論 文 の 内 容 の 要 旨

応用生命化学専攻
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論文題目　Structure and enzymatic characterizations of yam storage protein dioscorin from *Dioscorea japonica*
(ヤムイモ貯蔵タンパク質 Dioscorin の構造・機能解析)

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. Some of the beneficial properties of yam have been attributed to the storage protein dioscorin.

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), dehydroascorbate (DHA) reductase, and monodehydroascorbate (MDA) reductase activities. CAs 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. Proteinaceous protease inhibitors are important in regulating and controlling endogenous proteases

and in acting as protective agents against insect and pathogen proteases in plants. Both of DHA reductase and MDA reductase 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).

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, purified and crystallized dioscorin from *Dioscorea japonica*.

Firstly, dioscorin was cloned, and the DNA sequencing showed that four similar genes of dioscorin were obtained. The dioscorins were expressed in *E. coli* and purified using affinity resin and anion-exchange chromatography. The recombinants were compared with native dioscorin using SDS-PAGE and CD measurement, and no obvious structure change was detected (Fig. 1).

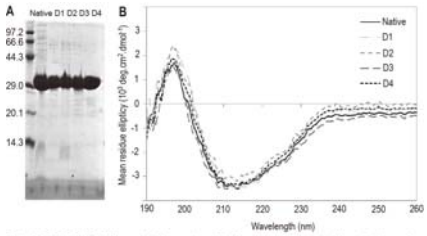

Fig.1 SDS-PAGE (A) and CD spectra (B) of native and recombinant dioscorins

Recombinant dioscorins obtained clearly showed CA, TI, DHA reductase and MDA reductase activities. These activities were not affected by the amino acid substitutions, which suggest that these dioscorins were isoforms of one another. Although the recombinants had the same CA activity and TI activity as native dioscorin, The DHA and MDA reductase activities of recombinants decreased greatly (Fig. 2). Since glycosylation is a post-translational process and very rarely occurs in bacteria, the recombinant dioscorins were not glycosylated. In order to investigate the

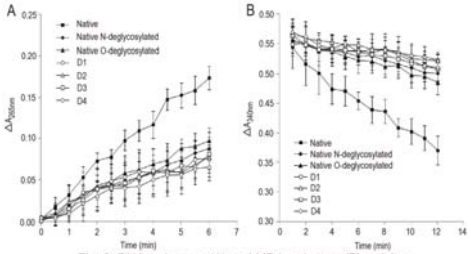

Fig. 2 DHA reductase (A) and MDA reductase(B) activity of native and recombiant dioscorins

activities of native dioscorin without glycans, PNGase F was used to eliminate the N-glycans, and neuraminidase and endo-α-N-acetylgalactosaminidase were used to eliminate the O-glycans from native dioscorin. After the cleavage by the deglycosyslation enzymes and the purification using a Superdex75 gel filtration column, the DHA reductase activity and MDA reductase activity of deglycosylated dioscorin were tested again. The results showed that the DHA reductase activity and the MDA reductase activity of both N-deglycosyslation and O-deglycosyslation dioscorins decreased greatly, and the differences between the deglycosylated and the recombinant dioscorins were not significant (Fig. 2), which means the glycans are closely related to the DHA reductase activity and the MDA reductase activity of native dioscorin. We also confirmed that native dioscorin is an N- and O-linked glycoprotein using enhanced concanavalin A-peroxidase staining method and N- / O-deglycosyslation treatments.

Dioscorin was crystallized, and after several rounds of optimization, a single crystal with dimensions of 0.5 × 0.3 × 0.06 mm was obtained using the reservoir solution consisting of 0.1*M* CAPS pH 10, 0.2 *M* lithium sulfate, 1.8 *M* ammonium sulfate at 278 K for 6 days. An X-ray diffraction data set was collected to 2.11 Å using a synchrotron X-ray source (PF NW12). The crystal belonged to the centered orthorhombic type and the space group was $C222_1$, with unit-cell parameters a =83.5 Å, b = 156.8 Å, c = 83.6 Å. A total of 222 696 reflections in the resolution range of 20.0-2.11 Å were collected with 99.3% completeness and an R_{sym} of 3.3%. After phase determination and refinement, the structure of dioscorin was resolved. Dioscorin has one big β-sheet that consists of nine β-strands, which was surrounded by six α-helices, two small β-strands and several loops (Fig. 3). Although dioscorin and CA from *N. gonorrhoeae* only share

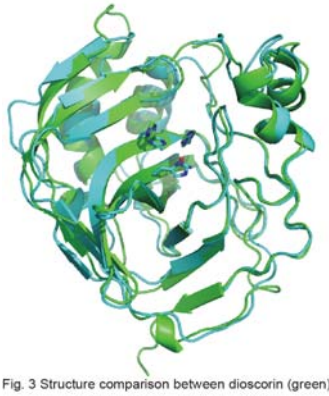

Fig. 3 Structure comparison between dioscorin (green) and CA form *N. gonorrhoeae* (cyan)

32% sequence identity and two of the active site residues are altered (Q114 mismatch, H95 different orientation), the three-dimensional structures are quite similar for the two proteins (Fig.3). Q114H mutant changed dioscorin to a typical α-CA and the CA activity was increased greatly in the presence of zinc ions.

The structures of dioscorin-DHA complex (2.28 Å) and dioscorin-ascorbate complex (2.20 Å) were also obtained using cocrystallization method, and some miner structure changes (side chain movements of His69 and Arg93) were identified, which were considered to be the structure changes happened during the reaction process. As a result of glycosylation site prediction using *YinOYang 1.2* server, two possible O-glycosylation sites (Thr188 and Thr228) were identified, and Thr188 was located near the active site of dioscorin. It is possible that the loss of O-glycosylation of Thr188 caused the change of structure, and then the DHA and MDA reductase activities of dioscorin were reduced. Based on the typsin docking model of dioscorin obtained from the *Cluspro 2.0* server, point mutations were introduced. After the TI activity determination of the mutants, the inhibitory loop (loop α2-α3) of dioscorin was identified.

References

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2. Xue Y.-L., Miyakawa T., Sawano Y., Tanokura M. Crystallization and preliminary X-ray crystallographic analysis of dioscorin from *Dioscorea japonica.* Acta Crystallographica Section F: Structural Biology and Crystallization Communications（in press）