Dioscorin, the Major Tuber Storage Protein of Yam (*Dioscorea batatas* Decne) with Carbonic Anhydrase and Trypsin Inhibitor Activities

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Dioscorin, the tuber storage protein of yam (*Dioscorea batatas* Decne), was purified successively by ammonium sulfate fractionation, DE-52 ion exchange chromatography, and Sephadex G-75 column. Two protein bands (82 and 28 kDa) were found under nonreducing conditions after SDS−PAGE; but only one band (32 kDa) was detected under reducing conditions. The first 21 amino acids in the N-terminal region of the 28 kDa form were VEDEFSYIEGNPNGPENWGNL, which was highly homologous to deductive sequence of dioscorin from cDNA of another yam species (*Dioscorea cayenensis* Lam) reported by Conlan et al. (*Plant Mol. Biol.* **1995**, *28*, 369−380). Hewett-Emmett and Tashian (*Mol. Phylogenet. Evol*. **1996**, *5*, 50 −77) mentioned that, according to DNA alignments, dioscorin from yam (*D. cayenensis*) was α-carbonic anhydrase (α-CA) related. In this report, we found that the purified dioscorin showed both CA dehydration activity using sodium bicarbonate as a substrate and CA activity staining after SDS−PAGE. A polyclonal antibody, which was raised against trypsin inhibitor (TI), a storage protein of sweet potato (*Ipomoea batatas* [L.] Lam var. Tainong 57), cross-reacted with dioscorin, which also showed TI activity determined by both activity staining after SDS−PAGE and trypsin inhibition determination.

Keywords: *Dioscorin; storage proteins; yam; Dioscorea batatas Decne; carbonic anhydrase; trypsin inhibitor*

INTRODUCTION

Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the simple interconversion of CO_2 and HCO_3^-. They are found in almost all organisms and are notable for the extremely high turnover numbers of the high-activity forms. In the hydration reaction, CO_2 reacts with a Zn−OH intermediate at the active site of the enzyme. In the reversed dehydration direction, HCO_3^- reacts with Zn−H_2O (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), CAs were encoded by three independent CA gene families (α-CA, β-CA, and γ-CA). Most CAs of mammals and green algae belonged to the α-CA family; those of plants and eubacteria belonged to the β-CA family, and those of archabacteria belonged to the γ-CA family. Surprisingly, according to cDNA alignment, the storage protein of yam (*Dioscorea cayenensis* L.) tuber, dioscorin, was α-CA related proteins (Hewett-Emmett and Tashian, 1996). However, the authors also mentioned that dioscorin was unlikely to have classical CA activity because of active site alternations.

Patatin, the potato tuber storage protein, has been demonstrated to have lipid acyl hydrolase and acyltransferase activities that are involved in tuber tissue response to wounding (Andrews et al., 1988). The soybean vegetative storage proteins VSP α and VSP β both have acid phosphatase activity (Dewald et al., 1992). We also demonstrated that the root storage proteins of sweet potato not only have trypsin inhibitor (TI) activity but also have both dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou and Lin, 1997b). Tarin, the storage protein of taro (*Colocasia esculenta* L. Schott), showed homologous to mannose-binding lectin and curculin 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 that YBP 2 showed sequence homology to protease inhibitors (Gomes et al., 1997). In this study, we found that the purified dioscorin from yam (*D. batatas* Decne) tuber showed both CA dehydration activity and positive CA activity staining after SDS−PAGE. We also found that a polyclonal antibody, which was raised against trypsin inhibitor, a storage protein of sweet potato (*Ipomoea batatas* [L.] Lam var. Tainong 57), cross-reacted with dioscorin, which also showed TI activity determined by both activity staining after SDS−PAGE and trypsin inhibition determination.

MATERIALS AND METHODS

Materials. Fresh tubers of yam (*D. batatas* Decne) were imported from Japan and purchased from a local market. Electrophoresis grade acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, ammonium persulfate, trypsin (TPCK-treated, 40 U/mg), and *N*-benzoyl-L-arginine-4-nitroanilide were from E. Merck Inc. (Darmstadt,

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Germany); Immobilon PVDF membrane was from Millipore (Bedford, MA); Seeblue prestained markers for SDS−PAGE including BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin (30 kDa), and lysozyme (16 kDa) were from Novex (San Diego, CA); other chemicals of reagent grade were purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of Dioscorin from Yam Tuber. Extraction and purification processes were performed at 4 °C and modified from those of Harvey and Boulter (1983). After cleaning with water, tubers were peeled and cut into strips immediately for dioscorin extraction. Samples 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 in the report of Harvey and Boulter (1983). After centrifugation at 12500*g* for 30 min, the supernatants were saved for further purification. The precipitates from 45 to 75% ammonium sulfate saturation of crude extracts of yam were collected. After dialysis against 50 mM Tris-HCl buffer (pH 8.3) overnight, this fraction was loaded onto a DE-52 ion exchange column (2.0 × 20 cm). The column was washed with 50 mM Tris-HCl buffer (pH 8.3) of three column volumes and then eluted batchwise with 150 mM NaCl in 50 mM Tris-HCl buffer (pH 8.3). Flow rate was 50 mL/h, and each fraction contained 5 mL. The eluted fraction was collected and concentrated with Centriprep 10 (Amicon, USA) and further purified with a Sephadex G-75 column (1.6 × 70 cm). The column was eluted with 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl. Flow rate was 27 mL/h, and each fraction contained 3.6 mL. The purified dioscorin (fractions between 23 and 33) was collected and concentrated with Centriprep 10 and desalted with a PD 10 column (Sephadex G-25 M, Pharmacia Biotechnology, Uppsala, Sweden) for further use.

Electroblotting and Protein Sequencing. The purified dioscorin was subjected to 15% SDS−PAGE according to Laemmli (1970). After electrophoresis, gels were equilibrated in 250 mM sodium borate, pH 9.6, and 0.5% (w/v) SDS and transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA) by a semidry electroblotting cell (Chang et al., 1990). Dioscorin band was excised with a sharp razor blade and then prepared for protein sequencing. Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid-phase model 470A/900A sequencer with an on-line model 120A phenylthiohydantoin-amino acid analyzer (Hsieh et al., 1988).

CA Activity and Activity Staining on 15% SDS−PAGE Gels of Dioscorin. CA activity of dioscorin was determined by the pH-stat technique from the direction of dehydration of sodium bicarbonate (Carter et al., 1984; Shelton and Chegwidden, 1996; Chegwidden and Spencer, 1996) using an assay system supplied by Radiometer, Copenhagen. The autotitration was done with 0.1 M H_2SO_4 to pH 7.1 set as a fixed end point. The activity staining of CA for dioscorin on 15% SDS−PAGE gels was achieved by color change of bromothymol blue (Edwards and Patton, 1966). Four volumes of samples were treated with one volume of sample buffer, 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 25% glycerol, and 0.1% bromophenol blue with or without 2-ME at a final concentration of 14.4 mM at room temperature overnight. After electrophoresis, the gel was cut into two parts; one was fixed by 12.5% trichloroacetic acid for protein staining with Coomassie Brilliant Blue G-250 (Neuhoff et al., 1985); the other was washed with 25% 2-propanol in 10 mM Tris-HCl buffer (pH 7.9) to remove SDS (Hou and Lin, 1998) before activity staining.

TI Activity, Activity Staining, and Immunostaining on 15% SDS−PAGE Gels and PVDF Membrane of Dioscorin. The determination of TI activity of dioscorin was according to the method of Lee and Lin (1995) by the inhibition of trypsin-catalyzed hydrolysis of *N*-benzoyl-L-arginine-4-nitroanilide at 0.1 M Tris-HCl buffer (pH 8.2). Different amounts of dioscorin were preincubated with 4 μg 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. Three determinations were averaged for TI activity and expressed as micrograms of trypsin inhibited. Activity staining of TI for dioscorin on 15% SDS−PAGE gels was according to the method of Hou and Lin (1998). Polyclonal antibody against 38 kDa TI from sweet potato (*I. batatas* [L.] Lam var. Tainong 57) was raised from rabbit. After electrophoresis, dioscorin was electroblotted onto an Immobilon PVDF membrane, and the immunostaining was achieved with alkaline phosphatase−naphthyl phosphate system (Lin and Lu, 1994).

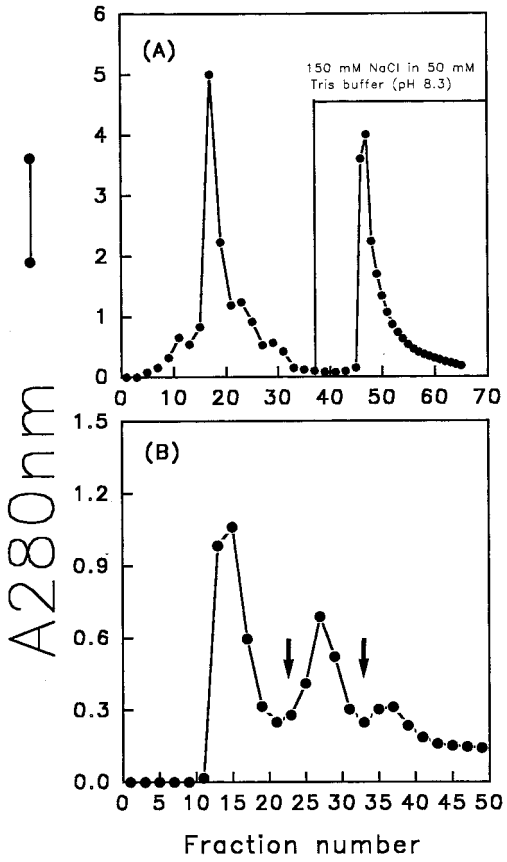

Figure 1. Chromatograms of dioscorin purified by (A) DE-52 ion exchange column and then by (B) Sephadex G-75 gel filtration after precipitation from 45 to 75% ammonium sulfate saturation. For DE-52 column (2.0 × 20 cm): washing buffer 50 mM Tris-HCl buffer (pH 8.3); eluting buffer, 150 mM NaCl in 50 mM Tris-HCl buffer (pH 8.3); flow rate, 50 mL/h; fraction size, 5 mL/tube. For Sephadex G-75 column (1.6 × 70 cm): eluting buffer, 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl; fractions between two arrows (fractions 23−33) were collected; flow rate, 27 mL/h; fraction size, 3.6 mL/tube.

RESULTS

Purification of Dioscorin from Yam Tuber. Dioscorin was purified from tubers of yam (*D. batatas* Decne) by a modified method based on Harvey and Boulter (1983) using only batch elution of discorin with salts for DE-52 ion exchange chromatography after sample extraction. After ammonium sulfate fractionation (45−75% saturation), DE-52 ion exchange chromatography (Figure 1A), and Sephadex G-75 chromatography (Figure 1B), two dioscorin protein bands with M_r 28 and 82 kDa, respectively, were found in SDS−PAGE gels without 2-ME (Figure 2A, lane 1), but only one band with M_r 32 kDa was found with 2-ME (Figure 2A, lane 2). Sporamin, the storage proteins of sweet potato, has been reported to have the same phenomena on SDS−PAGE gels showing two protein

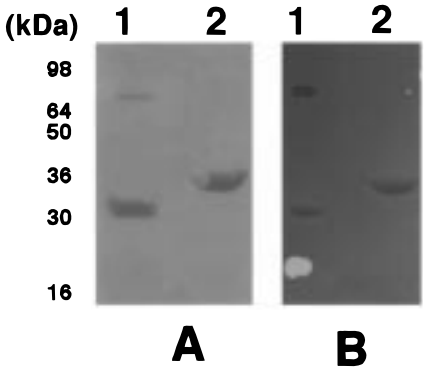

Figure 2. (A) Protein staining and (B) carbonic anhydrase activity staining on 15% SDS−PAGE gels of dioscorin. Lane 1, purified dioscorin without 2-ME treatment; lane 2, purified dioscorin with 2-ME treatment; SeeBlue prestained electrophoretic markers were labeled. A total of 5 μg of protein was loaded in each well.

bands (22 and 31 kDa) without dithiothreitol treatment, while only one protein band with the intermediate molecular weight (25 kDa) was reported in the presence of dithiothreitol (Maeshima et al., 1985). The M_r 28 kDa of dioscorin was the same as reported (Harvey and Boulter, 1983; Conlan et al., 1995), but the higher molecular weight of 82 kDa was new. This new 82 kDa band found in our preparations might be due to the covalent linking of the intra- and intermolecular disulfide bridges of dioscorin molecules to form oligomers in crude extracts of dioscorin from yam tuber while the reducing agent of 2-ME was absent. The intra- and intermolecular disulfide bridges to form oligomers have been reported in dioscorin at different pH values, ionic strength, and protein concentrations (Harvey and Boulter, 1983). The disappearance of the 82 kDa band under reducing conditions (Figure 2A, lane 2) may be due to disruption of interactions between monomeric units of dioscorin oligomers.

N-Terminal Sequence of Dioscorin. After electrophoresis, dioscorin was electroblotted onto a PVDF membrane for determination of the N-terminal amino acid sequence. The first 21 amino acids in the N-terminal region of the 28 kDa form were VEDEFSYIEGNPNGPENWGNL, which showed high homology to the deduced sequence of dioscorin from yam cDNA of another species (*D. cayenensis* L.) (Conlan et al., 1995) except at position 1 (alanine in the latter) and position 11 (serine in the latter).

CA Activity and Activity Staining on 15% SDS−PAGE Gels of Dioscorin. Figure 2B showed CA activity staining of dioscorin on 15% SDS−PAGE gels. Lane 1 was purified dioscorin under nonreducing conditions; lane 2 was purified dioscorin under reducing conditions. All dioscorin molecules on gels, with or without 2-ME treatment, showed CA activity with yellow bands against the blue background of bromothymol blue (Figure 2B) that matched the protein bands (Figure 2A). Figure 3 showed the time course of CA dehydration activity of 27 μg of purified dioscorin using sodium bicarbonate as a substrate. Linear CA dehydration activity of dioscorin was found in 10 min.

TI Activity, TI Activity Staining, and Immunostaining on 15% SDS−PAGE Gels and PVDF Membrane of Dioscorin. Protein staining (Figure 4A), TI activity staining (Figure 4B), and immunostaining (Figure 4C) on 15% SDS−PAGE gels or PVDF membrane of dioscorin were shown in Figure 4. Lane 1 was

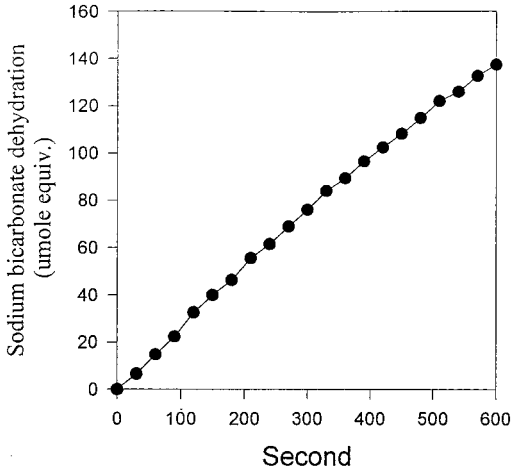

Figure 3. Time course of dehydration activity for carbonic anhydrase activity of dioscorin by the pH-stat technique. Substrate, 20 mL of 30 mM sodium bicarbonate in 10 mM HEPES buffer (pH 7.1); titrants, 0.1 M H_2SO_4; end point, pH 7.1; 27 μg of dioscorin added.

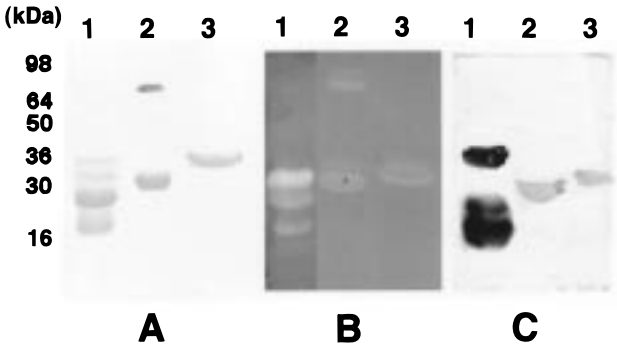

Figure 4. (A) Protein staining, (B) trypsin inhibitor activity staining, and (C) immunostaining of a polyclonal antibody against trypsin inhibitor from sweet potato on 15% SDS−PAGE gels and PVDF membrane, respectively, of dioscorin. Lane 1, purified trypsin inhibitors from sweet potato as positive controls (see Hou and Lin, 1997a); lane 2, purified dioscorin without 2-ME treatment; lane 3, purified dioscorin with 2-ME treatment. SeeBlue prestained electrophoretic markers were labeled. A total of 8 μg of protein was loaded on each well.

purified TIs from sweet potato roots after trypsin affinity chromatography (Hou and Lin, 1997a) without 2-ME treatment as positive controls; lane 2 was purified dioscorin without 2-ME treatment; lane 3 was purified dioscorin with 2-ME treatment. All dioscorin molecules with or without 2-ME treatments showed positive TI activity stainings (lanes 2 and 3, Figure 4B). Both molecular forms of dioscorin (28 and 32 kDa) could cross-react with a polyclonal antibody of TI from sweet potato roots (lanes 2 and 3, Figure 4C), but the 82 kDa form could not. TI activity was determined using different amounts of dioscorin and was expressed as micrograms of trypsin inhibited (Figure 5). A positive correlation ($r^2 = 0.992$) between TI activity and amounts of dioscorin was found. On average, 1.9 μg of trypsin was inhibited per 100 μg of dioscorin added.

DISCUSSION

This is the first report demonstrating that dioscorin, the storage protein purified from yam (*D. batatas* Decne) tuber, has both CA activity and TI activity. From the random amplified polymorphic DNA analysis for iden-

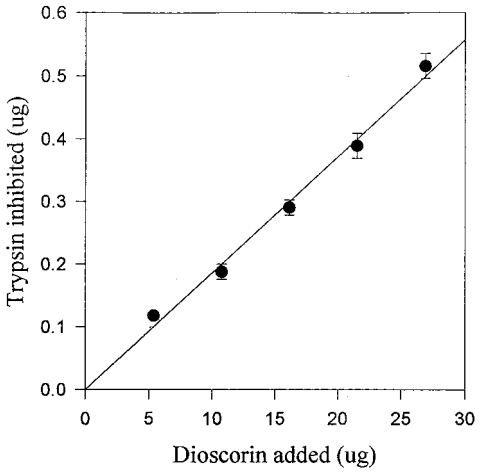

Figure 5. Determination of trypsin inhibition activity exhibited by dioscorin. Mean of three determinations was plotted against different amounts of dioscorin added.

tification of different yam species (Asemota et al., 1996), authors found many different DNA fragment patterns among yam species. Although Hewett-Emmett and Tashian (1996) mentioned that dioscorin was unlikely to have classical CA activity after comparing *D. cayenensis* deduced amino acid sequences of yam cDNA with the α-CA families, we obtained positive CA activity results using a different species of yam. In this report, we used activity staining on SDS−PAGE gels (Figure 2B) and sodium bicarbonate as a substrate to determine CA dehydration activity (Figure 3) to demonstrate that purified dioscorin from yam (*D. batatas* Decne) has CA activity. We do not know the physiological roles of this finding. Ubiquitin has been reported to have CA activity (Matsumoto et al., 1984). It is interesting that dioscorin was α-CA related, implying that they were more close to CAs of animal than those of plant that belong to the *β*-CA family (Hewett-Emmett and Tashian, 1996). It might be possible to find another CA belonging to the *β*-CA family in yam for facilitating CO_2 fixation during photosynthesis.

Protease protein inhibitors in plants may be important in regulating and controlling endogenous proteinases, in serving as storage proteins, and in acting as protective agents against insect and/or microbial proteinases (Ryan, 1973, 1989). Many storage proteins have been reported to have other functional properties than storage sinks, and most of them were shown to play protective roles against environmental stresses (Andrews et al., 1988; Bezerra et al., 1995; Hou and Lin, 1997b; Gomes et al., 1997). The N-terminal amino acid sequences of storage proteins purified from yam bean (*Pachyrhizus erosus* L. Urban), YGB1 and YGB2, showed high homology to cysteine protease, but both of them showed minor protease activities using azocasein as substrates (Gomes et al., 1997). We found that dioscorin had minor TI activity as compared with those from sweet potato roots, with an average of 1.9 *μ*g of trypsin inhibited per 100 *μ*g of dioscorin (Figure 5). Both activity staining and immunostaining for TI of our dioscorin samples were also positive (Figure 4). The 82 kDa form of dioscorin, which showed positive TI activity staining (lane 2, Figure 4B), did not cross-react with a polyclonal antibody of TI from sweet potato roots (lane 2, Figure 4C). However, this is not surprising because the active sites for TI activity may not necessarily be the same as the epitopes for the corresponding antibody. In addition, the disappearance of the 82 kDa protein band under reducing conditions (Figure 2A, lane 2) may be due to the disruption of interactions between monomeric units of dioscorin oligomers. These interactions might have a chance to mask the epitopes for polyclonal antibody of TI from sweet potato roots without affecting the active sites of TI activity at the same time. Although TI activity of dioscorin molecules is weak, the vast amounts of the storage protein could provide a significant protective role in yam tuber.

ABBREVIATIONS USED

CA, carbonic anhydrase; 2-ME, 2-mercaptoethanol; PVDF, poly(vinylidene difluoride); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TI, trypsin inhibitor.

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