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Cloning, Expression, Purification and Assay of *Sorbitol dehydrogenase* from "Feicheng" Peach Fruit (*Prunus persica*)

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ABSTRACT

A gene encoding NAD^+-dependent sorbitol dehydrogenase (SDH) in peach fruit was cloned and expressed in Escherichia coli. Recombinant SDH protein with 6×His-tagged was localized exclusively in the cytoplasmic soluble fraction of E. coli *when the strains were grown for 4-5 h at 37 ^oC. Highly pure protein was isolated by Ni^{2+}-resin chromatography with 150 mM imidazole in 50 mM Tris, pH 8.0, by elution. In order to ensure that the recombinant SDH could be used for further study, the fluorescence and ultraviolet spectrum of the recombinant SDH were detected. Recombinant SDH was confirmed to be capable of oxidizing sorbitol by enzymatic activity assay. The activity of the recombinant SDH was 2.73 U $mg^{-1}min^{-1}$, which was similar with that directly extracted from peach fruits. The activities of SDH extracted from the fruits in different periods (30, 60, 90 days after flowing) were 7.75, 5.95, 3.26 U $mg^{-1}min^{-1}$, respectively.*

Key words: sorbitol dehydrogenase, recombinant SDH, expression and purification, Ni-NTA, enzyme assay, peach fruit

INTRODUCTION

Sorbitol is the major photosynthetic product in peach (*Prunus persica* (L.) Batsch.) (Yamada et al. 1999). NAD^+-dependent sorbitol dehydrogenase (SDH) is one of the important enzymes in sorbitol metabolism in the *Rosaceae* plants, which catalyzes the reversible oxidation of sorbitol to fructose using NAD^+ as a cofactor, and plays important roles in regulating sink strength and controlling the quality of the fruit (Yamaki and Ishikawa 1986; Loescher and Everard 1996; Yamaguchi et al. 1996; Nosarzewski and Archbold 2007). In the peach fruit, SDH contributes to the production of fructose from sorbitol and is responsible for the regulation of fructose concentrations (Loescher et al. 1982). Similar is also found in the apple fruit (Yamaguchi et al. 1994). However, the accumulation of sugar during fruit development of peach is quite different from that of apple in which SDH plays an important role for sugar metabolism. It is known that sorbitol is important in the translocation of photosynthates in peach, but little is known about sorbitol metabolism in the peach fruit. The SDH distributes ubiquitously in the fruit and leaf of apple (Wang et al. 2009) and is expressed and active in the apple fruit during apple fruit set and early development (Nosarszewski et al. 2004; Nosarzewski and Archbold 2007).

The SDH enzyme was purified from the mature Japanese pear fruit (Oura et al. 2000) for the first

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time. The molecular weight of the native enzyme was estimated to be 160 kDa by gel filtration, whereas SDS-PAGE gave a subunit of 40 kDa, indicating that the native enzyme was a homotetramer (Oura et al. 2000). The cDNA encoding SDH protein from *Rosaceae* plants was firstly isolated from the apple fruit by using partial amino acid sequences of SDH protein (Yamada et al. 1998). The *SDH* gene has been successfully isolated from loquat fruit (Bantog et al. 2000), peach fruit (Yamada et al. 2001), apple fruit (Nosarszewski et al. 2004), and the bud of Japanese pear (Ito et al. 2005). Although sorbitol concentration is low in peach fruit mesocarp, *SDH* is also expressed during the first and third stage of peach fruit development in accordance with the changes of SDH activity (Lo Bianco and Rieger 2002; Morandi et al. 2008). Because it is still unclear about sorbitol metabolism and the role of *SDH* in peach fruit, it is important to understand the relation in more detail between the expression of *SDH* and development of peach fruit.

In this study, a gene encoding SDH was cloned from "Feicheng" peach fruit at physiological mature and expressed in *Escherichia coli*.

MATERIALS

Peach fruits (*Prunus persica* (L.) Batsch, cv. Feicheng) were obtained from Feicheng, Shandong province in China at a pre-climacteric but physiological mature stage, with a mean firmness of 85 N cm^{-2}. They were selected for uniformity of size and ground color and freedom from the defects and mechanical damage. Then they were frozen immediately in liquid nitrogen and stored at -80°C until further use. The sorbitol dehydrogenase genomic sequence was accessed from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov, GenBank nos: AB025969.1). *E. coli* BL21 (DE3) was kindly provided by the College of Life Sciences, Shandong Agricultural University.

METHODS

Gene cloning and construction of SDH expression vector

The frozen peach fruit samples were ground in liquid nitrogen to a fine powder using a mortar and pestle. Total ribonucleic acid (RNA) was extracted from the frozen peach fruit by the method of cetyltrimethylammonium bromide (CTAB) (Murray and Thompson 1980; Gasic et al. 2004). Sense and antisense primers of *SDH* were designed using the Primer Premier 5.0 and synthesized by Invitrogen (Beijing, China). Gene-specific primers were designed based on a partial sequence of peach *SDH* cDNA (GenBank nos: AB025969.1) Forward primer 5'–GGA TCC AAA ATG GGC AAG GGA GGG ATG-3' and reverse primer 5'-AAG CTT ATG TTC TCG CCG ACC TTT CTA-3'. RT-PCR was performed using RNA PCR Kit (AMV) (TaKaRa), following the manufacturer's guidelines. A fragment of *SDH* (1104 bp) was amplified using the primers to conserve *SDH* sequence at a 55.2°C annealing temp for 36 cycles. The resulting RT-PCR product was isolated by a quick Agarose Gel DNA Purification Kit (TaKaRa). The product was ligated into pMD^{TM}18-T Vector (TaKaRa), following the manufacturer's guidelines. The recombinant plasmids (pMD^{TM}18-T+*SDH*) were transferred into *E. coli* DH5α by the method of heat shock response as following. After incubating on ice for 30 minutes, the ligation product of pMD^{TM}18-T+*SDH* was heat-shocked at 42°C for 90s and then kept on ice for 5 minutes. The *E. coli* DH5α harboring the pMD^{TM}18-T+*SDH* vector was incubated in 1.0 mL of Luria-Bertani (LB) medium at 37°C for 1.5 h. Afterwards, 1.0 mL of *E. coli* cells suspension was centrifuged to 0.1 mL at 1,2000×g for 1 min and the cells were plated on LB agar with 50 μg mL^{-1} ampicillin (Inoue et al. 1990; Brooke et al. 2009; Singh et al. 2010). Positive clones were selected on LB solid media with ampicillin at 37°C overnight. Next day, all the bacterial colonies were removed from the plates and then grown to stationary phase in 3.0 mL of sterile liquid LB from which the plasmids were extracted and purified using alkaline lysis (Birnboim and Doly 1979; Birnboim 1983; Ehrt and Schnappinger 2003) as follows. All the studies were carried out at room temperature unless otherwise indicated. After 12 h incubation, 1.0 mL of culture was transferred to a 1.5 mL Eppendorf tube for plasmid extraction. The tube was centrifuged for 1 min at 12,000×g in a microcentrifuge to pellet the bacteria. The supernatant was carefully removed with a micro-adjustable pipette and the cell pellet was thoroughly suspended in 200 μL of solution I (9 g L^{-1} glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0). After incubation at 0°C for 30 minutes,

200 μL of solution II (1% SDS, 0.2 M NaOH) was added and the tube was gently vortexed, then the tube was maintained for 5 minutes at 0°C and then 300 μL of solution III (0.5 M CH_3COOK, 11.5% CH_3COOH) was added. The contents of the tube were gently mixed by inversion for a few seconds. After 5 minutes incubation at 0°C, the tube was centrifuged at 12,000×g for 7 minutes in a microcentrifuge and the supernatant was transferred to a fresh tube. At the same time, an equal volume of phenol: chloroform (1:1) was added and the contents of the tube were mixed by vortexing for 5 s. The tube was centrifuged at 12,000×g for 7 minutes in a microcentrifuge to achieve the phase separation. Then the top aqueous phase was transferred to a clean tube. After 700 μL 100% isopropanol was added, the tube was incubated at -20°C for 30 minutes. The precipitate was again collected by centrifugation as before. The supernatant was removed as completely as possible and the DNA pellet was dissolved in 50 μL of TE buffer containing 20 μg mL^{-1} of RNase A.

Following the recombinant plasmids, including *SDH* fragment being obtained, correct plasmids were identified by restriction digests with *Hind* III and *BamH* I. All the restriction digests were carried out for 3 h at 37°C with 1 μg of plasmid and 10 U of each enzyme. With 5 μL 6×loading buffer added to terminate reaction, the products of restriction endonucleases were electrophoresed through a 1% (w/v) agarose gel. The correct plasmids were then sequenced by Invitrogen (Beijing, China).

The *SDH* fragment digested by restriction enzymes was then ligated into pET-30a with T4 DNA Ligase at 16°C for 12 h. Then the ligation product was used to transform *E. coli* BL21 (DE3) by heat-shock response. Afterwards, positive clones were selected on LB solid media with kanamycin (50 μg mL^{-1}) at 37 °C overnight. Then the new construct recombinant plasmids (pET-30a+*SDH*) were extracted and purified by the methods mentioned above (Birnboim and Doly 1979; Birnboim 1983; Ehrt and Schnappinger 2003) and assayed by the PCR. Meanwhile, the new construct recombinant plasmids were sequenced by Sangon Biotech (shanghai) Co., Ltd.

Expression and purification of SDH

E. coli BL21 (DE3) harboring the pET-30a+*SDH* vector were grown at 37°C with shaking at 250 rpm to a maximum optical density at 600 nm. One milliliter was used to inoculate a new culture, which was grown at 37°C with shaking at 250 rpm until optical density at 600 nm reached 0.4~0.6. The cultures were then induced with 1 mM IPTG and shocked at 250 rpm and 37°C for 4 h. One hundred milliliter of cells was harvested by centrifugation (5,000×g at 4°C for 15 min) and lysed by re-suspension in 4.0 mL of 1×Binding Buffer (20 mM Na_3PO_4, 500 mM NaCl, pH 7.8). Lysozyme was added into lysate with the final concentration of 1.0 mg mL^{-1}. The lysate was incubated at 4°C for 30 min and then ultrasonicated in the presence of protease inhibitors. The resulting lysate was centrifuged to remove the cell debris. The supernatant was collected as a crude extract.

The recombinant SDH was purified from the soluble fractions with Ni-NTA chromatography. Most of the impurities were in the flow-through while His-SDH fusion protein was retained on the column. Target fusion protein appeared as the strongest band after Ni-NTA purification from SDS-PAGE gel. The cell pellet was washed with PBS and re-suspended in 1:5 (w/v) buffer A (50 mM Tris, 0.2 M NaCl, 5 mM b-mercaptoethanol, 5 mM imidazole, 1% glycerol, pH 8.0, 5°C) and stirred for 15 min at 5°C. The cells were disrupted by pulsed sonication and the lysate was spun at 15,000×g for 15 min. The soluble fraction was loaded onto a Ni–NTA-His bind column (Novagen), equilibrated with buffer A at 5°C. The column was washed with 300 mL of buffer B (50 mM Tris, 0.2 M NaCl, 50 mM imidazole, pH 8.0, 5°C) until the A_{280} reached 0.001. The second wash was done with 50 mL of buffer C (50 mM Tris, pH 8.0, 5°C) and the protein was eluted with buffer D (150 mM imidazole in 50 mM Tris, pH 8.0, 5°C). The protein-containing fractions were pooled and dialyzed exhaustively against 20 mM HEPES, pH 7.0 or 50 mM Tris, pH 8.0. The protein content was determined using Coomassie brilliant blue G-250 (Bradford 1976). From the total fresh mass extracted and the various protein concentrations, the extractable protein concentration of the tissue was estimated. SDH protein was subsequently analyzed by 10% SDS-PAGE (Laemmli 1970; Jose and von Schwichow 2004).

Fluorescence measurement of guanidinium hydrochloride (GdnHCl)-induced equilibrium unfolding

The fluorescence measurement was carried out according to Yadaiah et al. (2012). The samples for equilibrium unfolding were prepared by mixing appropriate volumes of stock solutions of native and unfolded protein prepared in 20 mM HEPES, pH 7.0. The former contained no denaturant and the latter was 6 M in GdnHCl. The initial volumes of the two stock solutions were identical and they were sufficiently uniform in *SDH* content. Therefore, samples prepared by mixing these two stocks were identical in terms of protein content (about 0.05 mg mL^{-1}). This procedure of samples preparation also provided a test for reversibility of the folding-unfolding equilibrium. The samples were incubated at 25°C for 24 h and the fluorescence emission spectra were recorded at 360-600 nm with an excitation wavelength of 340 nm.

Comparison of activities between recombinant SDH and SDH extracted from peach

SDH activity was assayed following the protocol described by Bianco et al. (1998). Mesocarp tissues (1.0 g) was homogenized on ice with 4.0 mL of 0.2 M Tris-HCl buffer (pH 9.0, 25°C) containing 20 mM β-mercaptoethanol, 8% (v/v) glycerol, 0.1 % (v/v) Tween 20 and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through a layer of miracloth and centrifuged at 3000×g for 15 minutes at 4°C. The supernatant was desalted at 4°C using a Sephadex G-25 column.

Assay mixture (5.0 mL) consisted of 1.0 mL 0.5 mg mL^{-1} recombinant *SDH* (or extracted *SDH*), 100 mM Tris-HCl buffer (pH 9.0, 25°C), 1 mM NAD^+ and 300 mM sorbitol. The increase in absorbance at 340 nm (UV-2450 spectrophotometer, Shimadzu) due to the reduction of NAD^+ to NADH during the conversion of sorbitol to fructose was recorded over 10 to 30 min at 25°C. From the increase in absorbance over the time and the difference between the assays with and without sorbitol, the rate of NAD^+ reduction was determined. The specific activity of *SDH* was expressed as units min^{-1} mg^{-1} of protein. One unit of *SDH* activity was defined as the amount of enzyme needed to oxidize 1 μM of substrate min^{-1} under initial velocity conditions.

Each experiment was repeated three times and the data was processed by the analysis of variance (ANOVA). The data values were expressed as mean ± SE (n = 3). Standard errors of the means were shown for the data points in each figure.

RESULTS AND DISCUSSION

Cloning of the gene for SDH

Total RNA was extracted from the frozen peach fruit and the RNA sample (approximately 1 μg each) was electrophoresed through 1% (w/v) agarose gel. Distinct 28S and 18S rRNA bands without degradation were observed in Figure 1. The brightness of 28S was more than two times higher than that of 18S. The RNA concentration was 3253.0 ng μL^{-1} and the $A_{260/230}$ ratio was 2.27. These indicated that the RNA was of high purity and was not contaminated by polyphenol and polysaccharide. The $A_{260/280}$ ratio was 2.10, indicating lack of protein contamination.

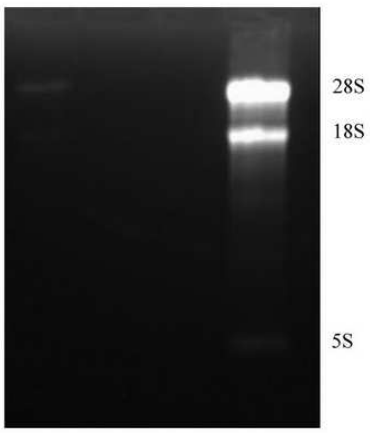

Figure 1 - Electrophoretogram of total RNA through a 1% (w/v) agarose gel. Total RNA was extracted from peach fruit, and analyzed throush 1% (w/v) agarose gel.

RT-PCR products of *SDH* were electrophoresed through 1% (w/v) agarose gel (Fig. 2). Eight samples were injected into gel holes. The *SDH* gene was successfully acquired in the eight samples,. The band of RT-PCR product appeared at 1100 bp and was in accordance with the target *SDH* gene.

The RT-PCR products were isolated from the agarose gel and ligated into pMD^{TM}18-T vector, then transformed into *E. coli* DH5α. The DH5α harboring the recombinant plasmids (pMD^{TM}18-T+*SDH*) was incubated at 37°C for 4 h and then detected by the PCR (Fig. 3). PCR products were electrophoresed through a 1% (w/v) agarose gel. Ten samples were analyzed by the PCR but lanes 1, 2, 5, 7, 8, 9, 10 only showed positive clone. There were obvious bands at about 1.1 kbp in Lanes 1, 7 and 10, in accordance with size of target *SDH* gene. Result from Figure 3 confirmed that the recombinant plasmids were successfully transformed into *E. coli*.

The recombinant plasmids (pMD^{TM}18-T+*SDH*) were digested with *Hind* III and *Bam*H I restriction enzymes for 4 h at 37 °C. Digested products were electrophoresed through a 1% (w/v) agarose gel (Fig. 4) and the gene fragments at 1.1 kbp were purified by TaKaRa Agarose Gel DNA Purification Kit Ver.2.0.

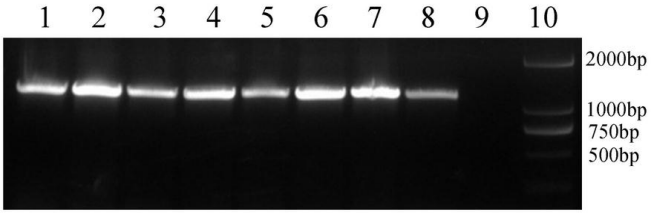

Figure 2 - Electrophoretogram of RT-PCR products of *SDH* gene through a 1% (w/v) agarose gel. Lane 1-8, RT-PCR products of *SDH* gene; Lane 9, negative reagent control; Lane 10, DL2000 DNA marker.

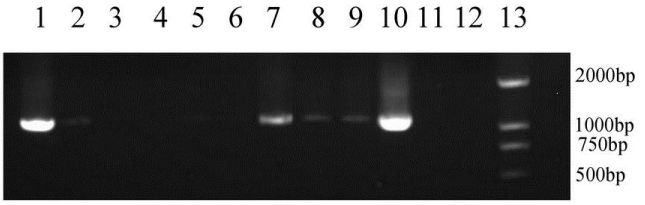

Figure 3 - Electrophoretogram of *E. coli* DH5α harboring the recombinant plasmids (pMD^{TM}18-T+*SDH*) through a 1% (w/v) agarose gel. Lane 1-10, PCR product; Lane 11 and 12, negative reagent control; Lane 13, DL2000 DNA marker.

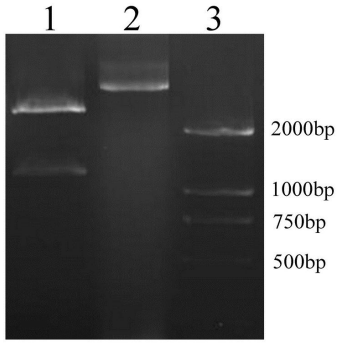

Figure 4 - Electrophoretogram of digested products in a 1 % (w/v) agarose gel. Recombinant plasmid (pMD^{TM}18-T+*SDH*) was digested by *Hind* III and *Bam*H I simultaneously. Lane 1 showed two bands appeared in 1100 bp and 2600 bp, it showed that cohesive end of target gene formed. Lane 1: digested products of recombinant plasmids (pMD^{TM}18-T+ *SDH*) by *Hind* III/*Bam*H I; Lane 2: digested products of plasmid (pET30a) by *Hind* III/*Bam*H I; Lane 3: DL2000 DNA Marker.

Digested products-*Hind* III/*Bam*H I were then ligated into pET-30a, which had been previously prepared by *Hind* III and *Bam*H I digestion, and then were transformed into BL21 (DE3). Recombinant plasmids (pET-30a+*SDH*) was certified by the restriction analysis and sequencing. *SDH* sequence identity was 96.74%.

Expression and purification of SDH

The recombinant plasmid pET-30a+*SDH* of *SDH* gene of 'Feicheng' peach by prokaryotic expression was transformed into BL21(DE3). Cells were lysed by sonication in the presence of protease inhibitors. Fusion protein was purified from the soluble fractions with Ni-NTA chromatography. Most of the impurities were in the flow-through while His-*SDH* fusion protein was retained on the column. Target fusion protein appeared as the strongest band after Ni-NTA purification from SDS-PAGE gel (Fig. 5).

Protein concentrations were determined by measuring the continuous changes in absorbance at 595 nm and 25°C with a UV-2450 spectrophotometer (Shimadzu, Japan) according to the Bradford method (Bradford 1976). The concentration of recombinant *SDH* was 0.4687 mg mL^{-1}.

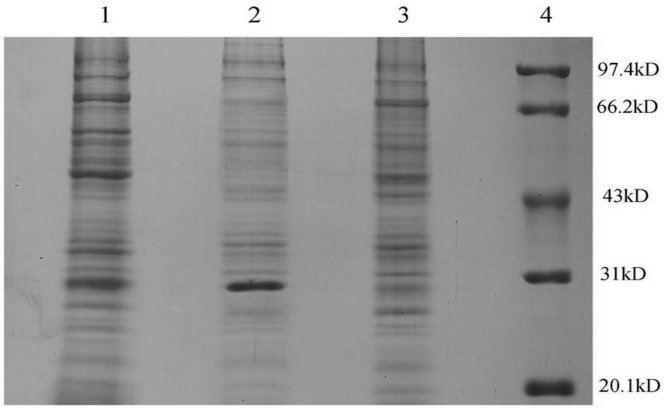

Figure 5 - Expression of His-tagged *SDH* fusion protein in *E. coli* and purification of the 6×His-tag recombinant *SDH* with Ni-NTA. The crude extract was applied to a HisTrap Chelating column (1 mL), washed with phosphate buffer (pH 7.4) containing 10 mM imidazole (pass-through, each 1 mL-scale fraction) and eluted with phosphate buffer (pH 7.4) containing 200 mM imidazole (w/Imidazole, each 1 mL-scale fraction). Sample was extracted with SDS sample buffer by boiling and then analyzed with 10% SDS-PAGE. Lane 1: crude extract of *SDH* protein induced with IPTG; Lane 2: Purification of *SDH* protein induced with IPTG; Lane 3: crude extract of *SDH* protein induced without IPTG; Lane 4: molecular mass marker. Positive band was visualized with Ni-NTA conjugate and Coomassie brilliant blue R-250 staining kit.

Reversibility of GdnHCl-induced unfolding of the purified SDH

In order to find whether the purified recombinant SDH was stable and folded, GdnHCl-induced equilibrium unfolding of the protein was checked. Figure 6 showed the fluorescence spectra of the native and 6 M GdnHCl-unfolded protein. The observed fluorescence was largely due to the three tryptophan residues, the enhancement of fluorescence in the unfolded state should serve as a probe for folding–unfolding studies. A quantitative treatment of the unfolding equilibria and the interpretation of the folding–unfolding could not be presented here. However, consideration of the strategy for the sample preparation for this experiment made it clear that the unfolding reaction was reversible.

Comparison of SDH enzyme activity between expressed and extracted

In order to find out whether the recombinant SDH had catalytic function like native SDH, native SDH was extracted from the fruits on 30, 60, 90 days after the flowering (DAF) and the activities were also determined. Figure 7 showed that native SDH activity decreased with increasing mature of peach fruit. However, SDH activity was low in young apple fruit and then increased gradually as fruit developed, with a rapid rise 144 DAF and SDH specific activity was lower between 88 and

144 DAF than either earlier, or later (Yamaguchi et al. 1996). The intensity and the seasonal profile of SDH activity in pear fruit were roughly the same as those of apple fruit. The activity of SDH rose once rapidly in immature stage until it attained the maximum activity on 10th July, then reduced with fruit enlargement and subsequently rose again with fruit maturation after 20th August (Moriguchi et al. 1990). The difference between the present work and the previous reports in native SDH activity could depend on the maturity of peach fruit. It was suggested that SDH activity increased and then decreased during the fruit development. The fruit types could also contribute to the difference in SDH activity in the fruits.

The activity of the recombinant SDH (2.73 U mg^{-1} min^{-1}) was significantly lower than that of the native SDH in the fruits 30 and 60 DAF (7.75 and 5.95 U mg^{-1} min^{-1}, respectively). However, there was no significant difference in the activity between the recombinant SDH and the native SDH in the peach fruits 90 DAF (3.26 U mg^{-1} min^{-1}). The previous reports showed that there were 11 cDNA encoding SDH proteins from the apple fruit and the expression product of *SDH* was provided with SDH activity *in vitro* (Yamada et al. 1998). The K_m value for sorbitol of this recombinant protein (247 mM) was higher than that of purified SDH from the apple fruit (40.3 mM) (Yamaguchi et al. 1994). This indicated that native SDH had a higher affinity to sorbitol than recombinant *SDH*, which was consistent with the difference of activity between the native SDH and recombinant SDH in the peach fruit. The conformation of recombinant SDH could be different compared to native SDH from the peach fruit since this recombinant protein contained thioredoxin as an extra-protein (Yamada et al. 1998).

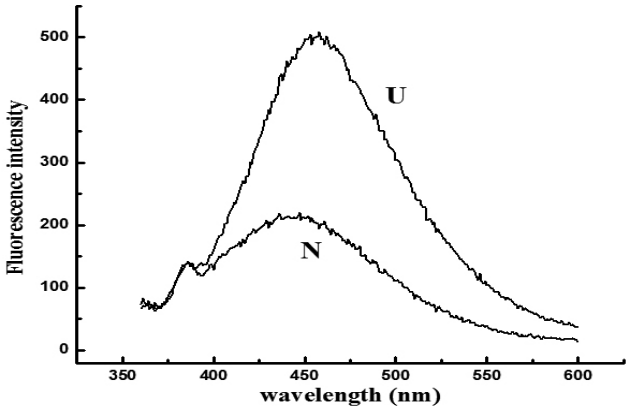

Figure 6 - Fluorescence spectra and reversible unfolding of recombinant SDH. Emission spectra of native (N) and 6 M GdnHCl-unfolded (U) SDH in 20 mM HEPES, pH 7.0, 25^{o}C. Excitation wavelength is 280 nm.

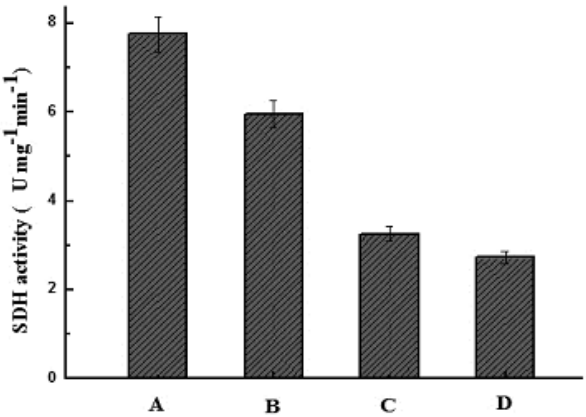

Figure 7 - Comparison of enzyme activity between the recombinant SDH and the native SDH extracted directly from peach fruit. A, B and C represented native *SDH* extracted from peach fruit in 30 days, 60 days, and 90 days after flowering, respectively. D represented the recombinant *SDH*. Each point is the mean ± SE of three experiments (n = 3).

CONCLUSION

This work cloned and characterized the expression of *SDH* and its product in peach fruit. Recombinant SDH protein with 6×His-tagged was localized exclusively in the cytoplasmic soluble fraction of *E. coli* when the strains were grown at 37^{o}C for 4-5 h. Highly pure protein was isolated by Ni^{2+}-resin chromatography using 150 mM imidazole in 50 mM Tris, pH 8.0, by elution. The activity of the recombinant SDH enzyme was similar with that of peach fruits assayed by monitoring the ultraviolet absorbance of the product of NADH from NAD^{+}.

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REFERENCES

Bantog N, Yamada K, Niwa N, Shiratake K, Yamaki S. Gene expression of NAD^{+}-dependent sorbitol dehydrogenase and $NADP^{+}$-dependent sorbitol-6-phosphate dehydrogenase during development of loquat (*Eriobotrya japonica* Lindl.) fruit. *J Japan Soc Hort Sci.* 2000; 69 (3):231-236.

Bianco RL, Rieger M, Sung S-JS. A simple, rapid extraction and assay procedure for NAD^{+}-dependent sorbitol dehydrogenase (SDH) in peach. *J Am Soc Hortic Sci.*1998; 123 (6):1065-1068.

Birnboim H. Rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol*. 1993; 100:243-255.

Birnboim H, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 1979; 7 (6):1513-1515.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72 (1-2):248-254.

Brooke R, Hopkins J, Laflamme E, Wark P. Effects of Temperature-induced changes in membrane composition on transformation efficiency in *E. coli* DH5α. *J Exp Microb Immunol.* 2009; 13:71-74.

Ehrt S, Schnappinger D. Isolation of plasmids from *E. coli* by alkaline lysis. *Methods Mol Biol.* 2003; 235:75-78.

Gasic K, Hernandez A, Korban S. RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Mol Biol Rep.* 2004; 22:437a-437g.

Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* 1990; 96 (1):23-28.

Ito A, Hayama H, Kashimura Y. Partial cloning and expression analysis of genes encoding NAD^{+}-dependent sorbitol dehydrogenase in pear bud during flower bud formation. *Sci Hortic.* 2005; 103 (4):413-420.

Jose J, von Schwichow S. Autodisplay of active sorbitol dehydrogenase (*SDH*) yields a whole cell biocatalyst for the synthesis of rare sugars. *Chembiochem.* 2004; 5 (4):491-499.

Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227 (5259):680-685.

Bianco RL, Rieger M. Partitioning of sorbitol and sucrose catabolism within peach fruit. *J Am Soc Hortic Sci*. 2002; 127:115-121

Loescher W, Everard J. Sugar alcohol metabolism in sinks and sources. in Zamski, E; Schaffer, AA (eds). Photoassimilate distribution in plants and crops: source-sink relationships, New York: Marcel Dekker; 1996. 185-208.

Loescher W, Marlow G, Kennedy R. Sorbitol metabolism and sink-source interconversions in developing apple leaves. *Plant Physiol.* 1982; 70 (2):335-339.

Morandi B, Grappadelli LC, Rieger M, Bianco RL. Carbohydrate availability affects growth and metabolism in peach fruit. *Physiol Plant.* 2008; 133 (2):229-241.

Moriguchi T, Sanada T, Yamaki, S. Seasonal fluctuations of some enzymes relating to sucrose and sorbitol metabolism in peach fruit. *J Am Soc Hortic Sci.* 1990; 115 (2): 278-281.

Murray M, Thompson W. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 1980; 8 (19):4321-4323.

Nosarszewski M, Clements AM, Downie AB, Archbold DD. Sorbitol dehydrogenase expression and activity during apple fruit set and early development. *Physiol Plant.* 2004; 121 (3):391-398.

Nosarzewski M, Archbold DD. Tissue-specific expression of *sorbitol dehydrogenase* in apple fruit during early development. *J Exp Bot.* 2007; 58 (7):1863-1872.

Oura Y, Yamada K, Shiratake K, Yamaki S. Purification and characterization of a NAD^{+}-dependent sorbitol dehydrogenase from Japanese pear fruit. *Phytochem.* 2000; 54 (6):567-572.

Singh M, Yadav A, Ma X, Amoah E. Plasmid DNA transformation in *Escherichia coli*: effect of heat shock temperature, duration, and cold incubation of $CaCl_2$ treated cells. *Int J Biotechnol Biochem.* 2010; 6 (4):561-568.

Wang XL, Xu YH, Peng CC, Fan RC, Gao XQ. Ubiquitous distribution and different subcellular localization of sorbitol dehydrogenase in fruit and leaf of apple. *J Exp Bot.* 2009; 60 (3):1025-1034.

Yadaiah M, Rao PN, Sudhamalla B, Ramakrishna D, Yasin UM, Bhuyan AK. Cloning, *Escherichia coli* expression, purification, characterization, and enzyme assay of the ribosomal protein S4 from wheat seedlings (*Triticum vulgare*). *Protein Expres Purif.* 2012; 81 (1):55-62.

Yamada K, Mori H, Yamaki S. Gene expression of NAD-dependent sorbitol dehydrogenase during fruit development of apple (*Malus pumila* Mill. var. domestica Schneid.). *J Japan Soc Hort Sci.* 1999; 68 (6):1099-1103.

Yamada K, Niwa N, Shiratake K, Yamaki S. cDNA cloning of NAD-dependent sorbitol dehydrogenase from peach fruit and its expression during fruit development. *J Hortic Sci Biotechnol.* 2001; 76 (5):581-587.

Yamada K, Oura Y, Mori H, Yamaki S. Cloning of NAD-dependent sorbitol dehydrogenase from apple fruit and gene expression. *Plant Cell Physiol.* 1998; 39 (12):1375-1379.

Yamaguchi H, Kanayama Y, Soejima J, Yamaki S. Changes in the amounts of the NAD-dependent sorbitol dehydrogenase and its involvement in the development of apple fruit. *J Amer Soc Hort Sci.* 1996; 121(5):848-852.

Yamaguchi H, Kanayama Y, Yamaki S. Purification and properties of NAD-dependent sorbitol dehydrogenase from apple fruit. *Plant Cell Physiol.* 1994; 35 (6):887-892.

Yamaki S, Ishikawa K. Roles of four sorbitol related enzymes and invertase in the seasonal alteration of sugar metabolism in apple tissue. *J Am Soc Hortic Sci.* 1986; 111 (1):134-137.

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