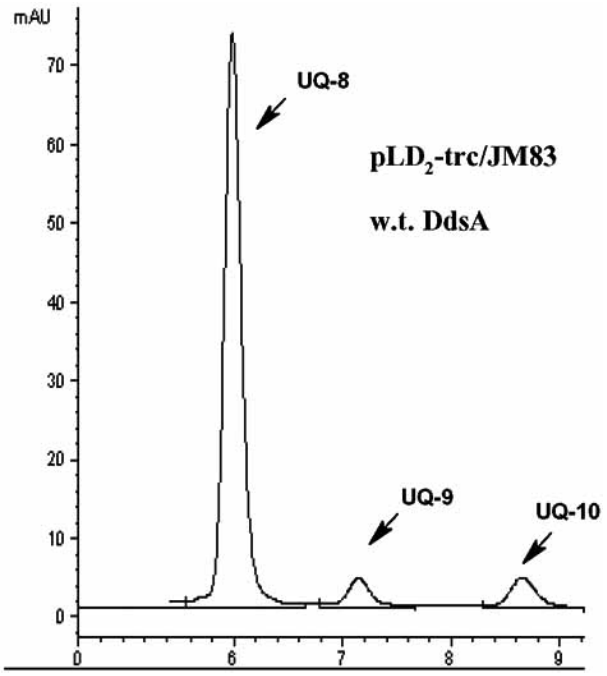

Figure 1. HPLC analysis of UQs UQs was extracted from pLD_2-trc/Jm83. The HPLC analysis was described in material and methods. UQ-8, UQ-9 and UQ-10 can be separated absolutely in this system.

MATERIALS AND METHODS

Enzyme and Medium

The restriction enzymes, DNA-modifying enzymes, *Taq* DNA and *Pyrobest*™ DNA polymerase were purchased from Takara. Tryptone and yeast extract were purchased

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0929-8665/07 $50.00+.00

from OXOID. The final concentration of ampicillin, glucose and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were 100 μg/ml, 1 %(w/v) and 1 mM.

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table **1**.

Site-Directed Mutagenesis of DdsA Protein

DdsA mutants were constructed by site-directed mutagenesis using PCR. PCR was performed in 50 μl volumes containing 1×PCR buffer, 200 μM dNTP, 50 pmol amplification primer pairs, 100 pg plasmid DNA (pLD_2-trc) and 2.5 u DNA polymerase. PCR was carried out for 30 cycles where each cycle consisted of 30 s at 95°C, 1 min at 58°C, and 2min at 72°C. The final cycle was followed by an extension at 72°C for 10 min. Oligonucleotides primers used for PCR are listed in Table **2**. The method of site-directed mutagenesis was described in Fig. **2**. Four different amplified fragments (UC25/TB2 UC70/TB1 UC186/TB1 UC226/TB2) containing partial unchanged *ddsA* gene were ligated with pMD18-T. These plasmids were digested by corresponding restriction enzymes and ligated with pTRC99a to form pUC25, pUC70, pUC186 and pUC226 as described in Table

Table 1. Strains and Plasmids Used in this Study

Strain/plasmid	Relevant characteristics	Source
Strains		
E. coli JM83	F⁻, ara, △(*lac-proAB*), *rpsL*, (Str^r), Φ80, △(*lacZ*), M15	Novagen, USA
Plasmids		
pMD18-T	*Ap^r*	Takara, Japan
pTRC99a	*Ap^r*, *lacZ*	Novagen, USA
pLD_2-trc	*Ap^r*, pTRC99a with 1.2-kb *Bam*HI-*Hin*dIIII fragment containing *dds*A gene	Ye (2004)
pUC25	*Ap^r*, pTRC99a with 1.0-kb *Sma*I-*Hin*dIII fragment containing partial unchanged *dds*A gene	this study
pUC70	*Ap^r*, pTRC99a with 0.4-kb *Nco*I-*Hin*dIII fragment containing partial unchanged *ddsA* gene	this study
pUC186	*Ap^r*, pTRC99a with 0.7-kb *Nco*I-*Hin*dIII fragment containing partial unchanged *ddsA* gene	this study
pUC226	*Ap^r*, pTRC99a with 0.6-kb *Eco*RV-*Hin*dIII fragment containing partial unchanged *ddsA* gene	this study
p25mt1	*Ap^r*, pTRC99a with mutated *ddsA* gene at H25E	this study
p25mt2	*Ap^r*, pTRC99a with mutated *ddsA* gene at H25F	this study
p25mt3	*Ap^r*, pTRC99a with mutated *ddsA* gene at H25G	this study
p25mt4	*Ap^r*, pTRC99a with mutated *ddsA* gene at H25W	this study
p70mt1	*Ap^r*, pTRC99a with mutated *ddsA* gene at A70C	this study
p70mt2	*Ap^r*, pTRC99a with mutated *ddsA* gene at A70G	this study
p70mt3	*Ap^r*, pTRC99a with mutated *ddsA* gene at A70W	this study
p186mt1	*Ap^r*, pTRC99a with mutated *ddsA* gene at A186D	this study
p186mt2	*Ap^r*, pTRC99a with mutated *ddsA* gene at A186G	this study
p186mt3	*Ap^r*, pTRC99a with mutated *ddsA* gene at A186P	this study
p186mt4	*Ap^r*, pTRC99a with mutated *ddsA* gene at A186W	this study
p186mt5	*Ap^r*, pTRC99a with mutated *ddsA* gene at A186Y	this study
p226mt1	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226A	this study
p226mt2	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226D	this study
p226mt3	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226G	this study
p226mt4	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226H	this study
p226mt5	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226I	this study
p226mt6	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226R	this study
p226mt7	*Ap^r*, pTRC99a with mutated *ddsA* gene at K226W	this study

Table 2. Primers Used for PCR

Primer	Sequence
TB1	5'-TGAAATGAGCTGTTGACAATTAATC-3'
TB2	5'-GACCGCTTCTGCGTTCTGAT-3'
TB3	5'-ACTCAACCAAGTCATTCTGAGAATAG-3'
TB4	5'-TACTGTTGTCGAAACCGTCGTT-3'
UC25	5'-TCGCCCCGGGAGGCA A-3'
UC70	5'-CGGTATGAA AGTACTCAACGC A-3'
UC186	5'-AGAGAGGCCTCTTCTGCTTCC-3'
UC226	5'-AAGGCGATATCACCCTGCC-3'
H25E	5'-GCCGCGACA AGTTCGGCG-3'
H25F	5'-GCCGCGACA AGGAAGGCG-3'
H25G	5'-GCCGCGACA AGACCGGCG-3'
H25W	5'-GCCGCG ACA AGCCAGGCG-3'
A70C	5'-TCATTCATACCTGCACACTGCTGCT-3'
A70G	5'-TCATTCATACCGGTACACTGCTGCT-3'
A70W	5'-TCATTCATACCTGGACACTGCTGCT-3'
A186D	5'-AAGATCTGGAGCGGTTTGG-3'
A186G	5'-AAGGTCTGGAGCGGTTTGG-3'
A186P	5'-AACCGCTGGAGCGGTTT-3'
A186W	5'-AATGGCTGGAGCGGTTTG-3'
A186Y	5'-AATACCTGGAGCGGTTTGG-3'
K226A	5'-AGCGCCTTCACGCATGTCA-3'
K226D	5'-ATCGCCTTCACGCATGTCA-3'
K226G	5'-ACCGCCTTCACGCATGTCA-3'
K226H	5'-GTGGCCTTCACGCATGTCA-3'
K226I	5'-GATGCCTTCACGCATGTCA-3'
K226R	5'-ACGGCCTTCACGCATGTCA-3'
K226W	5'-CCAGCCTTCACGCATGTCA-3'

Sites for *Sma*I, *Sca*I, *Stu*I and *Eco*RV (underlined bases) was introduced into the primers

1. The mutated PCR fragments (H25X/TB1 A70X/TB3 A186X/TB2 K226X/TB1; the letter X indicated different amino acid in this study) were amplified with *Pyrobest*™ DNA Polymerase and ligated with plasmid pUC25, pUC70, pUC186 and pUC226 digested by *Sma*I, *Sca*I, *Stu*I and *Eco*RV, respectively.

Polarity and ASA of the substitutes are considered. ASA of an atom is defined as the area over which the center of a water molecule can be placed while retaining van der Waals contacts with that atom and not penetrating any other atom [17]. The areas of 20 amino acids have been reported by Chothia [18] and are known to be simply proportional to the two-thirds power of their molecular weights. The ASA of amino acids used in this study is in the order: Gly(G)<Ala(A)<Pro(P)<Cys(C)<Ile(I)<Asp(D)<Lys(K)<Glu(E)<His(H)<Phe(F)<Arg(R)<Tyr(Y)<Trp(W).

All mutants were sequenced in Invitrogen Biotechnology Co., Ltd..

Expressing DdsA Protein in E. coli

E. coli JM83 harboring wild type or mutant *ddsA* gene grown at 37°C in LB containing 100 μg/ml ampicillin. Target protein was expressed after induction with 1 mM IPTG.

UQs Extraction and Analysis

After incubation overnight, the same amounts of biomass were collected by centrifugation 6000 rpm for 10 min and washed with 0.9% (w/v) NaCl. UQ was extracted using the methods of Lenaz G.. The analysis of UQ was carried out by HPLC on a C18 reserve-phase column (ZORBAX Eclipse XDB-C18; 4.6×250mm; Agilent) with ethanol as the mobile phase at a flow rate of 1ml/min. Detection was at 275nm. [19].

Prediction of 3D Structure

The 3D structures of DdsA mutants were predicted by SWISS-MODEL [20,21] (http://swissmodel.expasy.org) and imported into Swiss-PdbViewer [22].

RESULTS AND DISCUSSION

We generated nineteen mutants of DdsA via site-directed mutagenesis, focused on four residues, which are either inside (A70) or around (H25, A186 and K226) the predicted catalytic pocket, to study their roles in catalyzing the formation of side chain of UQs. All mutations were verified by DNA sequencing. The predicted 3D structures of all mutants

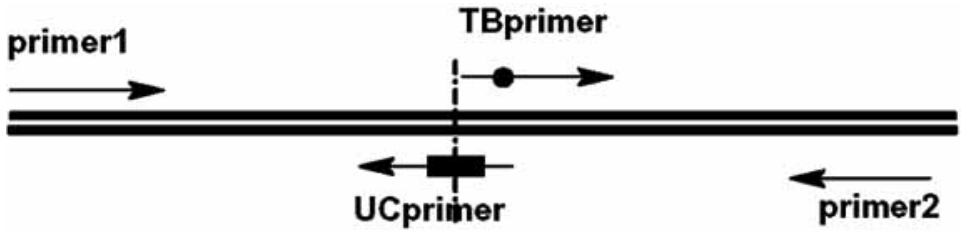

Figure 2. Site-directed mutagenesis contains two steps. UCprimer contain a **blunt end** restriction enzyme site (black frame). TBprimer has nucleotide substitutions (**black dot**). **Unchanged gene fragment** is amplified with primer1/UCprimer and digested by the **blunt end** restriction enzyme. TBprimer/primer2 is used to amplify the mutated fragment with Pyrobest™ DNA Polymerase. The mutant gene will be produced by ligating these two fragments.

were shown in Fig. **3**. Four a statistical purpose, the production of UQs of each mutant was tested in five individual experiments and evaluated by LIC values (Table **3**). In this study, an HPLC system was developed to analyze the production of UQs and each UQ component was separated clearly for calculating the relevant yield. An example was given in Fig. **1**.

His25 is located upstream of the first conserved region. Not any study on this position was reported before. Comparison of the His25 mutants (H25E, H25F, H25G and H25W) with wild type protein revealed that their average LIC values did not change significantly (Table **3**). Hydrophilic glutamic acid (E) and hydrophobic phenylalanine (F) have similar ASA as that of histidine (H), while hydrophilic glycine (G) and hydrophobic tryptophan (W) are obviously different with the histidine (H) in ASA. Neither polarity nor ASA of amino acid in His25 affects the production of UQs in chain length. These results suggest that the amino acid at this position is not important in the side chain formation of UQs. Accordingly, the 3D model of DdsA revealed His25 to be far from the catalytic pocket (Fig. **3** line1).

In light of previous evidence supporting the amino acids with small ASA upstream of the first DDxxD motif allowed the enzyme to synthesize products with longer chains [4,16], we sought to examine the role of Ala70 at this position. LIC values of two Ala70 mutants, A70G and A70C, were increased by 81% and 129%, respectively, whereas that of the mutant A70W was decreased by 69%. Yields of UQ-9 in these mutants were in the order of A70C>A70G> WT>A70W, while yields of UQ-10 were in the order of A70G> WT > A70C >A70W (Table **3**). Compared with the wild type protein, both A70C and A70G mutants have slightly increased LIC values. The A70C mutant produced more UQ-9 and less UQ-10 than the A70G mutant, although with similar LIC values. In 3D model of DdsA, Ala70 is located inside of the catalytic pocket and upstream of the first DDxxD motif (Fig. **3** line2). Amino acid with a larger ASA at this position prevented isoprenoid chain elongation. Consistent with the order of ASAs of amino acids (G<A<C), our studies revealed that A70C mutant produced more UQ-9, while A70G mutant produced more UQ-10, and the yield of UQ-10 by wild type protein is higher than that of A70C mutant but lower than that of A70G mutant. Thus, our results suggested that the ASA of amino acid residue at the position 70 is critical for determining the side chain length of produced UQs.

A186 is located at the export channel of catalytic pocket in the predicted 3D model (Fig. **3** line3). Thus, mutations at this position were also investigated for the influence on UQs production. In comparison with the type protein, LIC values of two mutants, A186D and A186G, were increased by 221% and 437%, respectively, whereas those of mutants A186W and A186Y were decreased by 77% and 89%, respectively. However, the LIC value of the mutant A186P was found to be similar to that of wild type protein. Given that the isoprenoid side chain of UQs is hydrophobic, our data suggested that the isoprenoid chain favored a hydrophilic amino acid residue at the position 186 (*e.g.* A186D

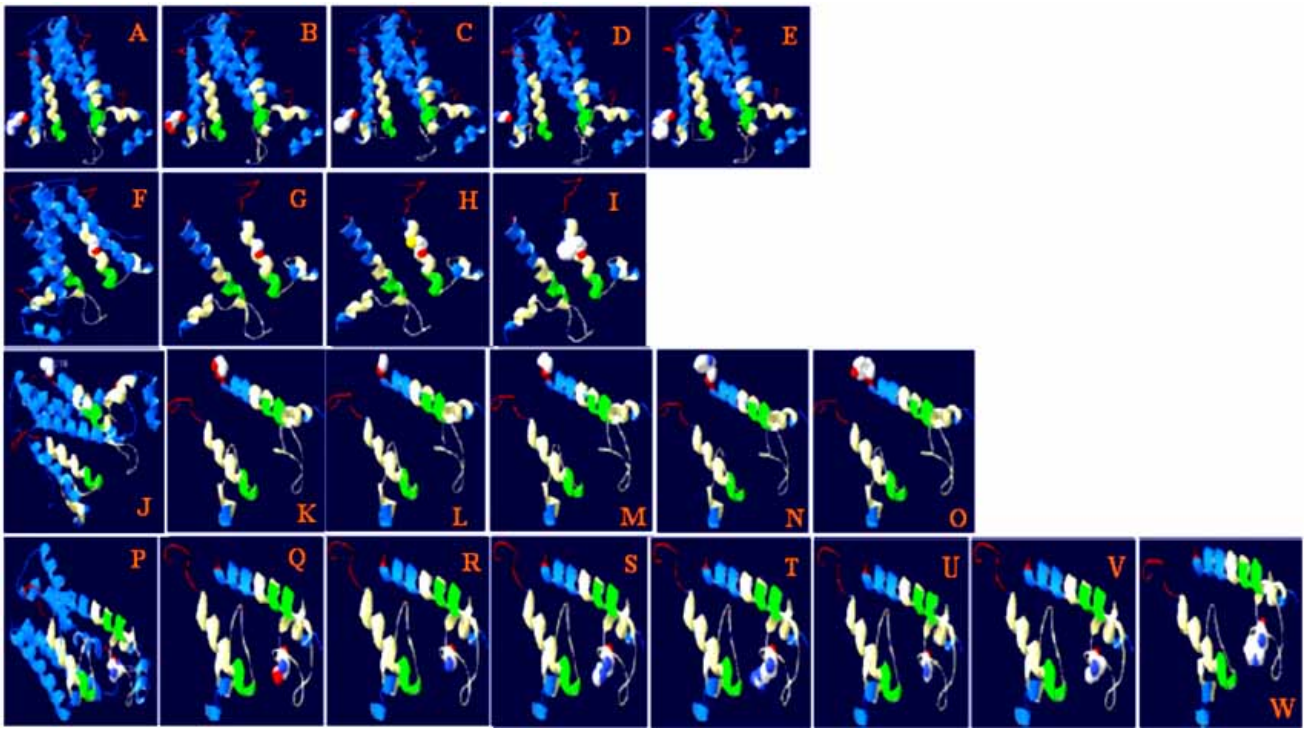

Figure 3. Predicted 3D models of wild type and mutated DdsA proteins A, F, J and **P** is wild type protein structure with His25, Ala70, Ala186 and Lys226 showed in sphere respectively. **B** (H25E)**; C** (H25F)**; D** (H25G)**; E** (H25W)**; G** (A70G)**; H** (A70C)**; I** (A70W)**; K** (A186D)**; L** (A186G)**; M** (A186P)**; N** (A186W)**; O** (A186Y)**; Q** (K226D)**; R** (K226G)**; S** (K226H)**; T** (K226R)**; U** (K226A)**; V** (K226I)**; W** (K226W). In the model, conserved regions II and VI were shown in white and two DDXXD motifs were shown in green, residues substituted were shown in sphere.

Table 3. Average UQs peak area and LIC value of *E.coli* JM83 harboring mutant *dds*A genes in five individual experiments

	UQ-8 (mAu)	UQ-9 (mAu)	UQ-10 (mAu)	LIC	Increase (+) or decrease (-) of the LIC compared to WT
WT	746.13±82.54	56.67±8.31	68.24±14.62	0.1268±0.0149	0
H25E	664.47±18.74	45.92±6.30	60.66±6.27	0.1226±0.0103	-3.3%
H25F	616.02±74.87	45.19±9.18	59.06±14.28	0.1291±0.0198	+1.8%
H25G	694.63±50.05	47.11±5.34	65.57±5.32	0.1240±0.0045	-1.7%
H25W	654.67±27.77	44.44±4.37	59.46±7.16	0.1216±0.0098	-2.2%
A70C	414.00±29.42	128.01±4.77	60.85±6.47	0.2902±0.2632	+129%
A70G	651.92±44.61	91.06±5.60	132.82±19.16	0.2298±0.0113	+81%
A70W	870.50±128.40	32.51±6.52	7.38	0.0397±0.0018	-69%
A186D	341.49±54.96	115.35±16.10	153.32±22.96	0.4075±0.0224	+221%
A186G	82.50±3.57	109.82±11.04	92.66±15.66	0.6814±0.0185	+437%
A186P	679.43±25.53	51.01±5.90	60.26±7.71	0.1253±0.0111	-1.2%
A186W	811.54±116.93	24.64±7.20	3.03	0.0296±0.0025	-77%
A186Y	802.95±44.84	12.61±1.00	0.00	0.0142±0.0010	-89%
K226A	167.49±26.03	218.15±38.58	110.21±25.03	0.6337±0.0115	+400%
K226D	634.33±36.12	45.40±3.81	56.89±8.78	0.1237±0.0105	-2.4%
K226G	579.46±75.69	45.07±7.31	51.77±10.26	0.1265±0.0122	-0.2%
K226H	652.43±39.61	41.08±4.51	65.97±20.74	0.1236±0.0182	-2.5%
K226I	212.75±40.86	121.61±18.08	164.42±47.22	0.5386±0.0076	+325%
K226R	662.66±21.00	47.19±4.21	60.10±4.30	0.1239±0.0063	-2.3%
K226W	257.12±37.20	113.97±12.18	154.66±20.47	0.4776±0.0213	+277%

and A186G) than a hydrophobic one (*e.g.* wild type). Thus, more UQs with longer isoprenoid chain were produced by the mutants A186D and A186G. However, the side chain length was not only dependent on the polarity of the amino acid residue at the position 186. Aspartic acid is more hydrophilic than glycine, while the LIC value of A186D (0.4075) is lower than that of A186G (0.6814). Thus, we assume that the ASA of a given amino acid at 186 also contributes to the side chain elongation. An amino acid residue with smaller ASA at the position 186 gave more space for products with a longer side chain to exit the catalytic pocket. Specifically, two mutants, A186Y and A186W, which had a residue with large ASA at 186, almost produced neither UQ-9 nor UQ-10 (Table **3**). These results indicated that a mutant with a hydrophilic amino acid and a small ASA at the position 186 was more liable for producing UQs with longer isoprenoid side chain. This was evident from the fact that mutant A186G gave the highest LIC value in this study.

The role of Lys226 in the catalytic property of DdsA was also investigated. Seven mutants (K226A, K226I, K226W, K226D, K226G, K226H and K226R) were generated and the LIC values were evaluated. Mutants with a hydrophilic amino acid residue (G, H, D and R) gave similar LIC values as the wild type protein. While LIC values of mutants with a hydrophobic amino acid residue (A, I and W) were increased by 277%-400%. Given that the substrate IPP is hydrophobic, a more hydrophobic amino acid residue at the position 226 will form a catalytic pocket (Fig. **3** line4) capable of attracting more IPP for side chain elongation. Thus, our data undoubtedly revealed that the polarity of the amino acid residue at the position 226 is a determinative factor for the side chain length of produced UQs. Conclusively, more UQs with longer isoprenoid chains were produced by mutants with a hydrophobic amino acid residue at the position 226 than a hydrophilic one.

We also found that mutants A70C, A186D, A186G, K226A and K226I produced more UQ-9 and UQ-10 but less UQ-8. We assume that the condensation of IPP with allylic substrates is dependent on energy consumption. When glucose was appended during the culture, the yield of each UQ increased (Fig. **4**). Octaprenyl diphosphate (C_{40}), solanesyl diphosphate (C_{45}) and decaprenyl diphosphate (C_{50}) are formed via condensation of FPP (C_{15}) with five, six and seven IPPs (C_5), respectively. Producing UQ-9 and UQ-10 will consume more substrates and more energy. This will prevent the cell from producing UQ-8. Certainly, this hypothesis needs to be proofed with some other experiments.

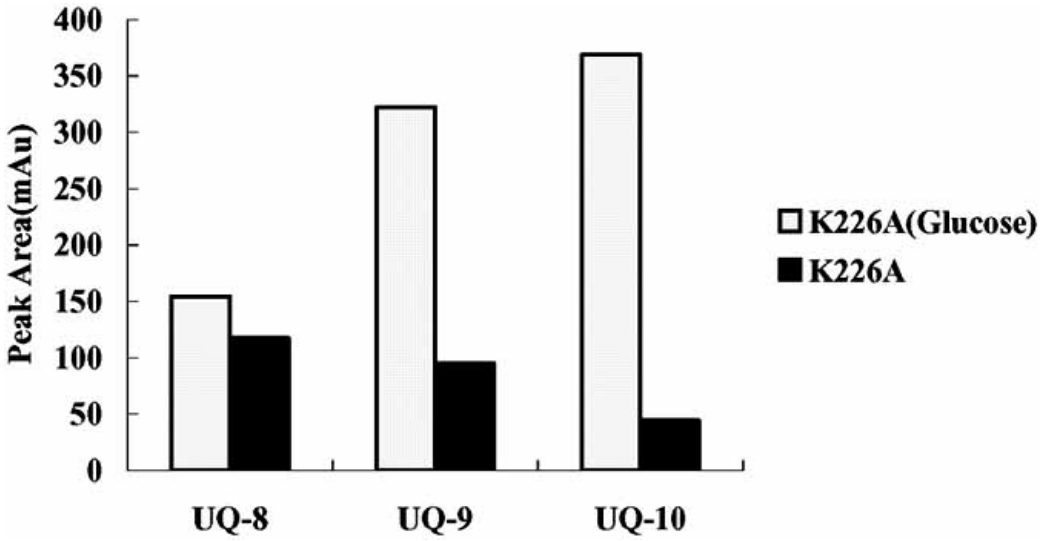

Figure 4. UQs produced by K226A/JM83 (append glucose during culturing) and K226A/JM83 X- axis is the species of UQs Y-axis is the yield of UQs.

The mechanism of isoprenoid side chain elongation in UQ biosynthesis is complicated. In this work, we investigated the roles of amino acid residues around the catalytic pocket of DdsA in side chain elongation though site-directed mutagenesis. Our experimental data revealed several aspects in producing longer side chains in UQ biosynthesis: hydrophobic amino acid in 226 and hydrophilic amino acid with small ASA in 186 are preferable for synthesizing UQs with longer isoprenoid chains. Given the relevant medicinal important of UQ-10, engineering a strain only producing UQ-10 would be of great industrial prospective. However, a single amino acid substitution may not result in the production of a single polyprenyl diphosphate side chain. Nevertheless, our study presented here sets the stage to pursue for a mutant producing a single product, particularly UQ-10, via the combination of multiple sites mutation.

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ABBREVIATIONS

ASA = Accessible Surface Area

DdsA = Decaprenyl diphosphate synthase

FPP = Farnesyl pyrophosphate

IPP = Isopentenyl pyrophosphate

LIC = Long Isoprenoids Chain

UQ = Ubiquinone

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