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Cloning of genes and enzymatic characterizations of novel dioscorin isoforms from *Dioscorea japonica*

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ABSTRACT

Dioscorin, the major tuber storage protein of yam, has been shown to possess carbonic anhydrase, trypsin inhibitor, dehydroascorbate reductase, and monodehydroascorbate reductase activities. In the present study, dioscorin from *Dioscorea japonica* was confirmed as a glycoprotein using the enhanced concanavalin A-peroxidase staining method, and the protein was shown to have both N- and O-glycans. Following the gene cloning, four full-length isoforms of dioscorin were expressed in *Escherichia coli* and purified by affinity purification and anion-exchange chromatography for structural and biochemical experiments. It was clearly observed that the recombinant dioscorins had carbonic anhydrase, trypsin inhibitor, dehydroascorbate reductase, and monodehydroascorbate reductase activities. However, the dehydroascorbate reductase and monodehydroascorbate reductase activities were markedly decreased in recombinant dioscorins compared with native dioscorin. The decreased activities were closely related to the loss of the glycosylation from the protein.

1. Introduction

Yam, the tuber of *Dioscorea* spp., is an important staple food in many tropical countries. More than 600 species of yam have been planted all over the world. In China, yam has been traditionally used as a health food and Chinese herbal medicine. It also receives much attention for its functional properties and pharmaceutical potential [1,2]. Some of the beneficial properties of yam have been attributed to the storage protein dioscorin [3–7].

Dioscorin is the major storage protein of the yam tuber and accounts for approximately 80–85% of the total soluble proteins. It has been reported that the protein has carbonic anhydrase (CA), trypsin inhibitor (TI) [4,8], dehydroascorbate (DHA) reductase, and monodehydroascorbate (MDA) reductase activities [9]. Carbonic anhydrases are zinc metalloenzymes that catalyze the interchange of CO_2 and HCO_3^-. In plants, they play an important role in photosynthesis and other biosynthetic reactions [8,10]. Proteinaceous protease inhibitors maintain compact and stable inhibitory domains that bind to the active sites of their target proteases and prevent access to the substrate molecules. In plants, they are important in regulating and controlling endogenous proteases and in acting as protective agents against insect and pathogen proteases [11,12]. DHA reductase catalyzes the reduction of DHA to ascorbate using glutathione as an electron donor, and MDA reductase catalyzes the reduction of MDA radical to ascorbate using NAD(P)H as an electron donor. Both of them are important enzymes in the glutathione-ascorbate cycle, which is a part of the antioxidative system for protecting plants from the toxicity of reactive oxygen species (ROS) [13,14].

Although the functional properties of dioscorin have been well explored, the mechanism for exerting the diverse functions remains unclear due to the lack of information about the structure–function correlation. In an effort to accelerate the structural and biochemical characterization of dioscorin, we cloned, expressed and purified dioscorin from *Dioscorea japonica*, and characterized this protein in terms of its enzymatic activities.

2. Materials and methods

2.1. Cloning, protein expression and purification

The gene cloning of dioscorin from *D. japonica* was performed according to a previous report [15] with some modifications. Briefly, the total RNA was isolated from 5 g of fresh yam tuber using Concert Plant RNA reagent (Invitrogen). After the elimination of polysaccharides (High-Salt Solution for Precipitation (Plant); Takara) and DNA (TURBO DNA-free Kit; Ambion), the first chain of cDNA was reverse-transcribed from the total RNA using the specific primers 5′-GGAATTCCATATGGTAGAGGATGAGTTTAGCTACATTG-3′ (the *Nde*I restriction site is underlined)

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and 5′-GTATCTCGAGTCATATGACACCAAGCTTCGAT-3′ (the *Xho*I restriction site is underlined) and an AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies) according to the manufacturer's protocols. The polymerase chain reaction (PCR) product was digested with *Nde*I and *Xho*I and inserted into the pET-28a (Novagen) vector, which was cleaved by the same restriction enzymes. The resulting plasmid was confirmed by DNA-sequencing analysis.

The expression vector for dioscorin was transformed into *Escherichia coli* Rosetta (DE3) cells (Novagen). The cells were cultured in Lysogeny Broth (LB) medium supplemented with 20 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C until the OD_{600} reached about 0.6, and then the protein was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C overnight. After harvesting by centrifugation at 5000 rpm for 10 min, the cells were resuspended in the purification buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl and 1 mM dithiothreitol (DTT)) containing 10 mM imidazole and disrupted by sonication on ice. The supernatant was separated from the cell fragments by centrifugation at 40,000 × *g* for 30 min at 4 °C and then loaded onto 3 ml of Ni Sepharose 6 Fast Flow resin (GE Healthcare). After washing the column with the purification buffer containing 20 mM imidazole, the $(His)_6$ tag was cleaved from dioscorin by incubation with thrombin protease (GE Healthcare) overnight at 4 °C. Following the elution with the purification buffer containing 10 mM imidazole and the dialysis against 20 mM Tris–HCl buffer (pH 8.0) with 1 mM DTT, the fraction containing the target protein was further purified by anion-exchange chromatography (Resource Q; GE Healthcare). The purity of the protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein solution was concentrated to 20 mg/ml in 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM DTT and stored at −80 °C for further use.

The purification of native dioscorin from yam tuber was conducted as reported previously [4] with slight modifications. Briefly, after cleaning with water, the tubers were peeled and immediately cut into strips for dioscorin extraction. Samples were homogenized with four volumes (w/v) of 20 mM Tris–HCl buffer (pH 8.0). After centrifugation at 20,000 × *g* for 30 min, the supernatant was applied to ammonium sulfate fractionation (45–75%). After dialysis against 20 mM Tris–HCl buffer (pH 8.0) overnight, this fraction was further purified by anion-exchange chromatography (Resource Q; GE Healthcare). The purified native dioscorin was collected and concentrated to 20 mg/ml and stored at −80 °C for further use. All chromatographic steps were performed using an AKTA FPLC system (GE Healthcare).

2.2. Circular dichroism (CD) measurements

The CD spectra for the native and recombinant dioscorins were recorded on a Jasco J-720 spectropolarimeter at wavelengths of 190–260 nm. 0.1 mg/ml of dioscorin in 5 mM phosphate buffer (pH 7.0) was put into a 0.1 cm path length cell under a nitrogen atmosphere at 20 °C for measurements. The software package Spectra Manager (Jasco) was used for data collection and analysis.

2.3. Enzymatic activity assay

The CA activity of dioscorin was determined by the pH-stat technique from the direction of dehydration of sodium bicarbonate [4,16] with some modifications using a 907 Titrando system (Metrohm). The autotitration was done with 0.1 M H_2SO_4 to pH 7.1, which was set as a fixed end point. The reaction mixture (10 ml) comprised 0.2 μM dioscorin, 10 mM HEPES-NaOH buffer (pH 7.1) and 30 mM sodium bicarbonate at 4 °C.

The TI activity of dioscorin was determined by the inhibition of trypsin-catalyzed hydrolysis of *N*-benzoyl-L-arginine-4-nitroanilide in 0.1 M Tris–HCl buffer (pH 8.2) [4]. Different amounts of dioscorin were preincubated with 20 μM of trypsin at room temperature for 15 min, and then the substrate was added for an additional 20 min. The absorbance at 405 nm was determined.

The DHA reductase activity of dioscorin was assayed according to a previous report [8] with some modifications. 10 mg DHA was dissolved in 5.0 ml of 100 mM phosphate buffer (pH 7.0). The reaction was carried out at 30 °C by adding 100 μl of 200 μM dioscorin solution to 900 μl DHA solution. The increase in absorbance at 265 nm was recorded for 6 min. Non-enzymatic reduction of DHA in phosphate buffer was measured in a separate cuvette at the same time. A standard curve was plotted using 0.1–6.0 nmol ascorbate (AsA).

The MDA reductase activity of dioscorin was assayed according to a previous report [8] by following the decrease in absorbance at 340 nm due to NADH oxidation. MDA free radicals were generated by AsA oxidase (EC 1.10.3.3; WAKO) in the assay system. The reaction mixtures contained 50 μM dioscorin, 50 mM phosphate buffer (pH 7.0), 0.33 mM NADH, 3 mM AsA and AsA oxidase (1 U) in a final volume of 1 ml. Dioscorin solution was replaced with distilled water for controls.

2.4. Deglycosylation treatment

To remove the N-linked carbohydrate residues from the protein, 5 μl of PNGase F (2500 units; Biolabs) was added to 100 μl of 5 mg/ml native dioscorin in the reaction buffer containing 1% NP-40 and 50 mM sodium phosphate (pH 7.5). To remove the O-linked carbohydrate residues from the protein, 4 μl of neuraminidase (200 units; Biolabs) and 6 μl of endo-α-*N*-acetylgalactosaminidase (240,000 units; Biolabs) were added to 100 μl of 5 mg/ml native dioscorin in the buffer containing 1% NP-40 and 50 mM sodium phosphate (pH 7.5). The solutions were incubated at 37 °C for 2 h.

2.5. Glycoprotein staining

The detection of glycoprotein was performed on 15% SDS–PAGE gels using the Periodic Acid Schiff (PAS) Staining method (Pierce Glycoprotein Staining Kit) and the concanavalin A-peroxidase staining method [17]. To strengthen the visualization of the concanavalin A-peroxidase staining, the binding of concanavalin A (WAKO) to the glycoprotein and the subsequent coupling of horseradish peroxidase (WAKO) to the glycoprotein–concanavalin A complex were performed twice. The complex was then visualized by the oxidation of 3,3′-diaminobenzidine (WAKO) with horseradish peroxidase-H_2O_2, forming a brownish stain at the gel surface.

3. Results and discussion

3.1. Cloning, expression and purification

Four genes of dioscorin isoforms (D1 through D4) were cloned without the signal sequences using the same primer pair, and four similar proteins (D1 through D4) were obtained. There are already twelve dioscorin precursors from *D. japonica* registered in the NCBI (National Center for Biotechnology Information) database. The results of a BLAST (Basic Local Alignment Search Tool) search showed that D1 was identical to the dioscorin-5 precursor (*D. japonica*; GenBank ID: CAO98738.1), and only slightly different from the other three proteins. The sequence alignment of the dioscorin-5 precursor and the four proteins is shown in Fig. 1. Although D2 through D4 were found in *D. japonica* for the first time, their similarity to the dioscorin-5 precursor was extremely