Dioscorins from different *Dioscorea* species all exhibit both carbonic anhydrase and trypsin inhibitor activities

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**Abstract.** Dioscorins, the storage proteins of yam tubers, from six cultivars of three *Dioscorea* species including *D. batatas*, *D. alata*, and *D. pseudojaponica* were detected in crude extracts of yam tubers but not leaves by immuno stainings on PVDF membrane with a polyclonal antibody which was raised against 28 kDa dioscorin from *Dioscorea batatas* Decene. It was demonstrated that the dioscorins from the above-mentioned yam species exhibited both carbonic anhydrase (CA) and trypsin inhibitor activities by activity stainings on 15% SDS-PAGE gels. Dioscorins were identified as glycoproteins by ConA-peroxidase activity staining. Dioscorins from *D. batatas* Decene contained no zinc atoms detectable by atomic absorption spectrophotometry. Neither 5 mM acetazolamide nor 2,6-pyridinedicarboxylic acid inhibited the CA activity of dioscorins.

**Keywords:** Acetazolamide; Carbonic anhydrase; Dioscorin; Pyridinedicarboxylic acid; Storage proteins; Trypsin inhibitor; Yam.

**Abbreviations:** CA, carbonic anhydrase; 2-ME, 2-mercaptoethanol; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TI, trypsin inhibitor.

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**Introduction**

Carbonic anhydrases (CAs) catalyze the simple interconversion of CO\(_2\) and HCO\(_3^-\). They are found in almost all organisms and are notable for extremely high turnover numbers (Tashian, 1989; Badger and Price, 1994). In cyanobacteria and plants, CAs facilitate the interchange of CO\(_2\) and HCO\(_3^-\) and play a key role in CO\(_2\) fixation of photosynthesis (Hatch and Burnell, 1990; Badger and Price, 1992). In mammals, CAs also facilitate the interchange of CO\(_2\) and HCO\(_3^-\) and play a key role in respiration (Tashian and Hewett-Emmett, 1984; Forster, 1988). According to Hewett-Emmett and Tashian (1996), CA is encoded by three independent CA gene families (\(\alpha\)-CA, \(\beta\)-CA, and \(\gamma\)-CA). Most CAs of mammals and green algae belonged to the \(\alpha\)-CA family, those of plants and eu bacteria to the \(\beta\)-CA family, and those of archaea bacteria to the \(\gamma\)-CA family. Surprisingly, based on the cDNA deduced amino acid sequence alignment, the storage protein of yam (*Dioscorea cayenensis* L.) tuber, dioscorin, was found to be a \(\alpha\)-CA related protein (Hewett-Emmett and Tashian, 1996). However, Hewett-Emmett and Tashian (1996) also mentioned that dioscorin was unlikely to have classical CA activity due to the active site alternations.

Patatin, the potato tuber storage protein, has been reported to contain both lipid acyl hydrolase and acyltransferase activities, both of which are involved in tuber tissue response to wounding (Andrews et al., 1988). The soybean vegetative storage proteins VSP \(\alpha\) and VSP \(\beta\) both have acid phosphatase activity (Dewald et al., 1992). We have demonstrated that the root storage proteins of sweet potato not only have trypsin inhibitor (TI) activity (Hou and Lin, 1997a), but also dehydroascorbate reductase and monodehydroascorbate reductase activities, which might be involved in response to environmental stresses (Hou and Lin, 1997b). Tarin, the storage protein of taro (*Colocasia esculenta* L. Schott), was homologous to mannose-binding lectin and curcumin in deduced amino acid sequences (Bezerra et al., 1995). Recently, it was reported that yam bean (*Pachyrhizus erosus* L. Urban) storage proteins YGB 1 and YGB 2 exhibited cysteine protease activities and YBP 2 showed sequence homology to protease inhibitors (Gomes et al., 1997). We reported recently that the purified dioscorins from tubers of *Dioscorea batatas* Decene (imported from Japan) showed both CA and TI activities (Hou et al., 1999a) and both dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou et al., 1999b). In this report, we extend the work to show that both CA and TI activities could be found in dioscorin samples from six cultivars of three *Dioscorea* species including *D. batatas*, *D. alata* and *D. pseudojaponica* grown in Taiwan by activity staining on 15% SDS-PAGE gels. Some properties of dioscorins were also included.

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Materials and Methods

Plant Materials and Chemicals

Fresh tubers and leaves of yam grown in Hualien, Taiwan—including D. batatas Decne (imported from Japan); D. batatas Decne var. Shoufeng; D. alata L. var. Tainong 1; D. alata L. var. Tainong 2; D. alata L. var. Zhongguo and D. pseudojaponica var. Keelung—were kindly provided by Dr. Peng Hwang, Research Fellow of Hualien District Agricultural Improvement Station. Electrophoresis grade acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethyl-ethylene diamine, ammonium persulfate, 2,6-pyridinedicarboxylic acid and trypsotin (TPCK-treated, 40 U/mg) were from E. Merck Inc. (Darmstadt, Germany); Immobilon PVDF membrane was from Millipore (Bedford, MA); SeeBlue prestained markers for SDS-PAGE were from Novex (San Diego, CA); other chemicals of reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO).

Soluble Protein Extractions from Tubers and Leaves of Different Yam Species

Extraction processes were performed at 4°C according to Harvey and Boulter (1983) with minor modifications. After cleaning with water, tubers were peeled and cut into strips immediately before extraction. Cleaned samples of tuber or leaf were homogenized with four volumes (W/V) of 50 mM Tris-HCl buffer (pH 8.3) instead of the same buffer containing 10 mM 2-ME (Harvey and Boulter, 1983). After centrifugation at 12,500 g for 30 min, the supernatants were saved as crude extracts.

Electroblotting and Immuno Staining of Dioscorins

The crude extracts from tuber or leaf of different yam species were subjected to 15% SDS-PAGE according to Laemmli (1970). After electrophoresis, gels were equilibrated in the buffer of 25 mM Tris and 192 mM glycine (pH 8.3) for at least 10 min (Towbin et al., 1979), and then transferred onto Immobilon PVDF membranes using a semi-dry electroblotting cell for immuno staining with peroxidase-hydrogen peroxide-aminophenol carbazole system. Polyclonal antibody against the 28 kDa Dioscorea batatas (Hou et al., 1999a) of Dioscorea batatas Decne was raised from rabbit.

CA Activity Staining of Dioscorins

The CA activity staining of Dioscorins from different yam species on 15% SDS-PAGE gels was achieved by color change of bromothymol blue (Edwards and Patton, 1966) having CA activity with yellow bands against a blue background of bromothymol blue. Four volumes of sample were mixed with one volume of sample buffer (60 mM Tris-HCl buffer pH 6.8 containing 2% SDS, 25% glycerol and 0.1% bromophenol blue) at room temperature overnight. After electrophoresis, the gel was cut into two parts. One was fixed with 12.5% trichloracetic acid for protein stain- ing with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1985); the other was washed with 25% isoproplanol in 10 mM Tris-HCl buffer (pH 7.9) to remove SDS (Hou and Lin, 1998) before activity staining. For CA activity inhibition experiments, the gel was placed in 100 mM Tris buffer (pH 8.5) containing 5 mM of acetazolamide (requiring protection against light) or 2,6-pyridinedicarboxylic acid with gentle shaking for 2 h or overnight. After washing with 100 mM Tris buffer (pH 8.5) several times, the gels were stained for CA activity.

TI Activity Staining of Dioscorins

Activity staining of TI for Dioscorins from different Dioscorea spp. on 15% SDS-PAGE gels was according to the method of Hou et al. (1999a) and Hou and Lin (1998).

Detection of Dioscorins as Glycoproteins on Gels

The detection of glycoprotein was performed on 15% SDS-PAGE gels according to the method of Spatz et al. (1985) using the ConA-peroxidase activity staining method.

Measurement of Dioscorin Zinc Contents

The zinc contents of purified Dioscorin (six determinations) from D. batatas Decne were measured by an atomic absorption spectrophotometer (Model 2380, Perkin-Elmer Co., Connecticut) after dry ashing according to the method of Osborne and Voogt (1978).

Results and Discussion

We were the first to report that Dioscorins purified from tubers of D. batatas Decne (imported from Japan) exhibited both CA and TI activities (Hou et al., 1999a). In this report, we extend the work to show that both CA and TI activities could be detected in Dioscorin samples from six cultivars of three Dioscorea species, including D. batatas, D. alata, and D. pseudojaponica, grown in Taiwan. The soluble proteins extracted from fresh tubers of different yam cultivars grown in Hualien, Taiwan—including D. batatas Decne; D. batatas Decne var. Shoufeng; D. alata L. var. Tainong 1; D. alata L. var. Tainong 2; D. alata L. var. Zhongguo and D. pseudojaponica var. Keelung—were analyzed with polyclonal antibody raised against the 28 kDa Dioscorin from Dioscorea batatas Decne (5000-fold dilution). In Figure 1A, the purified Dioscorins, a positive control, from tubers of D. batatas Decne are shown in lane 1. Lanes 2 to 6 are crude tuber extracts of D. batatas Decne var. Shoufeng; D. pseudojaponica var. Keelung; D. alata L. var. Tainong 1; D. alata L. var. Tainong 2, and D. alata L. var. Zhongguo and, respectively. A comparison of Figure 1A (protein staining) with Figure 1B (immuno staining) clearly shows the Dioscorins as the major soluble proteins (over 90%) in six cultivars from three species of yam tubers. Harvey and Boulter (1983) estimated that about 85% of the total soluble proteins in crude extracts were Dioscorins.
As Figure 1B shows, at least three major protein bands were detected in all species that cross-reacted with polyclonal antibody raised against the 28 kDa dioscorin. The intra- and inter-molecular disulfide bridges to form oligomers have been reported in dioscorin at different pH values, ionic strength, and protein concentrations (Harvey and Boulter, 1983). Conlan et al. (1998) used two-dimensional non-reduced/reduced electrophoresis and isoelectric focusing to show the existence of different dioscorin molecules in yam tuber. Based on these results, the multiple dioscorin bands detected in Figure 1 appear due to (a) the existence of different dioscorin molecules, (b) the formation of oligomers due to the intra- and inter-molecular disulfide bridges, or (c) both.

In Figure 2, the purified dioscorins, a positive control, from tubers of D. batatas Dence are shown in Lane 1. Lanes 2 to 7 are crude leaf extracts of D. batatas Dence; D. batatas Dence var. Shoufeng; D. pseudojaponica var. Keelung; D. alata L. var. Tainong 1; D. alata L. var. Tainong 2 and D. alata L. var. Zhongguochang, respectively. Comparing Figure 2A (protein staining) with Figure 2B (immuno staining), we conclude that dioscorins are undetectable in leaf samples. These results suggest that dioscorins are tuber-specific proteins in yam. This is in agreement with Conlan et al. (1995) who claimed that dioscorins were not expressed in leaf tissues according to the results of Northern blot hybridization. The tuber-specific expressions of yam dioscorin storage proteins are possibly used as vital nitrogen sources for tuber sprouting.

Figure 3 shows both CA and TI activity stainings of crude tuber extracts from various yam species. The purified dioscorins (Hou et al., 1999a) in Lane 1 were used as positive controls. Lanes 2 to 6 were crude tuber extracts of D. batatas Dence var. Shoufeng; D. pseudojaponica var. Keelung; D. alata L. var. Tainong 1; D. alata L. var. Tainong 2 and D. alata L. var. Zhongguochang, respectively. In Figure 3A, three CA activity bands (yellow
bands on a blue background) detected in all samples matched the immuno stained bands of dioscorins in Figure 1B, and all exhibited TI activity (Figure 3B). According to our previous report (Hou et al., 1999a), ca. 1.9 μg trypsin could be inhibited by 100 μg dioscorins added. Based on these results, we conclude that the dioscorins exhibiting both CA and TI activities are likely a general property of yam tubers.

Use of the Con A-peroxidase analysis system suggests yam dioscorins could be glycoproteins (Spatz et al., 1985) (Figure 4). Harvey and Boulter (1983), however, reported that dioscorins from another yam species Dioscorea rotundata were not glycoproteins. The reasons for the disagreement were not clear but are possibly due to (a) different types of dioscorins, (b) different cultivars or species of yam, or (c) both.

The CAs in general require zinc as the cofactor to achieve higher enzyme activity (Tashian and Hewett-Emmett, 1984; Tashian, 1989; Badger and Price, 1994). Several factors that could affect CA activity have been reported. Lower zinc content in Zn-deficient rice leaves, for example, reduced CA activity (Sasaki et al., 1998). Hewett-Emmett and Tashian (1996) pointed out that one of the three Zn-liganding sites (all three in a conserved active-site region containing 15 amino acid residues) was mismatched (histidine119 → glutamine119) when the amino acid sequence of dioscorin was compared with those of other α-CAs (some deduced from cDNA). In addition, a different amino acid residue (glutamine92 → arginine92) was found in the conserved active-site region of dioscorin; 18 different amino acid residues were found in a total of 36 in the whole active-site region. However, we did detect CA activity in dioscorin molecules from tubers of three Dioscorea species with no appreciable amount of zinc atom as determined by atomic absorption spectrophotometry (data not shown). The CA of bovine erythrocytes used as positive control (C-4831, lot 126H9401, Sigma Co., Louis, MO) was found to contain zinc atoms (data not shown). Matsumoto et al. (1984) reported that ubiquitin from bovine erythrocytes also exhibited CA
activity. Ubiquitin is a zinc-free protein. We do not know the real CA activity mechanisms of Dioscoria or ubiquitin in the absence of zinc. Since we used different Dioscorea species from those used by Conlan et al. (1995, 1997), the amino acid residues in the whole active-site region may not be the same among all Dioscorea species. We are now cloning the Dioscoria cdNAS of our Dioscorea species. Once the cDNA sequences are obtained, some questions regarding the real mechanisms for Dioscoria with CA activity could be answered.

We analyzed the influence of inhibitors on the CA activity of Dioscoria (Figure 5). These included sulfonamides (such as acetzolamide, ethoxzolamide and methazolamide), specific potent inhibitors of CAs (Baird et al., 1997; Chegwidden and Spencer, 1996; Maren and Sanyal, 1983), and pyridinedicarboxyl acid (or dipicolinic acid) (Chen et al., 1998; Soczafava et al., 1999), a Zn-chelating agent. Figure 5A is a control. In Figure 5B and 5C, samples were put in 100 mM Tris buffer (pH 8.5) containing either 5 mM of acetzolamide (requiring protection against light) or 2,6-pyridinedicarboxylic acid with gentle shaking for 2 h before CA activity staining. The results showed that neither 5 mM acetzolamide nor 2,6-pyridinedicarboxylic acid could inhibit the CA activity of Dioscoria. The time of incubation was extended overnight, and the result was the same (data not shown).

Consistent with the results of Maren and Sanyal (1983) found that the CAs from plant sources were 10-104 fold less sensitive than those from animals. Dioscoria purified from yam tubers were ox-CA related proteins (Hewett-Emmett and Tashian, 1996), meaning that Dioscoria were close to CAs of animal sources and not those of plants. Hence, the insensitivity toward acetzolamide (one kind of sulfonamide) of the CA activity of our Dioscoria samples is novel, interesting, and deserves further investigation.

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Literature Cited


Hou, W.C., H.J. Chen, and Y.H. Lin. 1999b. Dioscorins, the
major tuber storage proteins of yam (*Dioscorea batatas* Decne), with dehydroascorbate reductase and monodehydroascorbate reductase activities. Plant Sci. 149: 151-156.


不同山藥品種中的儲藏性蛋白質都具有
碳酸酐酶及胰蛋白酶抑制因子的活性

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以三種山藥品種（包括 *D. batatas, D. alata* 及 *D. pseudojaponica*) 中的六種栽培種的塊莖與葉子為材料進行可溶性蛋白質的抽取，利用免疫染色法發現儲藏性蛋白質（dioscorins）只存在於塊莖中而不存在於葉子中。在 SDS-PAGE 胶體上，利用活性染色法證實所有六種栽培種的儲藏性蛋白質都具有碳酸酐酶（carbonic anhydrase）及胰蛋白酶抑制因子（trypsin inhibitor）雙重活性。儲藏性蛋白質利用 ConA-peroxidase染色法實為醣蛋白質。利用原子吸光光度法分析發現從 *D. batatas Decne* 所純化的儲藏性蛋白質並不含有鋅原子。而儲藏性蛋白質所具有的碳酸酐酶活性不受 5 mM acetazolamide 及 2,6-pyridinedicarboxylic acid 抑制。

關鍵詞：Acetazolamide；碳酸酐酶；儲藏性蛋白質；Pyridinedicarboxylic acid；胰蛋白酶抑制因子；山藥。