Biochemical characterization of an alcohol dehydrogenase from *Pyrus ussuriensis* 'Nanguoli'

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Summary

Alcohol dehydrogenase (ADH) is an oxidation-reduction enzyme that catalyzes interconversion of alcohols and aldehydes and provides a substrate for the formation of esters. Although candidate genes encoding ADHs have been identified in pear fruits, the characterization of their encoded proteins remains unknown. In this study, an ADH was cloned from a cDNA of *Pyrus ussuriensis* 'Nanguoli' (PuADH2) and was expressed in *Escherichia coli*. The recombinant PuADH2 protein was purified and subject to biochemical characterization. PuADH2 was more likely an oxidative enzyme independent of substrate specificity. During oxidation reaction, no preference for coenzyme factors (NAD⁺ and NADP⁺) for PuADH2 oxidase activity. In addition, the oxidation activity of PuADH2 slightly increased with high K⁺ concentrations, but significantly decreased with Zn²⁺. These results provide insights into PuADH2 as an oxidase and promote future study on the synthesis and regulation of aromatic compounds in pear fruits.

Keywords

recombinant protein, oxidase, fruit aroma, enzyme activity

Significance of this study

What is already known on this subject?

- Alcohol dehydrogenases are key enzymes that catalyze the interconversion between aldehydes and the corresponding alcohols and participate in the process of ester-based aroma formation.

What are the new findings?

- Biochemical analysis suggested that PuADH2 be an oxidase. Its activity was enhanced by high K⁺ concentrations, but significantly decreased with Zn²⁺.

What is the expected impact on horticulture?

- Biochemical characterization of an alcohol dehydrogenase will help to understand its role in ester-volatile synthesis in pear fruits and promote future study on the synthesis and regulation of aromatic compounds in pear fruits.

Introduction

Fruit aroma is determined by a complex mixture of volatile compounds. Pear fruits have hundreds of aromatic compounds and volatile esters are the key compounds contributing to the development of their characteristic aroma (Qin et al., 2012). A previous report showed that ester production in pear fruits was limited to the concentration of precursors (Qin et al., 2017). Alcohol dehydrogenases (ADHs; EC 1.1.1.1) are key enzymes that catalyze the interconversion between aldehydes and the corresponding alcohols and participate in the process of ester-based aroma formation.

To date, all plants were found to carry multiple ADH genes, except for a few *Arabidopsis* species. Several ADH genes have been characterized in plants, including melons (Manriquez et al., 2006), apricots (González-Agüero et al., 2009), peaches (Zhang et al., 2010), and tomatoes (Longhurst et al., 1994). In melons, expression of *Candida maltose* (Cm) ADH1 and CmADH2 showed increasing patterns during ripening (Manriquez et al., 2006). In contrast, PpADH1, PpADH2, and PpADH3 in peaches (*Prunus persica*) were down-regulated during ripening (Zhang et al., 2010).

In pears (*Pyrus bretschneideri*), the total activity of eight *PbrADHs* and volatile ester production were strongly associated with increased *PbrADH6* transcription (Qin et al., 2017). However, the expression pattern of *PbrADH2* was opposite to that of *PbrADH6*, whose biochemical characteristics require further study.

Pyrus ussuriensis 'Nanguoli' has pleasant flavor produced by volatile esters, attractive color and exquisite flesh, therefore the cultivar was spreading in Northern China. Compared to *Pyrus ussuriensis* 'Nanguoli', *P. bretschneideri* 'Dangshansuli' produces many straight-chain alcohols and aldehydes, generating fruits a "green" flavor note. In this study, we cloned an ADH gene, PuADH2, from a cDNA of ripened 'Nanguoli' fruits and expressed PuADH2 in *Escherichia coli* BL21. The recombinant PuADH2 protein was characterized biochemically, including oxidant/reduction activity and effects of cofactors and metal ions, in order to understand its role in ester-volatile synthesis in pear fruits.

Materials and methods

Materials

Ripen pear (*Pyrus ussuriensis* 'Nanguoli') fruits were obtained from the orchard at Anhui Academy of Agricultural Sciences. All enzymes used in recombinant plasmid construction were bought from TaKaRa (Dalian, China). Polymerase chain reaction (PCR) product purification and plasmid mini-prep kits were purchased from Beijing TransGen Biotech Co.,

Ltd. (Beijing, China). The pET28a was bought from Novagen (Darmstadt, Germany).

Sequence analysis of *PuADH2*

Multiple sequence alignment of *PuADH2* and its homologs were performed with Clustal X (Thompson et al., 1997). A phylogenetic tree was constructed by using the Neighbor-joining (NJ) method (Saitou and Nei, 1987) of MEGA 5 software (www.megasoftware.net/index.html). Three-dimensional (3D) structure of *PuADH2* was predicted following Biasini (2014).

Amplification and cloning of *PuADH2*

Total RNA from the fruit of *P. ussuriensis* 'Nanguoli' was extracted according to Asif et al. (2000), which was then used to synthesize the cDNAs using the PrimeScript™ RT reagent kit (TaKaRa). A full-length cDNA fragment was amplified with the sequences *PuADH2*-specific-forward: 5'-CGGGATCCATGTCTAATACTGCTGGTCAGGT-3' and *PuADH2*-specific-reverse: 5'-CCGCTCGAGTTCCTCCATGTGGATTATGCAACG-3'. The thermal cycle program was: 3 min, 95°C; 30 cycles at (95°C, 30 s; 60°C, 45 s; 72°C, 60 s); and 72°C, 10 min. The *PuADH2* cDNA was gel purified and sub-cloned into the pMD19-T vector and verified by sequencing (Sangon Biotech).

Expression and purification of *PuADH2*

The coding sequence (excluding the stop codon) of *PuADH2* was cloned into the pET28a vector with His6-tag, the recombinant *PuADH2* was transformed into *E. coli* BL21 (DE3) cells. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 mM) at 37°C for 4 h. The cell cultures were harvested by centrifugation and re-suspended in 50 mL buffer (300 mM NaCl, 5% glycerol, 1 mM PMSF, 20 mM Tris-HCl, pH 8.0, and 5 mM imidazole) and sonicated for 30 min (300 W, with a 6-s pulse and an 8-s pause). The lysate was centrifuged and the pellet was washed using Tris-HCl (20 mM, pH 8.0) twice and re-suspended in 5 mL of solubilizing buffer (20 mM Tris buffer, pH 8.0, 7 M urea) to achieve complete solubilization. The supernatant was obtained by centrifuging and then was transferred to 50 mL refolding buffer (20 mM Tris buffer, pH 8.0, 10% (w/v) sucrose, 2 mM CaCl₂).

Following the method of Kumar et al. (2015) with modifications, the refolded protein sample was purified by chromatograph passing through a Ni-NTA affinity column containing 5 mL of nickel beads (Novagen). After the loading, the column was washed with 50 mL wash buffer in step-wise increments as follows: buffer 1 (150 mM NaCl and 20 mM imidazole); buffer 2 (150 mM NaCl and 40 mM imidazole); buffer 3 (150 mM NaCl and 60 mM imidazole); and buffer 4 (150 mM NaCl and 80 mM imidazole). His-tagged *PuADH2* was eluted then with 10 mL of wash buffer containing (150 mM NaCl, 400 mM imidazole). The elution was concentrated to 2 mL in a 10K Ultracel centrifugation filter (Merck Millipore, Billerica, MA, U.S.A.) and diluted with a low-salt buffer containing 20 mM Tris-HCl (pH 8.0).

Protein purity was verified by 10% SDS-PAGE, and the proteins were transferred to a nitrocellulose (NC) membrane. The NC membrane was incubated for 1 h with the rabbit anti-His monoclonal antibody. After washing with 1×PBST, the NC membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody for 1 h. The NC membrane was then washed five times with 1×PBST as described before exposure.

Measurement of enzyme activity

The reduction and oxidation activities of *PuADH2* were quantified by spectrophotometry following the method of Molina et al. (1987). Activity of *PuADH2* reductase was measured with 2.4 mM NADH, 33 mM acetaldehyde, 50 mM PBS (pH 7.0), and 12~15 μg *PuADH2*. Activity of *PuADH2* dehydrogenase was assayed in the presence of 2.4 mM NADH, 33 mM ethanol, 50 mM PBS (pH 7.0), and between 12~15 μg *PuADH2*. *PuADH2* was replaced with 50 mM PBS (pH 7.0) for the control. The OD was detected by spectrophotometry at 340 nm every 15 s until no change in the OD value was observed. All measurements were performed in triplicate. The results were expressed as specific activity (U mg⁻¹ protein).

Cofactor specificity

To evaluate cofactor preference, NAD⁺ and NADP⁺ were used to investigate the oxidation activity of *PuADH2* in a reaction mixture containing 2.4 mM NAD⁺ or NADP⁺, 33 mM ethanol, 50 mM PBS (pH 7.0), 12~15 μg *PuADH2*. Each assay was performed in triplicate.

Substrate specificity

Substrate specificity was studied with phenylacetaldehyde, ethanol, or hexanal as the substrate in reduction reactions. The oxidation activity of *PuADH2* was further analyzed using hexanol as a substrate.

Effect of metal ions on enzyme activity

PuADH2 is soluble in PBS, whereas it is insoluble in PBS with high concentrations of Ca²⁺, Mg²⁺, or Zn²⁺. To address this issue, 3 mg *PuADH2* was dialyzed in MES buffer (50 mM, pH 6.0) overnight for buffer exchange at 4°C. Effect of metal ions on *PuADH2* activity was conducted with reaction solutions with 33 mM ethanol, 2.4 mM NAD⁺ and different concentrations of Ca²⁺, Mg²⁺, K⁺ or Zn²⁺. The final concentrations of Ca²⁺ and K⁺ were 0 μM, 10 μM, 25 μM, and 50 μM, whereas the final concentrations of Mg²⁺ and Zn²⁺ were 0 μM, 1 μM, 2 μM, and 5 μM (Elleuche et al., 2014), respectively. The concentration of *PuADH2* was 7~8 μg.

Statistical analysis

Statistical analysis was done through SPSS software (IBM Corp., Armonk, NY, U.S.A.). The *t*-test (*p*<0.05) was used for two groups and Kruskal-Wallis one-way ANOVA (*p*<0.05) was used for more than two groups to test the significant differences.

Results

PuADH2 sequence analysis and phylogeny

PuADH2 (GenBank ID: KU507377) has an open reading frame of 1,140 bp and encodes a 380-amino acid protein with a predicted molecular weight of 46 kDa. An Interproscan (Philip Jones et al., 2014) of the sequence revealed that these sequences contained motifs characteristic of ADH. The alignment of the ADHs shared two major conserved domains, including a GroES-like domain (between residues 35–164) and a zinc-binding domain (between residues 206–340) (Figure 1). Gene Ontology prediction indicated that *PuADH2* may involve in oxidation-reduction processes (GO: 0055114) with zinc-binding (GO: 0008270) and oxidoreductase (GO: 0016491) activities. Additionally, there were high levels of sequence identity (84.7–96.8%) between *PuADH2* and zinc-medium-chain ADHs subfamily members of other plants (Figure 1). The phylogenetic tree revealed that the

FIGURE 1. Amino acid sequences alignment of *Pyrus ussuriensis* PuADH2 with closely related full length sequences of *Malus domestica* MdADH (CAA88271.1), *Prunus avium* PsADH (XP_021833624.1), *Fragaria x ananassa* FrADH (CAA33613.1), *Manihot esculenta*, MeADH (XP_021622038.1), *Dimocarpus longan* DladH (ABF61806.1), *Vitis vinifera* VvADH2 (NP_001268083.1), and *V. vinifera*, VvADH6 (AAF44335.1). Black shading indicates similar residues. Functional domains were predicted using Interproscan. A GroES-like domain and a structural zinc-binding domain were identified.

15 ADH family members were divided into two groups, with the closest neighbor of PuADH2 being MdADH from *Malus domestica* (Figure 2). As was predicted, the 3D structure of PuADH2 existed as a homodimer containing Zn^{2+} binding sites (Figure 3).

PuADH2 gene expression

Upon induction of PuADH gene expression, pE-T28a-transformed BL21 cells produced a 46-kDa PuADH2 protein (Figure 4A). The Western blot analysis revealed two specific bands of approximately 46 kDa and 92 kDa molecular masses (Figure 4B), representing the monomer and homodimer forms of the protein. The PuADH2 protein was mainly expressed in the form of inclusion bodies and the bioactive PuADH2 protein was obtained through solubilization and refolding procedures as described in methods.

PuADH2 oxidation/reduction activity

Purified PuADH2 was tested for reductase or oxidase activity. Although a higher reduction activity was shown by PuADH2 with acetaldehyde as a substrate comparing to its oxidation activity with ethanol serving as a substrate; however, its overall oxidation activity was higher than its reduction

FIGURE 3. 3D structure of PuADH2 predicted according to its translated protein sequence. Zn^{2+} ions are shown in pink.

activity (Figure 5A). This was confirmed by using hexanol as a substrate, suggesting that PuADH2 acts preferentially as an oxidase (Figure 5B).

FIGURE 2. Phylogenetic trees for PuADH2 with other ADH sequences. Numbers above the branches indicate bootstrap values (1,000 replicates). Sequences are from *Malus domestica* MdADH (CAA88271.1); *Fragaria × ananassa* FrADH (CAA33613.1), *Manihot esculenta* MeADH (XP_021622038.1), *Dimocarpus longan* DIADH (ABF61806.1), *Vitis vinifera* VvADH2 (NP_001268083.1), *V. vinifera* VvADH6 (AAF44335.1), *Solanum lycopersicum* SIADH (AAB33481.2), *Prunus armeniaca* PrADH (ABZ79222.1), *Prunus avium* PsADH (XP_021833624.1), *Arabidopsis thaliana* AtADH (AAS45601.2), *Prunus dulcis × Prunus persica* PdADH (AEF30408.2), *Musa acuminata colla* MaADH (AIU98498.1), *Diospyros kaki* DkADH2 (AGA15794.1), *Persea americana* PaADH3 (ALR87070.1).

FIGURE 4. SDS-PAGE and western blot of purified PuADH2. (A) M, marker; lane 1, supernatant before induction; lane 2, precipitant before induction; lane 3, supernatant after induction; and lane 4, precipitant after induction. (B) The proteins separated on non-reducing SDS-PAGE gels were transferred to NC membranes for western blot analysis. Band sizes were ~92 kDa and ~46 kDa, respectively. Lanes 2 and 3 were duplicates.

When phenylacetaldehyde, ethanal, hexanal, and hexanol were used as the substrates, *PuADH2* had the higher oxidase activity when hexanol was used as a substrate, indicating its preferential oxidase activity was not substrate-specific (Figure 5B).

The effect of cofactors on *PuADH2* activity

Upon addition of NADP⁺, reaction plateaued after 3 min, which was sooner than NAD⁺ addition. The final enzyme activity of *PuADH2* in the presence of NADP⁺ ($3,396 \pm 68.5$ U mg⁻¹) was lower than that with NAD⁺ ($4,237 \pm 45.7$ U mg⁻¹) (Figure 5C), but there is no significant difference between the two groups, suggesting no preference for coenzyme factors for *PuADH2* oxidase activity.

The effect of some metal ions on *PuADH2* oxidase activity

Minimal effects on *PuADH2* oxidase activity were observed when 10–50 μ M Ca²⁺ or 10–25 μ M K⁺ was present in the reaction. However, addition of 50 μ M K⁺ significantly enhanced the enzyme activity ($p < 0.05$) (Figure 6A). Additionally, the *PuADH2* oxidase activity was slightly elevated by Mg²⁺, but significantly suppressed by Zn²⁺ ($p < 0.05$) at all tested concentrations (Figure 6B).

Discussion

ADH genes can catalyze the reciprocal transformation between alcohols and aldehydes and are participates in ester-aroma formation, in additional ADH participate in plant development and stress responses (Singh et al., 2018). Eight *ADH* genes were identified in Chinese white pear, and the association between *ADH* activity and aromatic compounds was determined (Qin et al., 2017). Here, we cloned the *PuADH2* gene from *P. ussuriensis* determined 'Nanguoli', which reportedly has a "fruity" aroma (Wu et al., 2013). We also expressed the gene in *E. coli* and isolated and biochemically characterized the recombinant protein *PuADH2*. The *PuADH2* protein is a member of the zinc-medium-chain ADH subfamily (Chase, 1999), which has multiple genes involved in the production of aromatic compounds during fruit ripening. Transgenic tomato fruit expressing *MiADH1* gene showed a change in the level of several alcohols and aldehydes related to flavor (Singh et al., 2018). Lipoxxygenase

FIGURE 5. The X-axis represents detection frequency, and the Y-axis represents the activity units at each detection time. All values are the mean $\pm$ standard error of three replicates. (A) *PuADH2* oxidation-reduction reactions. Different lowercase letters indicate significant differences. (B) *PuADH2* oxidation-reduction reactions with different substrates. Different lowercase letters indicate significant differences. (C) *PuADH2* oxidation-reduction reactions with different cofactors.

FIGURE 6. (A) *PuADH2* oxidase activity in the presence of different concentrations of Ca²⁺ and K⁺. The X-axis represents detection frequency, and the Y-axis represents oxidase activity. All values are the mean $\pm$ standard error of three replicates. (B) *PuADH2* oxidase activity in the presence of different concentrations of Mg²⁺ and Zn²⁺. The X-axis represents detection frequency, and the Y-axis represents oxidase activity. All values are the mean $\pm$ standard error of three replicates. Asterisks indicate the significant differences in the oxidase activity compared to 0 μ M metal ion treatment condition in each group.

pathway is confirmed as an important biosynthetic mechanism for the generation of free and glycosylated volatile organic compounds during tamarillo ripening and this biosynthesis pathway is highly correlated with the activities of ADHs (Chen et al., 2020). A medium-chain protein encoding *CmADH1* was identified in melon and this gene can reduce the level of aldehydes such as 3-methylbutyraldehyde during fruit ripening, whereas the short-chain protein encoding *CmADH2* cannot; these two *ADH* genes work together to influence the production of aromatic compounds (Manriquez et al., 2006). Integrated transcriptome, qRT-PCR and GC-MS analyses revealed that the expression levels of three *ADH* genes were closely correlated with the content of aromatic compounds during fruit development, suggesting the roles of these three genes in catalyzing aldehydes to alcohols during pear fruit ripening stage (Zeng et al., 2020). We found that *PuADH2* shares $\geq 80\%$ amino acid sequence identity with *ADHs* from apple, cherry, strawberry, cassava, longan, and grape. Gene Ontology prediction indicated that *PuADH2* may involve in oxidation-reduction processes and *PuADH2* acts preferentially as an oxidase, which may catalyze the oxidation of alcohols to aldehydes to aromatic precursors during fruit ripening.

In this study, we showed that the *PuADH2* activity was higher on hexanol with NAD^+ as a cofactor than that on hexanal with NADH as a cofactor. We suggested that *PuADH2* be an oxidase, similar to the *ADHs* from olive and mango (Salas and Sánchez, 1998; Singh et al., 2010). Furthermore, its oxidase activity can be detected regardless of substrate specificity. Grape fruits produce higher levels of *ADH* with increasing activity in accordance with the ripening period (Tesniere and Verries, 2000). The *ADH* activity also resembles to the switch from a predominance of aldehydes to alcohols with the ripening of berry (Kalua and Boss, 2009). The pear aroma reportedly consists of >300 compounds, including esters, aldehydes, ketones, hydrocarbons, alcohols, and sulfur (Suwanagul and Richardson, 1997). The expression pattern of *PuADH2* was opposite to that observed based on the hexyl ester-volatile content with the ripening of pear fruit (Qin et al., 2017), indicating that *PuADH2* may oxidize alcohols to their corresponding aldehydes to be released or further oxidized during ester-volatile biosynthesis.

Many types of ions are capable of inhibiting or activating *ADH* activity (Zhu et al., 2012). The *PuADH2* activity decreased significantly along with increased Zn^{2+} concentrations (Figure 6B), which was corresponding with the effects of Zn^{2+} on *ADH* from *Desulfovibrio gigas* (Hensgens et al., 1993). One explanation for this might be that *PuADH2* obtains Zn^{2+} ions from the culture medium of gene expression experiment. Despite dialysis against MES, it was difficult to eliminate Zn^{2+} from the buffer. The higher the concentration of Zn^{2+} in the reaction mixture, the more Zn^{2+} - NAD^+ complexes are formed (Vallee and Hoch, 1955), resulting in fewer NAD^+ cofactors available for *PuADH2*. We found that $50\text{ }\mu\text{M}$ K^+ had enhanced *PuADH2* enzyme activity, which was consistent with the effects observed in *Thermus thermophilus* (Pennacchio et al., 2008). Furthermore, *PuADH2* showed a minimal activity when Ca^{2+} was present, although an upregulated *ADH* expression was reported following calcium treatment (Wei et al., 2016).

Conclusion

In this study, a *PuADH2* gene was cloned from *P. ussuriensis* 'Nanguoli' and expressed in *E. coli* cells to produce a protein (*PuADH2*) of 380-amino acids with a predicted molec-

ular weight of 46 kDa. Biochemical analysis suggested that *PuADH2* be an oxidase, no preference for coenzyme factors (NAD^+ and NADP^+). Its activity was enhanced by $50\text{ }\mu\text{M}$ K^+ , minimally affected by Ca^{2+} and Mn^{2+} , but inhibited by Zn^{2+} under all conditions tested. These results indicated that *PuADH2* may involve in aroma biosynthesis by catalyzing alcohol formation, however, its function in pear fruits requires a further study.

Acknowledgments

This work was supported by the China Agriculture Research System (CARS-28-33) and the National Natural Science Foundation (31301739). We are thankful to Professor Pan Yongbao (Agricultural Research Services, U.S. Department of Agriculture) for the help in polishing language.

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Received: Sep. 4, 2020

Accepted: Jul. 21, 2021

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