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FULL PAPER

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Overexpression and Characterization of a Novel Plant Carotenoid Cleavage Dioxygenase 1 from *Morus notabilis*

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Synthesis of β-ionone in microbial cell factories is limited by the efficiency of carotenoid cleavage dioxygenases (CCDs). To obtain genes responsible for specific cleavage of carotenoids generating β-ionone, a novel carotenoid cleavage dioxygenase 1 from *Morus notabilis* was cloned and overexpressed in *Escherichia coli*. The MnCCD1 protein was able to cleave a variety of carotenoids at the positions 9, 10 (9′, 10′) to produce β-ionone, 3-hydroxy-4-oxo-β-ionone, 3-hydroxy-β-ionone, and 3-hydroxy-α-ionone *in vitro*. MnCCD1 could also cleave lycopene and β-carotene at the 9, 10 (9′, 10′) bind bond to produce pseudoionone and β-ionone, respectively, in *E. coli* accumulating carotenoids. The enzyme activity of MnCCD1 was reached 2.98 U/mL at optimized conditions (temperature 28 °C, IPTG 0.1 mM, induction time 24 h). The biochemical characterization of MnCCD1 revealed the optimal activities were at pH 8.4 and 35 °C. The addition of 10% ethanol could increase enzyme activity at above 15%. However, an obvious decline was observed on enzyme activity as the concentration of Fe^{2+} increased (0–1 mM). The V_{max} for β-apo-8′-carotenal was 72.5 U/mg, while the K_m was 0.83 mM. The results provide a foundation for developing the application of carotenoid cleavage dioxygenases as biocatalysis and synthetic biology platforms to produce volatile aroma components from carotenoids.

Keywords: *Morus notabilis*, carotenoid cleavage dioxygenases, CCD1, β-ionone.

1. Introduction

Apocarotenoids are important compounds generated from a series of carotenoid oxidative cleavage in organisms by CCDs (carotenoid cleavage dioxygenases), widely distributed in bacteria, fungi, plants, and animals.[1] Among them, an exciting subfamily of these compounds is plants apocarotenoids, such as volatile scent compounds (α-ionone, β-ionone), pigments (bixin, crocin), and regulatory hormones (abscisic acid).[2] Notably, β-ionone is a highly valued chemical. It has a warm, woody, berry, characteristic violet odor, and lower odor threshold (0.007 ppb in air and 1 ppb in water).[3] Therefore, it is widely applied in the food, cosmetics, and perfume industry.[4,5] Besides, it also has various biological activities, including anticancer, chemopreventive, cancer-promoting, melanogenesis, anti-inflammatory, and antimicrobial activity.[6] However, the development of β-ionone is mainly focused on chemical synthesis[7] due to their lower abundance in plants, complicated extraction, and isolation.[8] Nevertheless, more importantly, the synthesis product cannot be sold as a natural flavor which is difficult to meet the needs of spices, cosmetics, food, medicine, and other industries. With the development of biotechnology, microbial cell factories have been success-

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fully applied to the synthesis of aroma components. Beekwilder et al. first expressed carotenoid cleavage dioxygenase (RiCCD1) from raspberry to produce β-ionone in *Saccharomyces cerevisiae* accumulating β-carotene and obtained β-ionone of 0.22 mg/g DCW.[3] In recent years, heterologous synthesis of β-ionone using plant-derived CCDs has been successfully engineered in *Escherichia coli* which reached 32.4 mg/L of β-ionone in shake flasks,[9] the highest β-ionone titer in *E. coil*. In addition, β-ionone production in other microorganisms has also been attempted. For example, in *S. cerevisiae*, titers of 33 mg/L and 184 mg/L in shake flasks have been reported;[10,11] while in the oleaginous yeast *Yarrowia lipolytica*, its yield reached up to 358 mg/L in shake-flask fermentation and 1 g/L in fed-batch fermentation, the highest β-ionone titer of engineered strain reported to date.[12]

Nevertheless, the major problem restricting the efficient synthesis of β-ionone is that the catalytic activity of CCDs in the synthetic pathway is low, and specificity is not strong leads to the low production of β-ionone. As a result, the diversion of pathway flux and the unwanted product formation occur during the synthesis process of β-ionone. The biosynthesis of β-carotene and β-ionone by recombinant *Y. lipolytica* constructed by Czajka et al., the yield of β-carotene was as high as 2.5 g/L. However, when OfCCD1 (*Osmanthus fragrans*) was introduced into strains, there was a sharp decline in the titer of β-ionone.[13] All studies have shown that the CCD enzyme is the rate-limiting enzyme in the whole synthesis pathway of β-ionone.

According to the function of carotenoid cleavage dioxygenases (CCDs), five known major subfamilies (CCD1, CCD4, CCD7, CCD8, 9′-*cis*-epoxycarotenoid dioxygenase (NCED)) were identified in plants.[14] The first CCD was cloned and functionally characterized as an enzyme from *Zea mays* known as viviparous-14 (VP14), which cleaved the C11-C12 double bond of substrate 9′-*cis*-epoxycarotenoid to synthesize xanthoxin, the precursor of abscisic acid.[15] The CCD1 and CCD4 prefer cleaved carotenoids at the C9-C10 (or C10′-C9′) double bond to generate the C13 products, contributing to the flavor, aroma, and pigmentation of fruits and flowers.[16] Subsequently, CCD7 and CCD8 appeared to be dedicated to the biosynthesis of strigolactones releasing 10′-apo-β-caroten-10′-al.[16–18]

In addition, in view of the current research on CCDs, most of the studies on CCDs mainly focus on plant metabolism; only the qualitative data of the enzyme function was presented by expression CCD in *E. coli* strain accumulating carotenoids. However, there is a lack of relevant data on the catalytic activity and enzymatic properties. Secondly, there is no relatively standard reaction system for investigating the properties of CCDs *in vitro*, and the reaction systems used in reports are various. As a result, it is difficult to compare and estimate the catalytic ability and properties of CCDs from different sources. Therefore, it is of great significance to explore efficient CCD resources and establish a complete evaluation system for the biosynthesis of β-ionone.

In this study, we found a hypothetical MnCCD1 enzyme from *Morus notabilis* through gene mining. The function of MnCCD1 was investigated for carotenoids *in vivo* and *in vitro*. Subsequently, the expression of MnCCD1 was further optimized in cultures, and the detailed biochemical properties of the enzyme were also characterized.

Experimental Section

Chemicals

β-ionone, β-apo-8′-carotenal, lycopene, β-carotene, zeaxanthin, lutein, astaxanthin, all-*trans*-retinal were purchased from Aladdin (Shanghai, China). Isopropyl-β-D-thiogalactoside (IPTG), chloramphenicol, and ampicillin were purchased from Sangon Biotech (Shanghai, China). HPLC-grade ethyl acetate, methanol, acetone were purchased from Sigma-Aldrich. All other reagents used in this study were analytical-grade chemicals.

Plasmid Construction and Sequence Analysis of MnCCD1

The putative gene CCD of *Morus notabilis* (MnCCD1, GenBank accession No. XP_024025901.1) was chemically synthesized with codons optimized for *E. coli* expression. The *BamHI* site was added to the 5′ end of the gene, and the *EcoRI* site was added to the 3′ end of the gene. MnCCD1 was digested with *BamHI* and *EcoRI*, and it was subcloned into the pETDuet-1 and pGEX-2T expression vector at the *BamHI* and *EcoRI* sites to generate pETDuet-1-MnCCD1 and pGEX-2T-MnCCD1, respectively. The neighbor-joining phylogenetic tree was obtained by using the MEGA 7 software. The Clustal X 2.0 was used for multiple sequence alignment.

Protein Expression and Purification

The recombinant plasmid pGEX-2T-MnCCD1 was transformed into *E. coli* BL21 (DE3) by heat stock. For

protein expression, recombinant *E. coli* cells were overnight cultured at 37 °C and then inoculated in 50 mL of Luria-Bertani (LB) medium at 37 °C, 180 rpm. When the optical density at a wavelength of 600 nm (OD_{600}) for cells reached about 0.6–0.8, isopropyl-β-D-thiogalactoside (IPTG) was added at a final concentration of 0.1 mM, and the cultures were further incubated at 28 °C for 21 h. Then, the culture conditions of BL21 (DE3) carrying recombinant plasmid PGEX-2T-MnCCD1 in LB medium were further optimized. The cells were harvested by centrifugation (10000 *g*, 20 min, 4 °C), resuspended in 5 mL of 1×PBS (pH 7.4) containing 5 mM Na-ascorbate, and lysed by ultrasonication on ice. The cell debris was removed by centrifugation (10000 *g*, 40 min, 4 °C). According to the manufacturer's protocol, the GST-tagged recombinant protein was purified with GST affinity chromatography (Sangon Biotech, Shanghai, China). The protein was examined by SDS-PAGE. Protein concentrations were determined using the protein Bradford assay (Sangon Biotech, Shanghai, China) with Bovine Serum Albumin (BSA) as a standard.

Preparation of Carotenoids for In Vitro *Assays*

The preparation of carotenoids was used according to the previously described method[19] and slightly modified. Carotenoid substrates were applied at a final concentration of 40 μM. To prepare carotenoid micelles, the substrates were first dissolved in 50 μL of dichloromethane and mixed with 50 μL of Triton X-100 (100 molar ratio with carotenoid substrates) in ethanol, then dried in a vacuum centrifuge. The residues were resuspended in ultrapure water and stored at 4 °C before being used.

Substrate Specificity Assays In Vitro

Substrate specificity experiments for MnCCD1 were conducted based on the previous method and slightly modified.[20] The reactions were filled to 1 mL with an appropriate sodium phosphate buffer, substrate, 100 μL of MnCCD1, and incubated at 35 °C overnight. After the incubation, 0.5 mL of chloroform was added to the reaction mixture as an enzyme inhibitor and extractant of assay products. For gas chromatography-mass spectrometer of the C13 compounds, a Trace ISQ-LT GC/MS (Thermo Fisher, USA) and a DB-5MS column (30 m×0.25 mm×0.25 μm) with flame ionization detector were used. Interface temperature was 250 °C, EI-MS with 70 eV (electron impact ionization) ionization energy, and swath range was 50–450 *m/z*. The spectra were evaluated with the Xcalibur software version 1.4.

Two-Plasmid System for In Vivo *Rests on Carotenoid Cleavage Activity*

E. coli strains engineered to accumulate lycopene (pAC-LYCipi, addgene plasmids # 53279),[21] β-carotene (pAC-BETA, addgene plasmids # 53272),[22] and zeaxanthin (pAC-ZEAX, addgene plasmids # 53274)[23] were transformed with the expression plasmid pETDuet-1-MnCCD1 or the control plasmid pETDuet-1.

The recombinant protein production was induced by 0.1 mM IPTG, and the volatiles was collected by SPME and analyzed by GC/MS, respectively, as described above based on the previous studies.[24]

Carotenoids were extracted from bacterial cells or growth medium as described previously[9] and then analyzed by HPLC. For HPLC (1200 Infinity II, Agilent Technologies, America), a reversed-phase Zorbax Eclipse Plus C18 column (5 μm, 1.5×4.6 mm, Agilent Technologies, Littlefalls, DE) was used. The mobile phase consisted of methanol/ethyl acetate/water (50%/48%/2%, v/v). The isocratic elution was used and maintained at 1 mL/min for 20 min at 30 °C. The DAD wavelength of 450 nm was used for carotenoids detection.

Enzyme Characterization

The CCD activity was measured as described previously and slightly modified.[25] The activity of MnCCD1 was determined HPLC using β-apo-8′-carotenal as substrate. The assay was performed with 250 μL of a 160 μM substrate solution in a total volume of 1 mL of 100 mM sodium phosphate buffer at pH 7.2 initiated via the addition of 100 μg of the purified enzyme at 35 °C water bath for 30 min. After the incubation, 1 mL of ethanol was added to the reaction mixture as an enzyme inhibitor. A control solution was prepared against β-apo-8′-carotenal under the same reaction conditions without purified enzyme. One unit of activity was defined as the amount of enzyme that catalyzed the formation of 1 nM β-ionone per minute.

Determination of the Optimal Temperature

A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg purified MnCCD1 in 100 mM sodium phosphate buffer, pH 7.2. The reaction was

performed under temperatures ranging from 25 to 65 °C for 30 min.

Determination of the Optimal PH

A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg MnCCD1 in 100 mM sodium acetate buffer with pH ranging from 3.6 to 5.6; or in 100 mM sodium phosphate buffer with pH ranging from 6.0 to 8.0; or in 100 mM glycine-NaOH buffer with pH ranging from 8.4 to 10.4. The reaction was performed under 35 °C for 30 min.

Determination of the Temperature Stability

The temperature stability of the MnCCD1 was determined by measuring their remaining activity after incubating the enzyme at a temperature ranging from 35 to 45 °C for 20–120 min. Then, the residual activity of the enzyme incubated at variant temperature and time was determined immediately. A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg incubated purified MnCCD1 in 100 mM sodium phosphate buffer, pH 7.2. The reaction was performed under 35 °C for 30 min. The activity of the enzyme without pre-incubation was defined as 100 %.

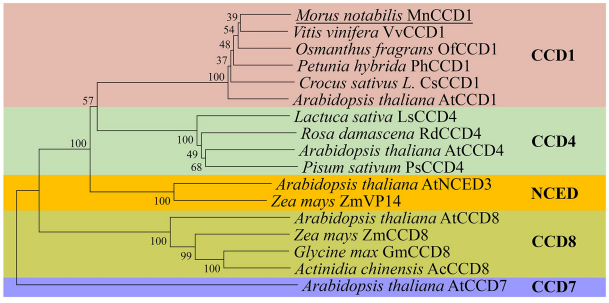


Figure 1. Phylogenetic tree of deduced amino acid sequences of carotenoid cleavage oxygenases from *Morus notabilis* and other plant species. The tree was generated using the MEGA 7.0 program and neighbor-joining algorithm. The resulting tree was bootstrap analyzed with 1000 replicates. The black bold underline indicates the MnCCD1 gene identified in this study. The GenBank accession numbers for the sequences are as follows: *Vitis vinifera* VvCCD1, AGT63320.1; *Osmanthus fragrans* OfCCD1, BAJ05401.1; *Petunia hybrida* PhCCD1, AAT68189.1; *Crocus sativus* L. CsCCD1, CAC79592.1; *Arabidopsis thaliana* AtCCD1, NP_191911.1; *Rosa damascena* RdCCD4, ABY60886.1; *Lactuca sativa* LsCCD4, BAE72094.1; *Arabidopsis thaliana* AtCCD4, NP_193652.1; *Pisum sativum* PsCCD4, BAC10552.1; *Arabidopsis thaliana* AtNCED3, NP_188062.1; *Zea mays* ZmVP14, AAB62181.2; *Arabidopsis thaliana* AtCCD8, NP_001329787.1; *Zea mays* ZmCCD8, NP_001183929.1; *Actinidia chinensis* AcCCD8, ADP37984.1; *Glycine max* GmCCD8, NP_001242715.2; *Arabidopsis thaliana* AtCCD7, BAF01693.1.

Determination of the PH Stability

The pH stability of the MnCCD1 was determined by measuring their remaining activity after incubating the enzyme at 4 °C for 8 h in 50 mM sodium acetate buffer with pH ranging from 3.6 to 5.6, or in 50 mM sodium phosphate buffer with pH ranging from 6.0 to 8.0, or in 50 mM glycine-NaOH buffer with pH ranging from 8.4 to 10.4. Then, the residual activity of the enzyme incubated at variant pH was determined immediately. A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg incubated MnCCD1 in 100 mM sodium phosphate buffer, pH 7.2. The reaction was performed under 35 °C for 30 min. The activity of the enzyme without pre-incubation was defined as 100 %.

Determination of the Fe^{2+} Effect

CCD belongs to the non-heme iron oxygenases, and Fe^{2+} showed the obvious influence on CCD enzyme activity.[26,27] So, we evaluated the effect of Fe^{2+} on MnCCD1. A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg MnCCD1 in 100 mM sodium phosphate buffer, pH 7.2. The Fe^{2+} (100–1000 μM) was added to the reaction, and the reaction was performed under 35 °C for 30 min.

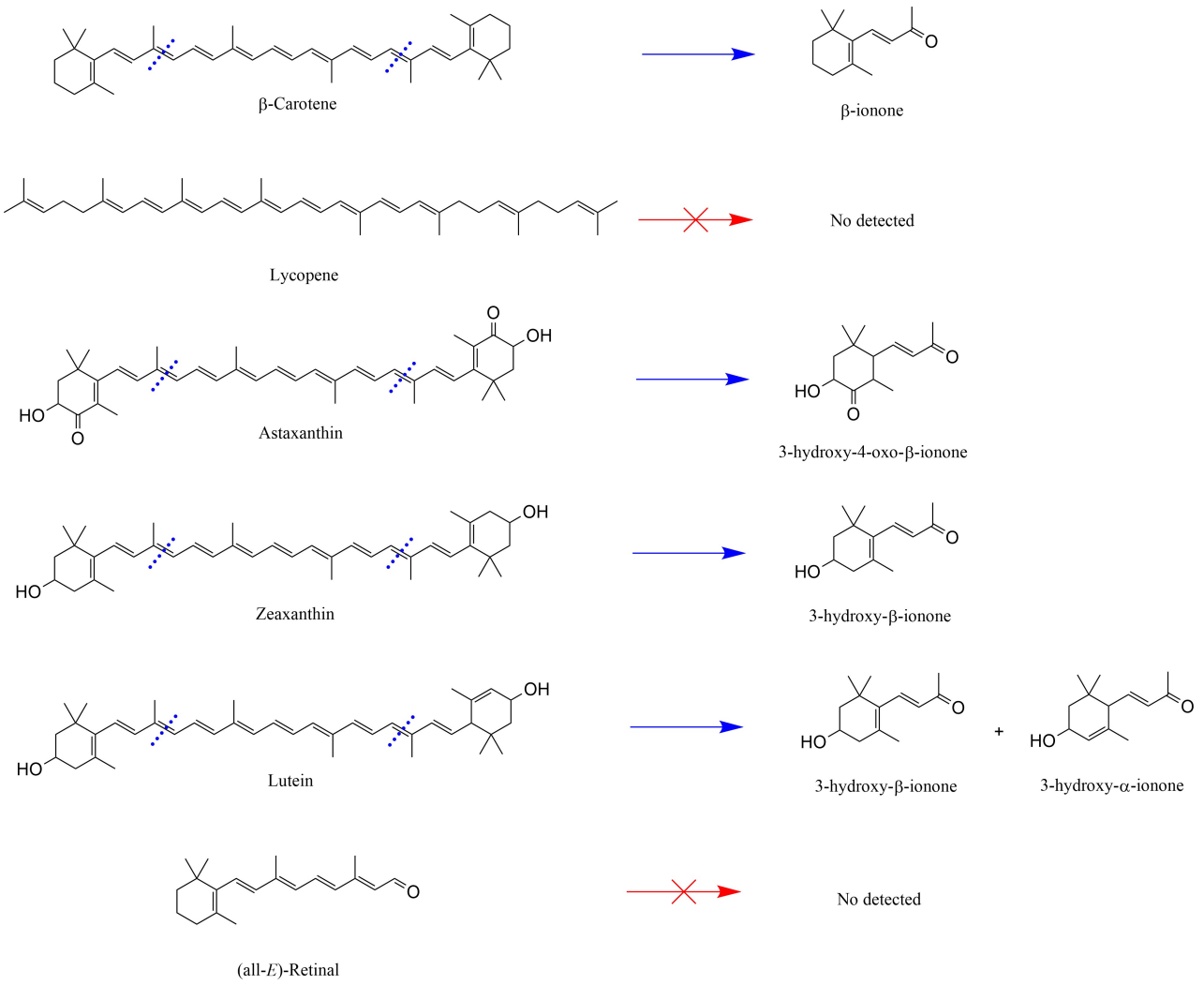
Determination of the Ethanol Effect

A total reaction volume of 1 mL contained 40 μM β-apo-8′-carotenal, 100 μg MnCCD1 in 100 mM sodium phosphate buffer, pH 7.2. The ethanol concentration ranging from 0 to 25 % (v/v) was added to the reaction. The reaction was performed under 35 °C for 30 min.

Determination of the Metals and Chemical Agents Effect

Other metals ions were assayed at the final concentrations of 1 mM in the reaction mixture. The chemical agents EDTA were assayed at concentrations of 10 mM in the reaction mixture.

The kinetic constant on MnCCD1 was processed via a proportional weighted fit using a nonlinear regression analysis program based on Michaelis-Menten enzyme kinetics.[28] The reactions were determined based on incubation of purified enzyme in the



Scheme 1. Carotenoid substrates (left) are oxidatively cleaved by MnCCD1 to yield the degradation products (right) *in vitro*.

presence of increasing 10 μM to 160 μM concentrations of the substrates β-apo-8′-carotenal. The reaction was performed at pH 7.2, 10% ethanol under 35 °C for 30 min.

β-ionone was analyzed using HPLC 1200 system (Agilent, USA) and a C18 column (4.6×250 mm; i.d., 5 μm) with distilled water(A) and methanol (B) at an A/B ratio of 15:85 for 15 min. The flow rate was 1 mL/min, the column temperature was 30 °C, and detection was performed by monitoring the absorbance at 300 nm. The results of the optimum temperature, optimum pH, temperature stability, pH stability, Fe^{2+} effect, and ethanol effect were displayed as percentages of the activity.

2. Results and Discussion

2.1. Sequence Analysis and Phylogenic Analysis of MnCCD1

β-Ionone was generated from β-carotene cleaved at C9-C10 (or C10′-C9′) double bond by CCDs. Among the CCDs subfamily, CCD1 and CCD4 could cleave β-carotene at C9-C10 (C9′-C10′) sites. However, it was reported that CCD4 showed low activities for carotenoids in *E. coli* compared to CCD1.[29] We decided to focus on the excavation of CCD1. According to the published functional CCD1amino acid sequence, We performed a GenBank search for a putative CCD from *Morus notabilis* (GenBank accession No. XP_024025901.1) by BLAST tool.[30] The full-length sequence of MnCCD1 contains an open reading frame of 1635 bp and encodes 544 amino acids.

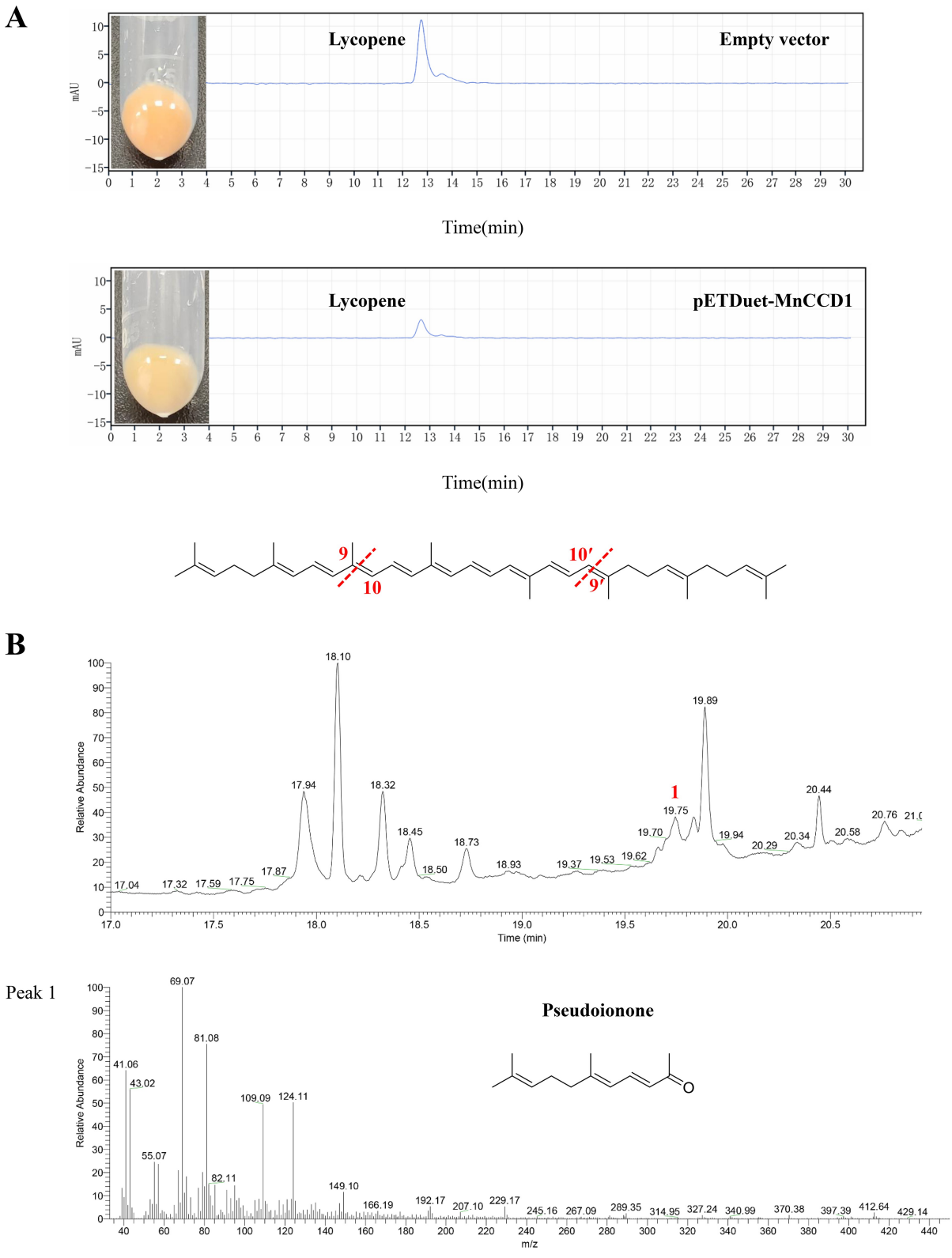


Figure 2. Functional expression of MnCCD1 in lycopene-producing *E. coli* cells. (A) HPLC analysis of lycopene cleaved by MnCCD1 in bacterial cultures. (B) GC-MS analysis of the cleavage products synthesized by MnCCD1. The mass spectra of peaks 1 were identified as pseudoionone with the standard.

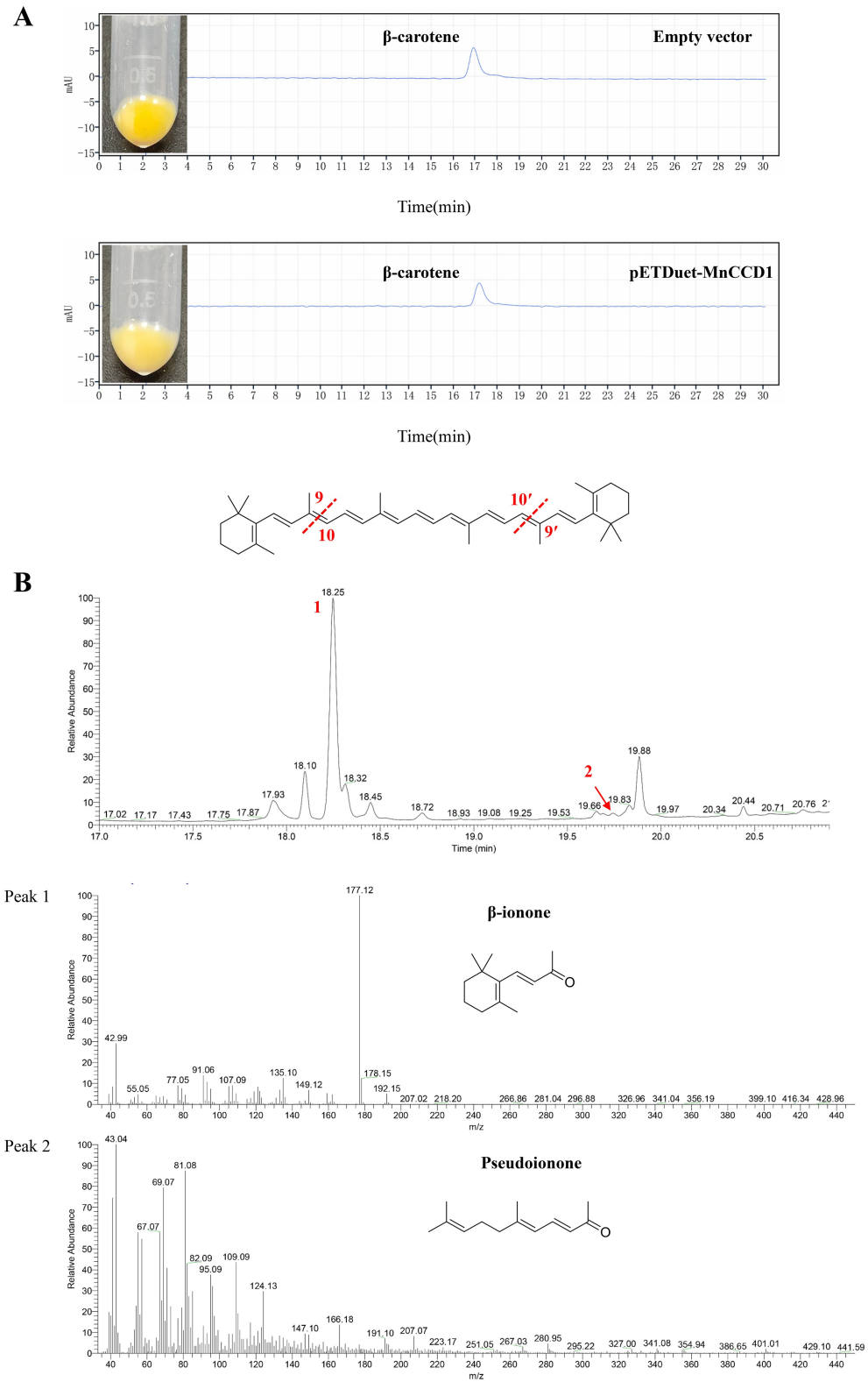


Figure 3. Functional expression of MnCCD1 in β-carotene-producing *E. coli* cells. (A) HPLC analysis of β-carotene cleaved by MnCCD1 in bacterial cultures. (B) GC-MS analysis of the cleavage products synthesized by MnCCD1. The mass spectra of peaks 1 and 2 were identified as β-ionone and pseudoionone with the standard.

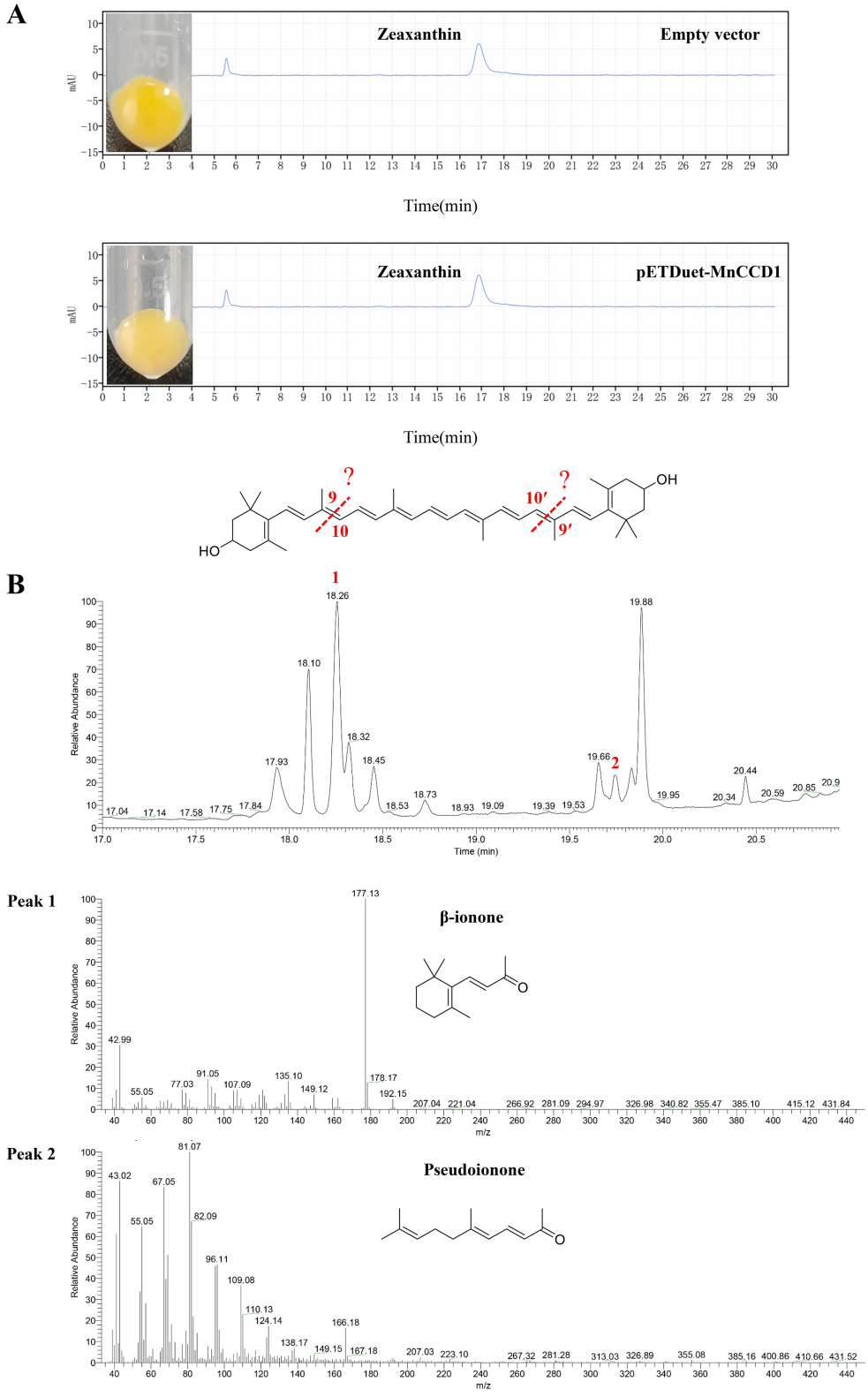


Figure 4. Functional expression of MnCCD1 in zeaxanthin-producing *E. coli* cells. (A) HPLC analysis of zeaxanthin cleaved by MnCCD1 in bacterial cultures. (B) GC-MS analysis of the cleavage products synthesized by MnCCD1. The mass spectra of peaks 1 and 2 were identified as β-ionone and pseudoionone with the standard.

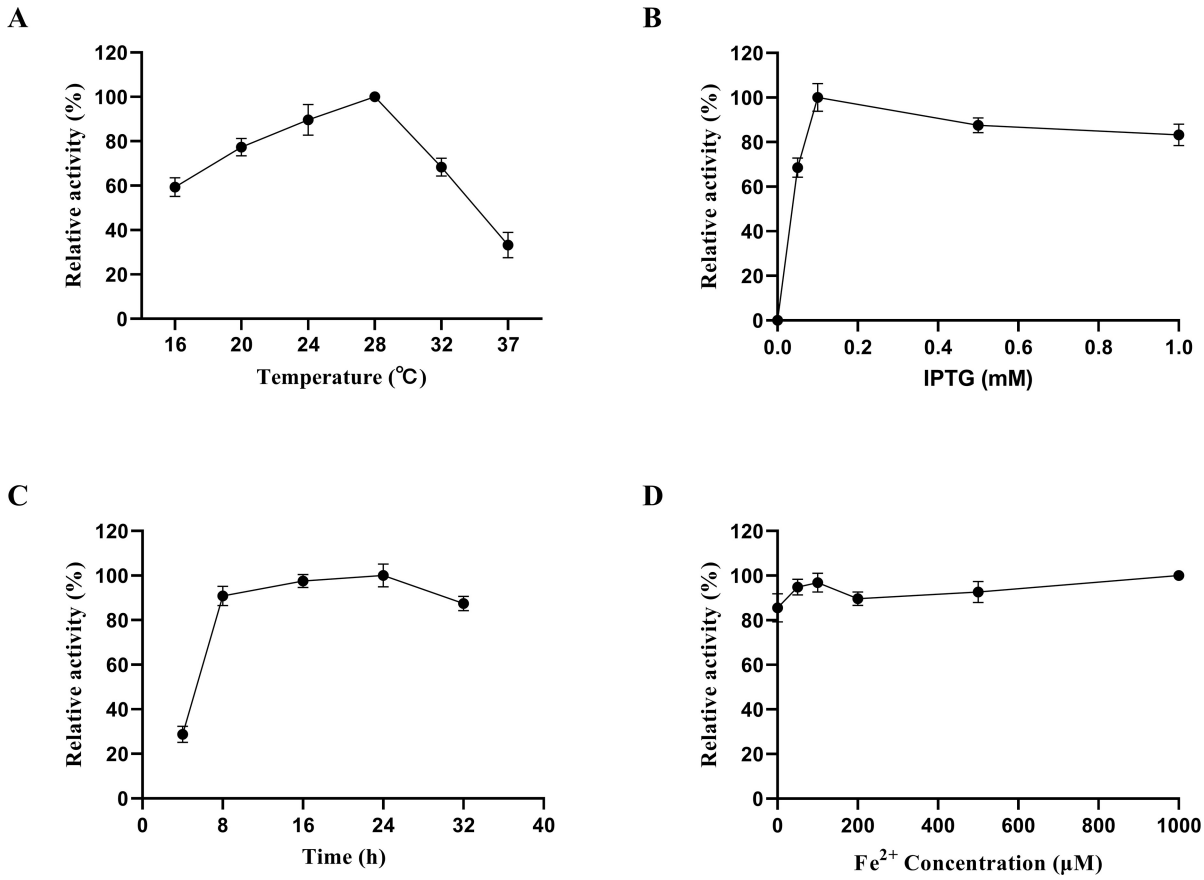


Figure 5. Optimal cultures for the expression of recombinant MnCCD1 in *E. coli*. (A) Effect of temperature on the expression of MnCCD1. (B) Effect of IPTG concentration on the expression of MnCCD1. (C) Effect of induction time on the expression of MnCCD1. (D) Effect of Fe^{2+} concentration on the expression of MnCCD1. The highest activity was defined as 100%. These activities were expressed as relative values. All the results were confirmed by three repeated experiments and data are shown as the means ± SD ($n = 3$).

MnCCD1 and CCDs from other plants were searched to build a tree by using the neighbor-joining (NJ) method (*Figure 1*).[31] The results revealed there were five CCDs families (CCD1, CCD4, NCED, CCD7, and CCD8). The protein encoded by MnCCD1 gene showed the closest evolutionary relationship with VvCCD1 (*Vitis vinifera*) and OfCCD1 (*Osmanthus fragrans*), which belong to the CCD1 family.

The first plant carotenoid cleavage enzyme was isolated from *Zea mays* named VP14, whose crystal structure was elucidated in 2010.[32] The amino acid sequence alignment shows that MnCCD1 displays 40.16% sequence similarity with VP14 from *Zea mays*. Moreover, MnCCD1 has a high sequence similarity with PhCCD1 (81.92% identity) from *Petunia hybrida*, VvCCD1 (83.76% identity) from *Vitis vinifera*, AtCCD1 (80.67% identity) from *Arabidopsis thaliana*, and OfCCD1 (84.37% identity) from *Osmanthus fragrans* (Figure S1). Based on the sequence alignment and the secondary structure prediction of MnCCD1, it contains four strictly conversed His residues that function cooperatively to coordinate a non-heme iron co-factor that is essential for (di)-oxygenase activity.[33] It also has the well-conserved, rigid β-propeller scaffold domains and the significant differences in α helical sections among six sequences that correspond to the structural feature of CCDs.[34]

2.2. Functional Characterization of MnCCD1

2.2.1. MnCCD1 Cleaves Carotenoids In Vitro

The substrate specificity of MnCCD1 was determined with five carotenoids (β-carotene zeaxanthin, lutein, astaxanthin, and lycopene) and two β-apocarotenals (β-apo-8′-carotenal, (all-*E*)-retinal) using 40 μM *in vitro*. After incubation, the reaction products were applied to GC/MS analyses (Figure S4–S7).

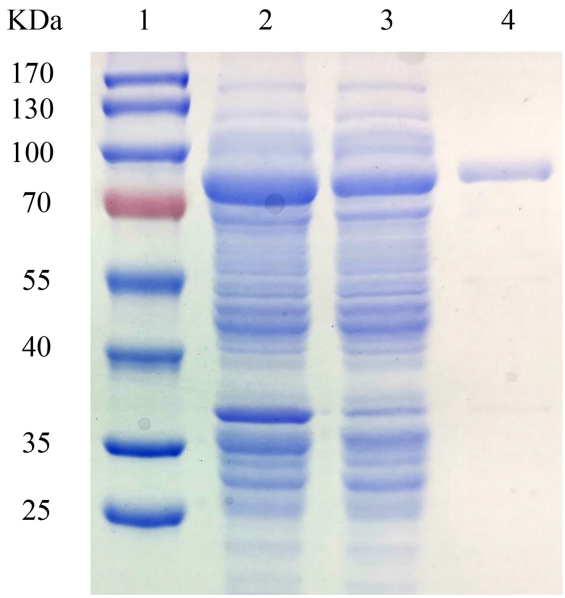


Figure 6. SDS-PAGE analysis of MnCCD1 expression in *E. coli*. Lane 1, protein marker; lane 2, the total cell protein of *E. coli* BL21(DE3) harboring PGEX-2T-MnCCD1; lane 3, the crude extract of *E. coli* BL21(DE3) harboring PGEX-2T-MnCCD1; lane 4, purified MnCCD1.

For symmetrical molecules containing β-carotene, zeaxanthin, lutein, astaxanthin, and lycopene, MnCCD1 could cleave all the symmetrical molecules except for lycopene. A variety of C13 volatile products is generated by 9, 10 (9′, 10′) bond cleavage of the symmetrical molecules (*Scheme 1*). β-Ionone, 3-hydroxy-4-oxo-β-ionone and 3-hydroxy-β-ionone were produced by cleavage of the 9, 10 (9′, 10′) positions of β-carotene, astaxanthin and zeaxanthin, respectively. In addition, MnCCD1 could also cleave the 9, 10 (9′, 10′) double bonds of lutein substrate to produce two different C13 products containing 3-hydroxy-β-ionone and 3-hydroxy-α-ionone.

For asymmetrical molecules including β-apo-8′-carotenal and (all-*E*)-retinal, MnCCD1 could only cleave β-apo-8′-carotenal to generate β-ionone resulting from cleavage at the 9, 10 (9′, 10′) positions (*Scheme 1*). It has been reported that RdCCD1 from *Rosa damascena* has a higher cleavage activity for β-apo-8′-carotenal than (all-*E*)-retinal.[35]

2.2.2. MnCCD1 Cleaves Carotenoids In Vivo

Considering the low solubility of carotenoid substrates in water, which is challenging to detect CCDs′ cleavage activity, we also used the tool of heterologous overexpression of carotenogenesis genes in *E. coli* to identify the function of MnCCD1 *in vivo*. We introduced pETDuet-1-MnCCD1 into *E. coli* strains accumulating β-carotene, lycopene, or zeaxanthin, respectively. Co-expression of MnCCD1 in strains of *E. coli* accumulating β-carotene, lycopene, or zeaxanthin led to the pigmentation fade comparing the control harboring an empty vector (*Figure 2A*, *3A*, *4A*), indicating that MnCCD1 showed catalytic activity for three substrates above or upstream carotenoids in the biosynthetic pathway.

When MnCCD1 was overexpressed in *E. coli* producing lycopene, in contrast to the determination of substrate specificity *in vitro*, pseudoionone was the main product that SPME-GC/MS detected in the headspace of those cells. Thus, using carotenoids as the substrate for detecting CCD function *in vitro* may be limited by their solubility in water.

Pseudoionone was produced by cleavage of the 9, 10 (9′, 10′) positions of lycopene (*Figure 2B*). No extra cleaved products were detected in the headspace of those cells like 6-methyl-5-hepten-2-one (MHO), the 5, 6 (5′, 6′) bond cleavage product of lycopene. These results indicated that MnCCD1 could specifically cleave lycopene at 9, 10 (9′, 10′) bonds to generate pseudoionone. It also proved that using carotenoid substrates for the assay of cleavage activity of CCDs *in vitro* may not be accurate, making the activity of CCDs for carotenoids be omitted.

When MnCCD1 was overexpressed in *E. coli* producing β-carotene, β-ionone was the main product that SPME-GC/MS detected in the headspace of those cells. β-ionone was produced by cleavage of the 9, 10 (9′, 10′) positions of β-carotene (*Figure 3B*). However, a small amount of β-ionone was also detected in the headspace of the control cells, which had been transformed with the empty vector (Figure S2). This β-ionone could be derived directly from β-carotene autoxidation. Additionally, a trace amount of pseudoionone was also detected (*Figure 3B*) in β-carotene accumulated cells expressing the MnCCD1. Pseudoionone is proposed as a breakdown product of lycopene, which is an intermediate of the β-carotene biosynthesis pathway. These results indicated that MnCCD1 could specifically cleave β-carotene at 9, 10 (9′, 10′) bonds to generate β-ionone. The presence of C13 products above was unequivocally confirmed by comparing their retention indices (RI) and mass spectra to authentic pseudoionone and β-ionone.

Interestingly, the norisoprenoid volatiles derived from zeaxanthin accumulating *E. coli* strains revealed a different characterization. The zeaxanthin of the *E. coli* strain expressing MnCCD1 displayed no significant

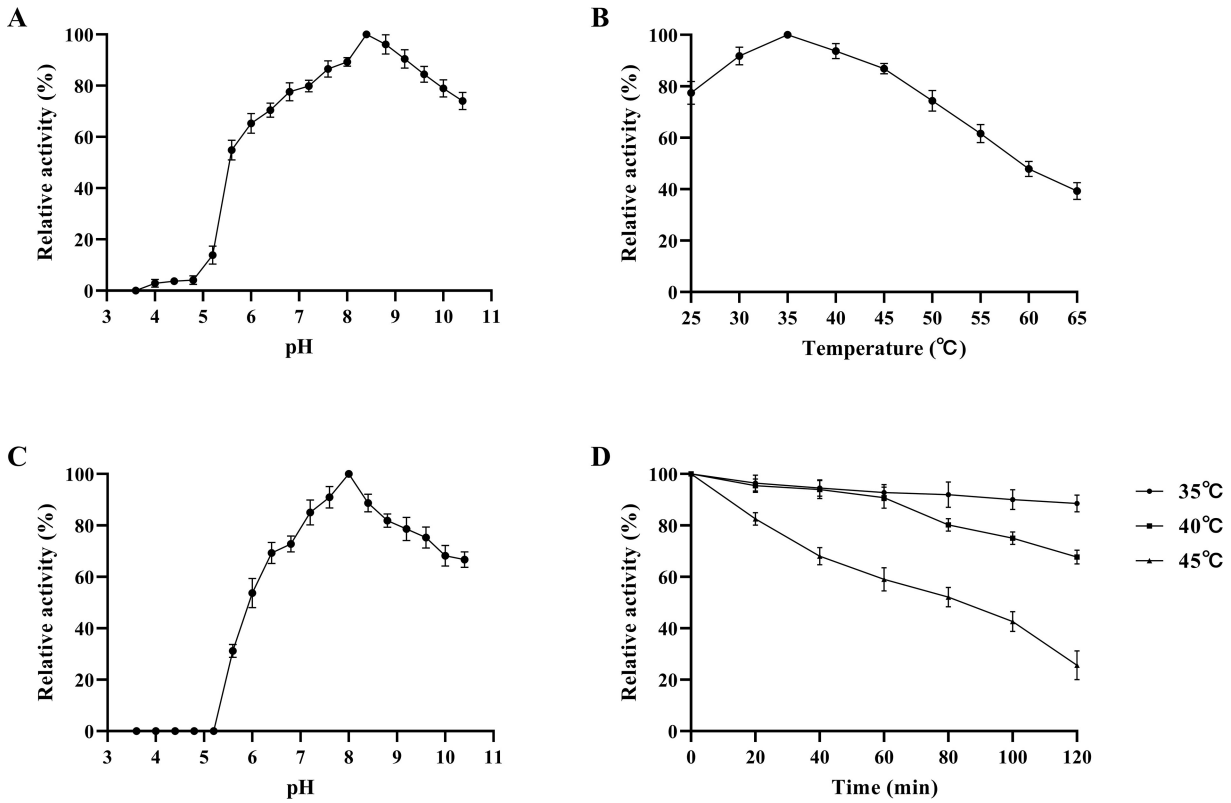


Figure 7. The effects of pH and temperature on the activity and stability of the recombinant MnCCD1. (A) Effect of pH on MnCCD1 activity. (B) Effect of temperature on MnCCD1 activity. (C) The pH stability of the enzyme MnCCD1. (D) The thermostability of the enzyme MnCCD1; the residual activity was monitored, while the enzyme was incubated at 35, 40, and 45 °C. The initial activity was defined as 100 %. These activities were expressed as relative values. All the results were confirmed by three repeated experiments and data are shown as the means ± SD ($n = 3$).

decrease via HPLC analysis compared with the strain harboring an empty vector (*Figure 4A*). Additionally, the GC/MS data showed β-ionone was also the main cleaved product of zeaxanthin-accumulating *E. coli* strains accompanied by a trace amount of pseudoionone (*Figure 4B*). 3-Hydroxy-β-ionone, the expected oxidative cleavage product of zeaxanthin, could not be detected in the headspace of these cultures, which was consistent with the cleaved characteristic of LnCCD1 from *Laurus nobilis* L. (Bay Laurel) Fruits,[36] DcCCD1 from carrot roots,[37] and some plant CCD4 enzymes.[17] On the one hand, β-carotene and lycopene are intermediates in the zeaxanthin biosynthesis pathway;[38] on the other hand, MnCCD1 could catalyze zeaxanthin in the reaction system *in vitro*. We hypothesize that substrate specificity of MnCCD1 for β-carotene is better than that of zeaxanthin in the case of both zeaxanthin and β-carotene exist in the carotenoids biosynthetic pathway. Any potential cleavage products were absent in *E. coli* cells that accumulate lycopene and zeaxanthin transformed with control plasmids devoid of the MnCCD1 gene (data not shown).

2.3. Expression Optimization of MnCCD1

An additional obstacle is the strong tendency of CCDs to form insoluble aggregates upon recombinant expression in *E. coli*.[38] So, we introduced the GST tagged at the N-terminal of MnCDD1. GST-tag has no apparent influence on the activity and properties of MnCCD1 (data not shown). By adding different concentrations of IPTG and incubating under different temperatures for different times, the optimal induction conditions were identified, and the results are shown in *Figure 5*. The optimal concentration and optimal temperature of IPTG were 0.1 mM and 28 °C, respectively. The optimal induction time of IPTG was 24 h. During the 24 h incubation, cultures grown on IPTG reached a maximum MnCCD1 activity of 2.98 U/mL. The expression of MnCCD1 was increased two times by optimizing the growth and induction conditions in

the tube. It is reported that adding ferrous iron in cultures could influence the activity of CCD.[39] However, after the cultivation of the *E. coli* cells in ferrous iron (0–1000 μM), there was no significant difference in MnCCD1 activity.

2.4. Purification and Characterization of MnCCD1

The molecular weight of purified GST-MnCCD1 was approximately 87 kDa (*Figure 6*). The relative enzymatic properties of recombinant MnCCD1 were further characterized by using purified MnCCD1 with β-apo-8′-carotenal as the substrate, which has relatively good solubility in micelle reaction compared to other carotenoids.[25]

The optimal temperature for MnCCD1 was 35 °C, and the activity was more than 60% of the maximum activity in the 25–55 °C temperature range (*Figure 7B*). When the purified MnCCD1 was incubated at 35 °C and 40 °C for 2 h in the absence of the substrate, its residual activity was kept above 60% (*Figure 7D*).

The optimal pH of MnCCD1 was determined to be pH 8.4 (*Figure 7A*). It is not unexpected, as the optimum pH for other plants′ carotenoid cleavage enzymes was also in the same range.[27,40] Moreover, the pH stability of MnCCD1 was excellent, while the activity of MnCCD1 was still more than 60% of its initial activity in the pH range from 6 to 10.4 after incubation at 4 °C for 8 h (*Figure 7C*).

Furthermore, the effects of Fe^{2+} and ethanol on the MnCCD1 enzyme activities were investigated. The ethanol showed activation on AtCCD1 enzyme activities by using a micellar reaction system.[25] The activity of MnCCD1 was measured at different concentrations of ethanol (0–25%) and reached its highest value at 10% ethanol. Nevertheless, the reason for activation in the physicochemically complex ternary water/surfactant/alcohol mixtures is still hard to explain. It may be that ethanol activates enzyme activity or increases the solubility of carotenoids in the micellar system. The K_m and V_{max} values of MnCCD1 with β-apo-8′-carotenal as the substrate were 0.83 mM and 72.5 U/mg, respectively. The Fe^{2+} is located in the active center of CCDs and interacts with four conserved His residues.[14] There was a significant decrease in MnCCD1 enzyme activity with iron concentration (Figure S3–A). The MnCCD1 maintained only 60% of its initial activity, while the iron concentration reached 1 mM. Finally, The effects of other metal ions and some chemicals on the activity of MnCCD1 were determined (*Table 1*). At 1 mM, almost all metal ions tested showed a slight enhancement effect on MnCCD1 except Hg^{+}. The residual enzyme activity was approximately decreased by 20% with Hg^{+}. Besides, the chelating agent EDTA (10 mM) revealed no significant inhibitory effects on MnCCD1 activity.

Table 1. Effects of cations and reagents on purified MnCCD1 activity.

Cation of reagent[a]	Residual activity (%)
Control	100
Fe^{3+}	132.64
Ni^{2+}	124.41
Na^{+}	117.35
Sr^{2+}	119.50
Ca^{2+}	116.81
Cu^{2+}	98.48
Li^{+}	107.54
$Co2^{+}$	113.55
Zn^{2+}	124.69
Mn^{2+}	100.76
Mg^{2+}	110.30
Ba^{2+}	109.61
K^{+}	104.36
Hg^{2+}	84.72
Al^{3+}	102.70
NH_4^{+}	121.30
EDTA (10 mM)	118.67

[a] Final concentration, 1 mM or as indicated. The values shown are the mean of duplicate experiments and the variation about the mean was less than 5%.

3. Conclusions

In summary, a putative CCD1 from *Morus notabilis* was cloned and overexpressed in *E. coli*. The function characterization showed that MnCCD1 could cleave a variety of carotenoids at the 9, 10 (9′, 10′) sites and form C13 flavor products. The expression of MnCCD1 in *E. coli* BL21 was 2.98 U/mL in LB medium. The biochemical characterization showed that MnCCD1 had a high enzyme activity and stability under alkaline conditions. In addition, an appropriate amount of ethanol proved to be most effective in obtaining maximum MnCCD1 activities and adding Fe^{2+} could not influence the activity. While a limited quantitative characterization of CCDs has been available, our study provides a potential CCD for flavor synthesis, including bioconversion and metabolic engineering.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Author Contribution Statement

Zhipeng Qi and Xianyu Fan performed the experiments, analyzed the data, and wrote the article. Chunyi Zhu and Dongsheng Chang contributed to the analysis tools and analyzed the data. Jianjun Pei and Linguo Zhao conceived and designed the experiments.

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