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Molecular cloning and enzymatic characteristics of chalcone isomerase from *Rheum palmatum*

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Abstract: *Rheum palmatum* has been widely developed in traditional Chinese medicine. One of the major active compounds in *R. palmatum* is flavonoids. Until now, few genes involved in flavonoid biosynthesis have been isolated from this medicinal plant. In this study, a new chalcone isomerase gene (RpCHI) was cloned from an RNA-seq library of *R. palmatum*. The 714bp RpCHI cDNA was cloned and sequenced. The RpCHI recombinant protein was successfully overexpressed in a prokaryotic expression system and purified to homogeneity by a nickel-nitrilotriacetic acid (Ni-NTA) prepacked column, and the enzymatic activity was investigated. The results have clarified that RpCHI was assembled into the type I CHI group based on phylogenetic analysis. Activity examination revealed that RpCHI exhibits high substrate affinity for naringenin chalcone, and it can also use isoliquiritigenin in relatively low activity. Optimum pH and temperature for enzymatic reaction was determined to be 7.0 and 40 °C, respectively. RpCHI was successfully cloned in the experiment, and the enzymatic activity revealed RpCHI uses various substrates to produce antioxidant flavonoids.

Key words: *Rheum palmatum*, chalcone isomerase, sequence analysis, molecular cloning, enzymatic activity

1. Introduction

Rheum palmatum has been a widely used medicinal herb in China and other Asian countries for thousands of years, containing high concentrations of anthraquinones, anthrones, and a mixture of flavonoids compounds in its root and rhizome (Matsuda et al. 2001; Chen et al. 2018). In traditional Chinese medicine, the root and rhizome of *R. palmatum* have been widely used for treatment of various diseases such as dysmenorrhea, hypermenorrhea, hematemesis, lower abdominal pain, jaundice, diarrhea, and constipation (Meng et al., 2018; Cheng et al., 2019; Tan et al., 2019).

Flavonoids are important secondary metabolites of plants and play vital roles in plant physiology and ecology, such as pathogen resistance, plant coloration, and nodulation (Tian et al., 2008; Falcone Ferreyra et al., 2012). Pharmacological research has shown that flavonoids from *R. palmatum* have several beneficial effects on human health, including protecting against barrier disruption, inhibiting inflammation, and exhibiting anti-fungal activity (Gillmeister et al., 2019; Zhuang et al., 2019). Thus, genes from plants, especially from medicinal plants, that are involved in flavonoid biosynthesis are attracting extensive attention for their potential biotechnological applications.

In plants, the main pathway of flavonoid biosynthesis that occurs through the phenylpropanoid pathway is well known. Chalcone isomerase (CHI) is a major rate limiting enzyme in the pathway described above. At the beginning of flavonoid biosynthesis, 4-Coumaroyl-CoA and Malonyl-CoA are combined into chalcone that is catalyzed by the chalcone synthase (CHS, EC 2.3.1.74). This is followed by chalcone isomerase (CHI; EC5.5.1.6) converting chalcone to (2S)-flavanone, which is a precursor of many downstream flavonoids like dihydroflavonols, anthocyanidins, and anthocyanins (Wang et al., 2010). However, the molecular mechanisms of flavonoid biosynthesis in *R. palmatum* have not yet been clarified.

Flavonoids are major active compounds in *R. palmatum*. However, few flavonoid biosynthesis genes have

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been cloned from *R. palmatum*. Two isoform chalcone isomerase genes by previous transcriptome sequencing were investigated. In this project, we isolated a new CHI gene from *R. palmatum* for the first time. We further carried out the heterologous expression, purification, and defined characteristics of this CHI from the medicinal plant *R. palmatum*. Our positive results are significant for the molecular mechanism of flavonoids biosynthesis research.

2. Materials and methods

2.1. Plant samples

R. palmatum root tissue samples were collected from the Medical Botanical Garden of Hebei University, Baoding, China. Tissues were washed in distilled water and quick frozen with liquid nitrogen for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from *R. palmatum* root tissue by using the RN38-EASYspin Plus Plant RNA kit with on–column DNase digestion (Aidlab Biotech, Beijing, China) according to the manufacturer's manual. RNA degradation and contamination were detected on 1.2% agarose gels. First–strand cDNA was prepared using a TRUEscript 1st Strand cDNA Synthesis Kit (Aidlab Biotech, Beijing, China).

2.3. RPCHI cloning, expression, and purification

Based on an isolated full length cDNA of an RpCHI sequence from our RNA-seq database, gene specific primers marked CHI-F (5'-ATGGCGTCATCGCTCGCT-3') and CHI-R (5'-TCATTCTTTGATCTCCACTC-3') were designed. The PCR was carried out using a Phanta Max Master Mix (Vazyme, Nanjing, China) under the following conditions: 95 °C for 4 min; 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; followed by a final extension of 72 °C (10 min). Amplified PCR products were electrophoretically checked on 1.2% agarose gel. The obtained amplicon production was then cloned in a Zero Background pTOPO vector (Aidlab Biotech, Beijing, China) and sequenced.

The open reading frame (ORF) of RpCHI was recombined into a pET-28a vector (Novagen, Madison, WI, USA) using primers pET RpCHI F(5'-atgggtcgcggatccgaattcATGGCGTCATCGCTCGCT-3') and pET RpCHI R(5'- gtggtggtggtggtgctcgagTCAT TCTTTGATCTCCACTCCGT-3') at EcoR I and Xho I sites using the CV19-One Step Seamless Cloning Kit (Aidlab Biotech, Beijing, China) to construct a RpCHI expression plasmid. The RpCHI expression plasmid was transformed into an *Escherichia coli* BL21(DE3) strain. Cells were grown in a Luria-Bertani medium containing kanamycin (100 μg/mL) at 37 °C in a shaking incubator (180 rpm) until the optical density at 600 nm was 0.5. β-D-1-thiogalactopyranoside (IPTG) (0.10 mM) was used for inducing recombinant protein expression at 25 °C for 4 h. Cells were harvested by centrifugation at 10000 rpm at 4 °C for 20 min. The precipitation was resuspended using lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, pH 8.0). We used ultrasonication to break the cell wall for 15 min at 45% amplitude at 4 °C, and the supernatant was collected by centrifugation at 14,000 rpm for 1 h at 4 °C. The supernatant was purified by using a HisPur Ni-NTA Kit (Changzhou Smart-lifesciences Biotechnology Co., Ltd., Changzhou, China). Ni-Sepharose 6 bound protein was washed with wash buffer (10 mM Tris-HCl, 300 mM NaCl, pH 8.0, 10 mM imidazole, pH 8.0) and eluted with elution buffer (10 mM Tris-HCl, 500 mM NaCl, pH 8.0, 300 mM imidazole, pH 8.0). The purity of purified protein RpCHI was analyzed on 12% SDS–PAGE.

2.4. Bioinformatics analysis

The DNASTAR software was used to predict the protein molecular weight. We used the online server SignalP 5.0 to analyze signal peptide of this protein, and the TMHMM Server v 2.0 was used to calculate the transmembrane domains of this protein.

A phylogenetic tree was constructed to analyze the evolutionary position with other known CHIs of plants. CHI sequences were downloaded from the NCBI GenBank database and were aligned using the Clustal Omega online tool (Madeira et al. 2019). MEGA 7.0 software (Kumar et al. 2016) was used to construct the phylogenetic tree with the Neighbor–Joining method (NJ). The reliability of the tree was evaluated by a bootstrap analysis with 1000 replicates.

2.5. Enzymatic activity testing

Enzymatic activity was measured using spectrophotometry based on the isomerization of naringenin isoliquiritigenin at 390 nm as described by Park et al. (2018). The reaction solution of the testing included 100 mM of phosphate buffer at pH 7.6, 1 mg bovine serum albumin, 50 μM substrate in DMSO, and a certain amount of enzyme and the reaction was carried out at 25 °C. We subtracted the spontaneous reaction rate from the total reaction rate for accuracy. The kinetic constants k_{cat} and K_m for the substrate (1–64 μM) were measured by adapting the data to the Michaelis–Menten equation by nonlinear regression using Origin 9.0 (Origin, San Diego, CA, USA). All experiments were carried out in triplicate.

2.6. Temperature optimum and stability of RpCHI

Optimum temperature on the activity of RpCHI1 was determined under the standard method described above at different temperatures (0–70 °C). To determine the temperature stability of RpCHI, RpCHI was preincubated at different temperatures (0–60 °C) for 30 min, and enzymatic activity was assayed using the standard assay method as mentioned above.

2.7. pH activity characteristics

To define the optimal pH of RpCHI, activity reactions were performed using different pH values from 3.0–10.0 (acetate: pH 3–6; phosphate: pH 6–7; Tris-HCl: 8–9; glycine NaOH: 10–12) as buffers.

3. Results

3.1. Molecular isolation and characterization of the RpCHI gene

The open reading frame (ORF) of RpCHI (Genebank ID: MN251616) was 714 bp, encoding a 237–amino acid protein (Figure 1). The calculated molecular mass of RpCHI was 25.5 kDa and the predicted isoelectric point (pI) was 6.01. RpCHI shows identity with *Fagopyrum dibotrys* (81.2%), *F. esculentum* (83.19%), *Chenopodium quinoa* (67.09%), *Pyrus pyrifolia* (66.37%), and *Lonicera japonica* (68.53%) (Figure 2). The Conserved Domains Database analysis of RpCHI has shown the characterization of a typical Pfam domain like 02431 (Chalcone, 12-214). These domains are defined to encode chalcone–flavanone isomerase protein family, which characterized the super family cl03589. The Pfam 02431 (12-214) domain is in charge of the isomerization of chalcone to naringenin, a vital procedure in the biosynthesis of flavonoids. The hydrophilic nature of RpCHI enzyme and lack of transmembrane domain confirm their action site directly in the cytoplasm.

To analysis the evolutionary relationship among RpCHI and other CHI proteins from plants, a phylogenetic tree was established based on the amino acid sequence alignment (Figure 3). All the CHIs were divided into 2 groups. Phylogenetic analysis demonstrated that RpCHI had a closer relationship with CHI from *Fagopyrum dibotrys* and *F. esculentum*.

3.2. RpCHI1 protein purification

The ORF of RpCHI1 was recombined and expressed in pET-28a vector (Novagen, Madison, WI, USA), a prokaryotic expression vector with a T7 promoter and His tag sequence. After purification from the supernatant, the obtained soluble recombinant fusion proteins were detected on 12% SDS-PAGE gel. The SDS–PAGE detection showed that the expressed single protein with an apparent molecular weight of 25 kDa, which falls in the range of respective protein family (Figure 4, Figure 5).

3.3. Enzyme assay

The enzymatic activity experiment was implemented to detect the substrate affinity of RpCHI1 based on various concentrations of 2 natural substrates, namely naringenin chalcone and isoliquiritigenin. The kinetic parameters of RpCHI are listed in Table 1. As expected, the RpCHI possessed a high affinity for naringenin chalcone. To our interesting, the enzyme can also lead to the isomerization of a type II CHI substrate, namely isoliquiritigenin. The K_m and k_{cat} values at 25 °C for isoliquiritigenin were 15.323 ± 0.36 μM and 2006 ± 13.82 min^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) value was $2.18 \times 10^6\ M^{-1}\ s^{-1}$.

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1          ATGGCGTCATCGCTCGCTGTAACCTCCGTCTCCGTAGAGGACTTCGTCTTCCCTCCCTCC
1           M  A  S  S  L  A  V  T  S  V  S  V  E  D  F  V  F  P  P  S
61         GTCCGCCCGCCCGCCACCGACAACTCTTTCTTCCTAGGCGGTGCAGGGGTTAGGGGGCTG
21          V  R  P  P  A  T  D  N  S  F  F  L  G  G  A  G  V  R  G  L
121        ATGATTGAAGGAAAGTTTATAACTTTCACGGCGATCGGAGTTTACTTGGAGGAAGGCGCG
41          M  I  E  G  K  F  I  T  F  T  A  I  G  V  Y  L  E  E  G  A
181        GTGGCGTCGCTCGCTGACAAGTGGAAGGGAAAATCCGCCACCGAGCTGGCTGAATCTGTT
61          V  A  S  L  A  D  K  W  K  G  K  S  A  T  E  L  A  E  S  V
241        GAGTTCTTCAGAGATATTGTTACCGGTCCATTTGAAAAATTCATTCAGATTACCATGCTC
81          E  F  F  R  D  I  V  T  G  P  F  E  K  F  I  Q  I  T  M  L
301        AAGCCCTTGACAGGGGCACAGTACTCTGAAAAGGTAGCCGAGAATTGTGTTGCTATCTGG
101         K  P  L  T  G  A  Q  Y  S  E  K  V  A  E  N  C  V  A  I  W
361        AAAGCAATCGGAATCTACACAGAGGCTGAAGAGAAGGCTATTGAGAAGTTCATGGAGATC
121         K  A  I  G  I  Y  T  E  A  E  E  K  A  I  E  K  F  M  E  I
421        TTCAAAGACGAGAAATTCCCCCCTGGATGCTCTATCATGTTCAAGCAATGTCCCCCGAAA
141         F  K  D  E  K  F  P  P  G  C  S  I  M  F  K  Q  C  P  P  K
481        TCACTAAGGATAGCATTTGGGAAGCATGATGCGATACCAGAAGCTGATGTGGCGGTGATA
161         S  L  R  I  A  F  G  K  H  D  A  I  P  E  A  D  V  A  V  I
541        GAGAACGGGCCCTTGTCGCAGTCTGTTCTGGAATCAATAATCGGTAAGAGTGGCGTTTCT
181         E  N  G  P  L  S  Q  S  V  L  E  S  I  I  G  K  S  G  V  S
601        CCAGCAGCCAAGGAGAGCTTGGCTGCCAGACTCCATCAACTGCTGAATGCCACAAAAGCT
201         P  A  A  K  E  S  L  A  A  R  L  H  Q  L  L  N  A  T  K  A
661        TCAAATGGAGAAGCTGAAACCAAAGAGAACAACGGAGTGGAGATCAAAGAATGA
221         S  N  G  E  A  E  T  K  E  N  N  G  V  E  I  K  E  *
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Figure 1. The nucleotide sequence and its deduced amino sequence of the RpCHI gene.

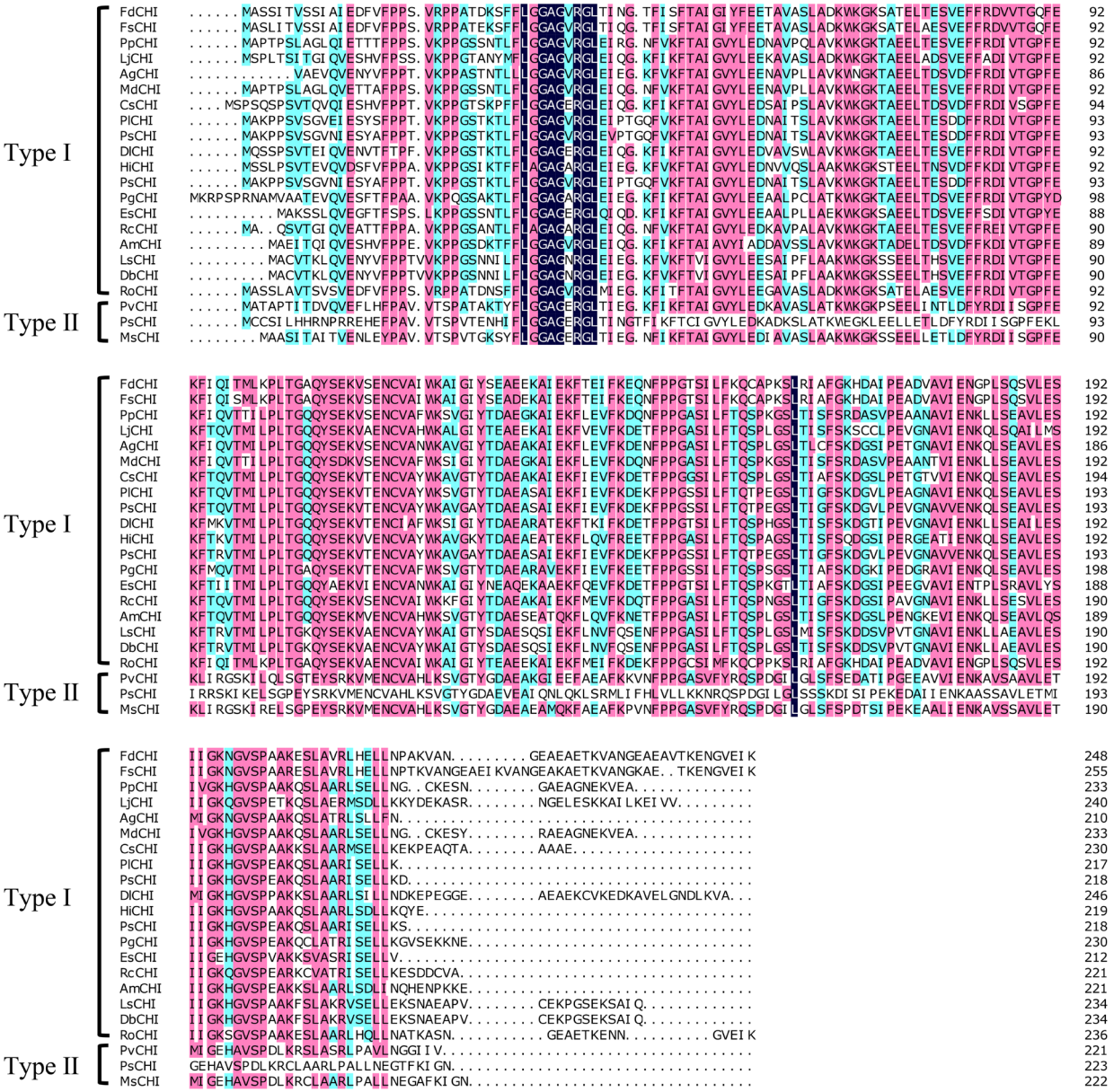

Figure 2. Multiple alignment of deduced protein sequence of RpCHI with CHIs from other species. The same amino acids are shaded in capital letters against a dark blue. Less identical amino acids are shaded in capital letters against a pink or light blue. Different amino acids are shaded in black capital letters against a white background. *Fagopyrum dibotrys* (AHH84790.1), *Fagopyrum esculentum* (ADT63063.1), *Pyrus pyrifolia* (ADP09377.1), *Lonicera japonica* (AGE10599.1), *Apium graveolens* (AGM46640.1), *Malus domestica* (XP_028956651.1), *Camellia sinensis* (ASU87415.1), *Paeonia lactiflora* (AEK32592.1), *Paeonia suffruticosa* (ADK55061.1), *Dimocarpus longan* (AEO36980.1), *Handroanthus impetiginosus* (PIN01245.1), *Punica granatum* (AHZ97871.1), *Epimedium sagittatum* (AHG95985.1), *Rosa hybrid* cultivar (AYU56554.1), *Antirrhinum majus* (BAO32070.1), *Iochroma squamosum* (AMQ48689.1), *Dunalia brachyacantha* (AMQ48685.1), *Phaseolus vulgaris* (XP_007142690.1), *Pisum sativum* (UniProtK B code P41089), *Medicago sativa* (UniProtK B code P28012).

3.4. Effects of temperature and pH on RpCHI activity

In this study, we determined the effects of temperature on RpCHI activity using isoliquiritigenin as the substrate at varying temperatures from 0–70 °C. The optimum temperature for the RpCHI activity was 45 °C (Figure 6A). When the temperature is above 50 °C, enzymatic activity rapidly decreases. When the temperature reaches 70 °C, enzymatic activity is almost lost. We also examined the effects of temperature and pH on the stability of RpCHI. The enzyme had higher than 50% activity after

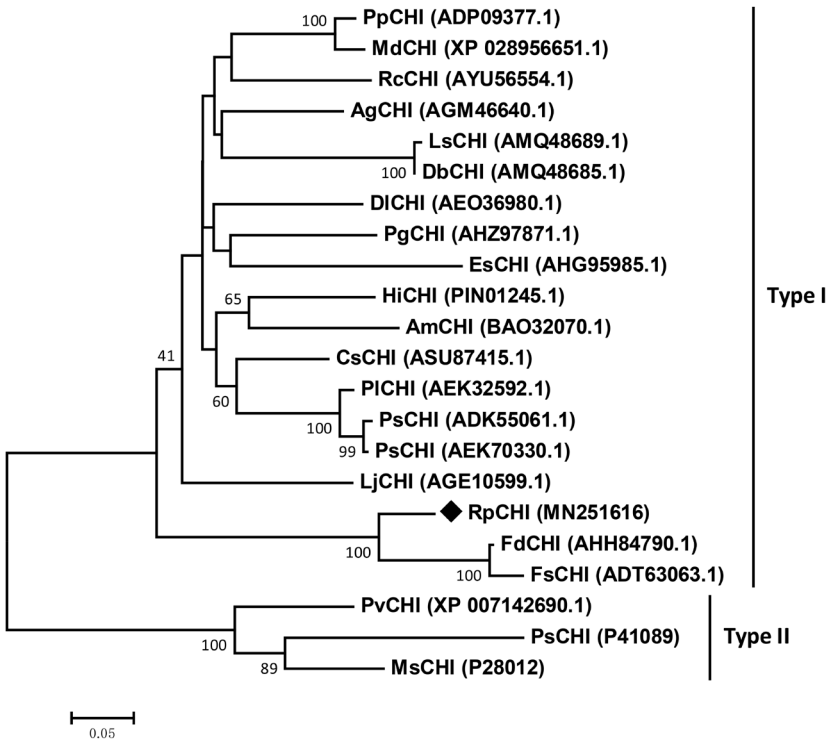

Figure 3. Phylogenetic tree of RpCHI protein together with CHIs from other species. *Fagopyrum dibotrys* (AHH84790.1), *Fagopyrum esculentum* (ADT63063.1), *Pyrus pyrifolia* (ADP09377.1), *Lonicera japonica* (AGE10599.1), *Apium graveolens* (AGM46640.1), *Malus domestica* (XP_028956651.1), *Camellia sinensis* (ASU87415.1), *Paeonia lactiflora* (AEK32592.1), *Paeonia suffruticosa* (ADK55061.1), *Dimocarpus longan* (AEO36980.1), *Handroanthus impetiginosus* (PIN01245.1), *Punica granatum* (AHZ97871.1), *Epimedium sagittatum* (AHG95985.1), *Rosa hybrid* cultivar (AYU56554.1), *Antirrhinum majus* (BAO32070.1), *Iochroma squamosum* (AMQ48689.1), *Dunalia brachyacantha* (AMQ48685.1), *Phaseolus vulgaris* (XP_007142690.1), *Pisum sativum* (UniProtK B code P41089), *Medicago sativa* (UniProtK B code P28012).

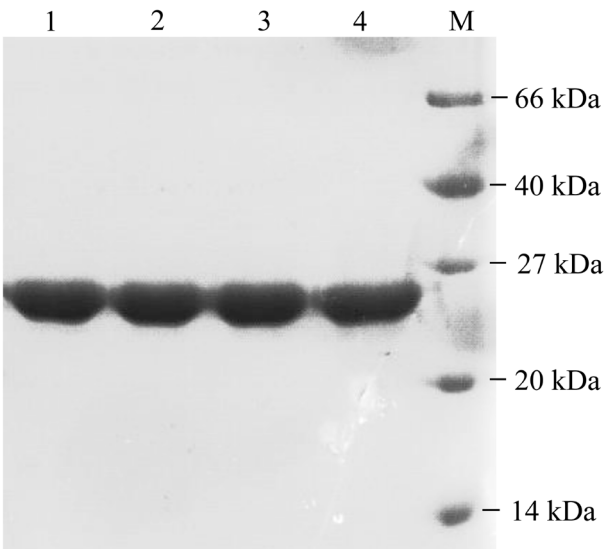

Figure 4. SDS-PAGE analysis of fractions obtained at each purification step of overexpressed recombinant RpCHI protein. Lane M: protein molecular weight marker, Lane 1–4 Ni-NTA purified RpCHI protein.

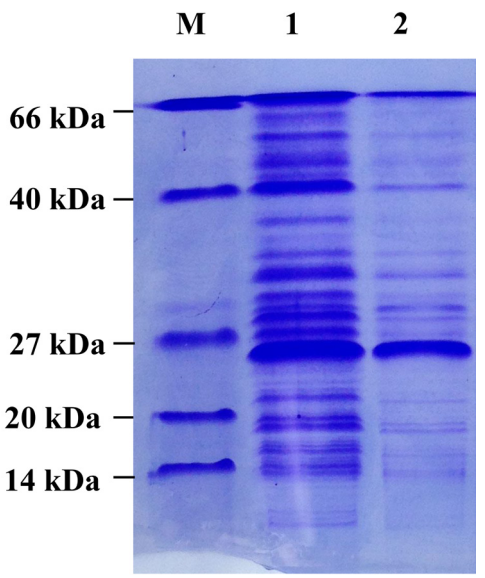

Figure 5. SDS-PAGE analysis of the RpCHI protein expression information. Lane M: protein molecular weight marker, Lane 1: whole cell lysis, Line 2: supernatant.

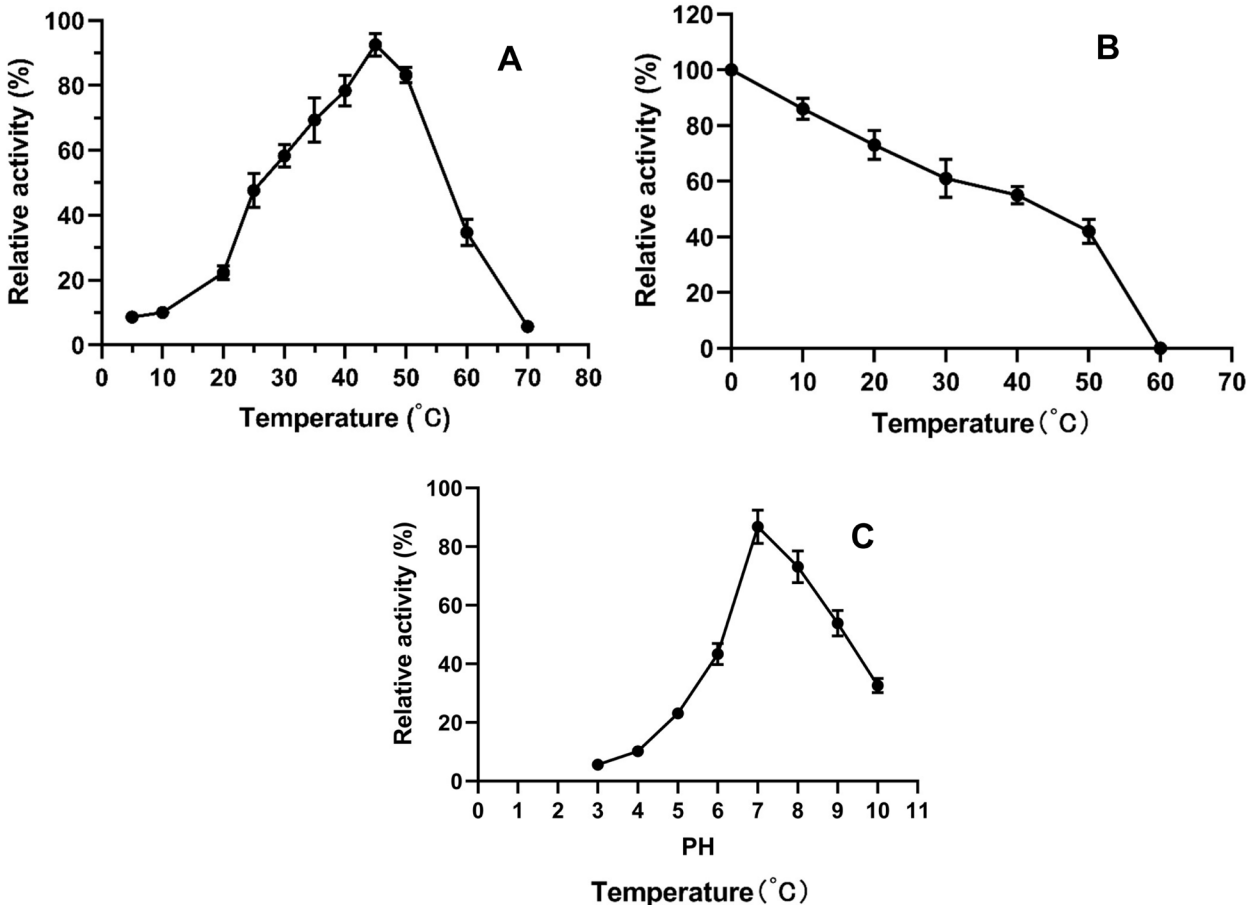

Figure 6. Activity and stability of RpCHI. (A) Effects of temperature on RpCHI activity. Enzymatic activity was evaluated in the temperature range of 0–70 °C in 100 mM potassium phosphate buffer (pH 7.6). Activity is expressed as a percentage of the maximum activity (100%). (B) Effects of temperature on the stability of RpCHI. The enzyme was preincubated for 30 min at temperatures ranging from 0–60 °C. Residual activity was measured at 25 °C. (C) pH dependence of DaCHI1 activity. The reaction was carried out at 25 °C in buffers with pH ranging from 3.0 to 10.0. Values are the means ± standard deviation of 3 replicates and the standard bars stand for standard deviation.

Table 1. Steady state kinetic parameters for RpCHI.

Substrates	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1} s^{-1}$)
Naringenin chalcone	5719 ± 342	5.74 ± 1.49	1.67×10^7
Isoliquiritigenin	2006 ± 139	15.323 ± 2.36	2.18×1^06

All K_m and k_{cat} values are expressed as the mean ± standard deviation of 3 parallel experiments.

preincubation at 40 °C for 30 min, whereas the activity of RpCHI was completely lost after preincubation at 60 °C for 30 min (Figure 6B). Figure 6C shows the pH stability profile; the enzymatic activity was highest at pH 7.

4. Discussion

Many plant species have been discovered to possess the chalcone–flavanone isomerase protein. To our best knowledge, several CHI genes have been isolated from many kinds of plants like soybean, corn, alfalfa, peanut, carnation, and *Ginkgo biloba* (Bednar and Hadcock, 1988; Grotewold and Peterson, 1994; McKhann and Hirsch, 1994; Qin et al., 2011; Shoeva et al., 2014; Liu et al., 2015). In our project, we first cloned the chalcone isomerase gene (RpCHI) from *R. palmatum*. Previous research has clarified that overexpression of the CHI gene can lead

to an increase of flavonoids in various kinds of plants including *Glycyrrhiza uralensis*, onion, and tobacco (Kim et al., 2004; Nishihara et al., 2005; Zhang et al., 2009). Vu et al. (2018) analyzed GmCHI gene expression (CHI gene, type II of soybean) in *Talinum paniculatum* and found that overexpression of the GmCHI gene enabled *T. paniculatum* plants to improve their total flavonoid content. Therefore, we assume that overexpression of *R. palmatum* CHI can result in increased flavonoid production.

Phylogenetic analysis has shown that RpCHI had a closer relationship with CHI than *Fagopyrum dibotrys* and *F. esculentum*, which all clustered into the type I CHIs group. Type I CHIs are common in most plants that isomerize naringenin chalcone into 5'-hydroxy–flavonoid (Shimada et al., 2003). In this study, we also expressed and purified the recombinant protein RpCHI, then carried out the enzymatic activity assay further. As expected, our enzyme assay results clarified that RpCHI has shown a strong preference for naringenin chalcone, the natural substrate of CHI type I, and it also is able to catalyze the CHI type II substrate (isoliquiritigenin) with weak enzymatic activity. The K_m value for isoliquiritigenin was 15.323 ± 0.36 μM, which is higher than type II CHIs from *M. sativa* and *Glycine max* for isoliquiritigenin (8.4 μM and 10 μM, respectively). The catalytic efficiency (k_{cat}/K_m) value was $2.18 \times 10^6\ M^{-1}\ s^{-1}$. The catalytic efficiency (k_{cat}/K_m) value of RpCHI is considerably lower than that of *M. sativa* (8.1 $\times 10^6\ M^{-1}\ s^{-1}$) but higher than that of *G. max* ($183\ M^{-1}\ s^{-1}$) (Bednar and Hadcock, 1988; Jez and Noel, 2002).

We also investigated the effects of temperature and pH on RpCHI activity. The optimum temperature for RpCHI activity was 45 °C, which is higher than that of *G. biloba* (30 °C) and consistent with that of bacterium *E. ramulus* (45 °C) (Cheng et al., 2011; Gall et al., 2014). Moreover, the enzyme activity was highest at pH 7.0, consistent with the optimum pH of CHIs previously reported (pH 6.6 ± 8.2) (Mol et al., 1985; Cheng et al., 2011).

In this project, we isolated and analyzed the chalcone isomerase gene (RpCHI) from *R. palmatum*. The phylogenetic analysis has clarified that RpCHI belongs to type I CHIs. Furthermore, enzymatic activity analysis revealed that RpCHI utilizes various substrates including type I substrates (naringenin chalcone) and type II substrate (isoliquiritigenin). Thus, these research achievements not only add to the current cognition of the enzymatic characteristics of RpCHI, but also hold significance in developing potential ways for establishing a transformed *R. palmatum* plant with an overexpressed RpCHI gene for accumulated production of medicinally important flavonoids.

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