

Characterization of the Yam Tuber Storage Proteins from *Dioscorea batatas* Exhibiting Unique Lectin Activities*

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Four major proteins designated DB1, DB2, DB3, and DB4 were isolated and characterized from the yam tuber *Dioscorea batatas*. The ratios of their yields were 20:50:20:10. DB1 was a mannose-binding lectin (20 kDa) consisting of 10-kDa subunits and was classified as the monocot mannose-binding lectin family. DB2, accounting for 50% of the total protein, was the storage protein, commonly called dioscorins consisting of a 31-kDa subunit. On the basis of amino acid sequence, DB2 was classified to be dioscorin A. DB3 was a maltose-binding lectin, having an apparent molecular mass of 120 kDa and composed of a 66-kDa subunit and two 31-kDa subunits (DB3S). The 66-kDa subunit was further composed of two 31-kDa subunits (DB3L) cross-linked by disulfide bonds. DB3L and DB3S (242 and 241 amino acid residues, respectively) were homologous with each other with 72% sequence identity. They showed a sequence homology to dioscorin B and dioscorin A from *Dioscorea alata*, with 90 and 93% identity, respectively, and to carbonic anhydrase from *Arabidopsis thaliana* with about 45% identity. DB3S had one intrachain disulfide bond located at Cys²⁸–Cys¹⁸⁷, whereas DB3L had one interchain disulfide bond (Cys⁴⁰–Cys^{40'}) in addition to the intrachain disulfide bond (Cys²⁸–Cys¹⁸⁸) to form a 66-kDa subunit. DB1 and DB3 agglutinated rabbit erythrocytes at 2.7 and 3.9 $\mu\text{g}/\text{ml}$, respectively. Despite the structural homology between DB2 and DB3, DB2 had no lectin activity. The 66-kDa subunit itself revealed the full hemagglutinating activity of DB3, indicating that DB3L but not DB3S was responsible for the activity. The hemagglutinating activity of DB3 required Ca^{2+} ions and was exclusively inhibited by maltose and oligomaltoses (e.g. maltopentaose and maltohexaose) but not by D-glucose. DB3 could not be classified into any known plant lectin family. DB4 was a chitinase, homologous to an acidic chitinase from *Dioscorea japonica*. DB1, DB2, and DB3 did not show any activity of carbonic anhydrase, amylase, or trypsin inhibitor activity. These results show that two of the four major proteins isolated from the yam tubers *D. batatas* have unique lectin activities.

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Yam is the common name applied to plants of about 500 species of the genus *Dioscorea* of the Dioscoreaceae family. Yam is an important staple crop, which is basically made up of carbohydrates but also constitutes an important source of proteins accounting for 1–3% of the fresh tubers (1). Over 80% of the protein represents the storage protein. Dioscorins, the major storage proteins in tubers of *Dioscorea rotundata*, consist of polypeptides of 31 kDa (2). The storage proteins are not structurally related to other storage proteins such as patatin from potato (*Solanum tuberosum*) (3) and sporamin from sweet potato (*Ipomoea batatas*) (4).

Storage proteins have been accepted to be the storehouses of nutrients. They enable the plant to survive periods of adverse conditions or to survive between growing seasons and provide nutrients to support the growth of new plants as seedlings or shoots. It has also been shown that tuber storage proteins exhibit biological activities that could contribute to resistance to pests, pathogens, or abiotic stresses (1). For example, patatin exhibits activity as an acylhydrolase and esterase (5); sporamin acts as a trypsin inhibitor (6), and dioscorin has antioxidative properties (7). It is therefore probable that storage proteins play a dual role in storage and defense.

Plant tubers are known to contain other defense-related proteins such as chitinase and lectins. Chitinase, which acts as fungicide and insecticide, exists in the yam tubers of *Dioscorea japonica* (8, 9), but the function of lectins in yam tubers has not yet been established. Lectins are carbohydrate-binding proteins that are widespread in the biosphere and occur in almost every living organism. The defensive role of plant lectins, such as anti-insect, anti-fungal, anti-microbial, as well as being toxic to birds and mammals, has been proposed on the basis of indirect and direct evidence (10). A rich source of lectins are the plants, in particular their storage organs such as seeds, tubers, bulbs, rhizomes, and bark, for example. Because many of the plants have been utilized as food resources by human beings, lectins are one of the most important physiologically active ingredients and potent exogenous biological signals in the diet (11).

In the present work we isolated and characterized tuber proteins from the tubers of a typical Japanese yam, *Dioscorea batatas*, and we demonstrated for the first time that mannose- and maltose-binding lectins are present as major proteins in the yam. Moreover, dioscorin-like storage protein itself proved to be a maltose-binding lectin, which could not be classified into any known plant lectin family.

EXPERIMENTAL PROCEDURES

Materials—The tubers of *D. batatas* were harvested in May at the Aomori prefecture, Japan, and were stored at 4 °C until used. For isolation of the proteins, whole tubers were used. Pyroglutamate aminopeptidase, endoproteinase Lys-C, and soybean trypsin inhibitor were purchased from Wako Chemical (Osaka, Japan). Chicken egg white

lysozyme (EC 3.2.1.17), bovine pancreatic trypsin (EC 3.4.21.4), carbonic anhydrase from bovine erythrocytes (EC 4.2.1.1), and α -amylase from *Bacillus* spp. (EC 3.2.1.1) were from Sigma. Dimethyl suberimide and dimethyl adipimide were from Nakalai Tesque (Kyoto, Japan). β -Amylase was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other reagents were the highest grade commercially available.

Purification of Tuber Proteins—Tubers (500 g) were homogenized in an equal volume (v/w) of 50 mM sodium acetate buffer (pH 4.0). The homogenate was centrifuged at $15,000 \times g$ for 40 min. The supernatant was adjusted to pH 7.0 with 6 M NaOH. Ammonium sulfate was added to 25% saturation with stirring. The solution was centrifuged at $15,000 \times g$ for 40 min, and the supernatant was subjected to hydrophobic chromatography on a phenyl-Toyopearl 650M column (3.5×20 cm) (Tosoh, Tokyo, Japan) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing ammonium sulfate to give 25% saturation. Elution was performed by a decrease of ammonium sulfate concentration from 25 to 0% in 50 mM Tris-HCl buffer (pH 7.0). The fractions yielding absorbance at 280 nm were pooled, dialyzed against 50 mM Tris-HCl buffer (pH 8.0), and subjected to an anion-exchange chromatography on a HiTrap Q HP column (5 ml) (Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Fractionation of proteins was performed by a linear salt gradient (0–0.3 M) NaCl in equilibrium buffer.

Gel filtration of purified proteins was performed on a HiLoad 16/60 Superdex 200 column (1.6×60 cm, Amersham Biosciences) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl or a TSKgel 3000G SW column (0.75×30 cm, Tosoh) equilibrated with 0.25 M sodium phosphate buffer (pH 7.0).

Chemical Cross-linking and Gel Electrophoresis—The purified proteins (0.1 mg) were dissolved in 0.1 ml of 0.1 M triethanolamine (pH 8.0), reacted with 1 mg of dimethyl suberimide (DMS)¹ and dimethyl adipimide (DMA) homobifunctional cross-linkers for 1 h at 20 °C, and stopped by adding 20 μ l of 0.2 M ammonium acetate. Cross-linked proteins were precipitated by ice-cold acetone and subjected to SDS-PAGE by using 12% (w/v) acrylamide gel as described by Laemmli (12). Native PAGE was performed according to the Davis system (13). For glycoprotein detection proteins were stained using periodic acid-Schiff staining (14). Protein concentration was estimated by the BCA assay (Pierce) using bovine serum albumin as a standard.

Mass Spectrometry—Molecular masses were measured by a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager-DE STR, Applied Biosystems) equipped with a pulsed nitrogen laser (337 nm; 4 ns) and delayed extraction unit. Aqueous solutions of protein samples (1 mg/ml) were diluted 1:1 in 0.1% trifluoroacetic acid containing sinapinic acid (1 mg/ml) matrix. One microliter of the mixture was deposited on the target and air-dried. The MALDI-TOF spectrometer was run in linear mode with an acceleration voltage of 25 kV. Mass spectra were obtained as averages of 100 laser shots, and several independent MALDI-TOF mass measurements were made for each sample to evaluate reproducibility. Insulin (M_r 5,733.5) (bovine pancreas, Sigma) and myoglobin (M_r 16,949.5) (horse heart, Sigma) were used for external calibration.

CD Spectroscopy—CD spectra were measured at room temperature using a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan) in the range of 210–260 nm with a 0.5-cm path length. Protein solutions were prepared at 0.1% (w/v) in 50 mM Tris-HCl buffer (pH 7.5) containing various concentrations of urea and were left for 2 h at room temperature before measurements.

Amino Acid Sequencing—Each purified protein was reduced with 10 mM dithiothreitol in 0.25 M Tris-HCl (pH 8.6) containing 10 mM EDTA and 6 M guanidine hydrochloride at 37 °C for 2 h, and reacted with 20 mM iodoacetamide for 20 min at room temperature in the dark. Excess reagent was removed by dialysis against distilled water and lyophilized. Reduced and carboxamidomethylated (CAM) proteins were directly applied to N-terminal sequencing on a protein sequencer (PPSQ-10, Shimadzu, Kyoto, Japan). Proteins having blocked N termini were further digested with pyroglutamate aminopeptidase according to the manufacturer's instructions. In some experiments, proteins were transferred electrophoretically to Trans-Blot polyvinylidene difluoride membrane (Bio-Rad) by the method of Lauriere (15). Polyvinylidene difluoride membrane was stained with 0.1% Coomassie Blue R-250. The protein bands were cut out, destained, and subjected to N-terminal sequencing.

¹ The abbreviations used are: DMS, dimethyl suberimide; BAEE, *N*-benzoyl-L-arginine ethyl ester; DB, *Dioscorea batatas*; DMA, dimethyl adipimide; GNA, *Galanthus nivalis* lectin; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

TABLE I
The yields of *D. batatas* tuber proteins

Purification step	Protein	Recovery	Activity
	g^a	%	$\mu g/ml^b$
Homogenate	2.3	100	
Phenyl-Toyopearl 650M	2.0	87	
HiTrap Q	1.2	53	
DB1	0.22		2.7
DB2	0.60		ND ^c
DB3	0.27		3.9
DB4	0.13		ND

^a Values were calculated per 1 kg of fresh tubers.

^b Minimum concentration required to agglutinate 2% rabbit erythrocytes.

^c ND, not detected.

CAM-DB3 subunits were digested with endoproteinase Lys-C (Wako) (substrate/enzyme, 100:1), endoproteinase Arg-C (Takara, Kyoto, Japan) (substrate/enzyme, 100:1), endoproteinase Asp-N (Takara) (substrate/enzyme, 250:1), or *Staphylococcus aureus* V8 protease (Wako) (substrate/enzyme, 50:1) according to the manufacturers' recommendations. Each digest was separated by reversed phase-high performance liquid chromatography on a TSKgel ODS 120T column (4.6×250 mm) using a linear gradient increase of acetonitrile in 0.1% trifluoroacetic acid.

The positions of the disulfide bonds in DB3 subunits were identified by analyzing the peptide fragments, which were derived from unmodified proteins upon cyanogen bromide cleavage in 70% formic acid and isolated by reversed phase-high performance liquid chromatography. The amino acid sequences of isolated peptide fragments were determined by the combined use of a protein sequencer, MALDI-TOF mass spectrometer, and an amino acid analyzer as described previously (16). Homologous sequences were searched by the FASTA program accessed by Genome Net.

cDNA Sequencing of DB3—Oligonucleotide primers (DB3F/DB3R) specific to each subunit were designed based on the amino acid sequence of DB3 to amplify cDNA fragments of the lectin by means of RT-PCR as follows (F and R indicate sense (forward) and antisense (reverse) primers, respectively): DB3F, 5'-TGGAARACNTGYGGNAA-YGGNATGGARCA-3'; DB3R, 5'-GCRTTTRTDTATNGCRTTYTCRTT-3'.

Total RNA was extracted using Concert Plant RNA Reagent (Invitrogen) according to the manufacturer's instructions. Poly(A)⁺ RNA was purified with a Micro-FastTrack mRNA Isolation kit (Invitrogen) and reverse transcribed with oligo(dT) primer using Access Quick RT-PCR System (Promega, Madison, WI). PCR was done with a combination of DB3F and DB3R primers and first strand cDNA as template on PCR Express™. DNA was denatured at 95 °C for 2 min, followed by three step cycles (35 cycles) as follows: 95 °C for 0.5 min, 45 °C for 0.5 min, 72 °C for 1 min, and further extended at 72 °C for 5 min. Amplified DNA and fragment (0.8 kbp) generated by PCR with DB3F/DB3R-specific primers was subcloned into the pCR-Blunt II TOPO vector (Invitrogen). To obtain the full sequence of cDNAs, the flanking regions of cDNA encoding DB3 were obtained by the 5'- and 3'-rapid amplification of cDNA ends methods using the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA). DNA was sequenced on an Applied Biosystems DNA sequencer (model 377) by cycle sequencing using T7, SP6, and M13 forward (–20) primers and the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences).

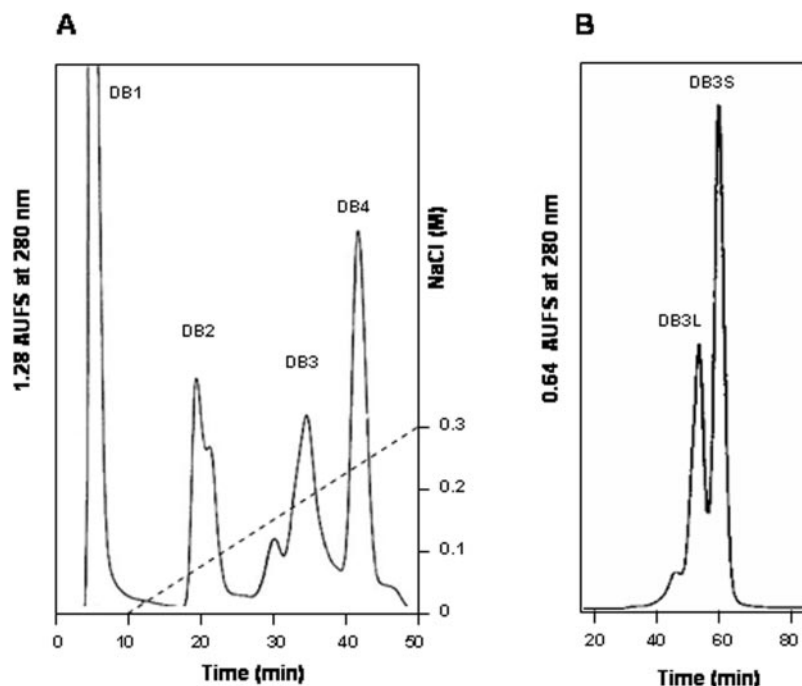
Hemagglutination and Inhibition Assays—Hemagglutination assays were carried out in 96-well microtiter plates in a final volume of 100 μ l containing 50 μ l of serially diluted lectin solution and 50 μ l of 2% fresh suspension of rabbit erythrocytes. Agglutination was assessed visually after 30 min at room temperature.

Carbohydrate-binding specificity was determined by inhibition of agglutination of rabbit erythrocytes. Stock solutions of the inhibitors (25 μ l) were diluted 2-fold in 96-well microtiter plates, followed by addition of 25 μ l of lectin solutions. After 30 min of incubation, the erythrocyte suspension (50 μ l) was added to the mixture and incubated for 30 min. The inhibitory activities were estimated by the minimum concentration of inhibitor needed to cause negative hemagglutination.

To test the dependence of hemagglutination on divalent cations, lectins were kept in 0.1 M EDTA for 15 min at room temperature and then dialyzed against 0.15 M NaCl overnight at 4 °C. The lectin solution was tested for hemagglutination activity in the presence of Ca²⁺, Mg²⁺, and Mn²⁺ ions in 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl.

Enzymatic Activity Measurements—Chitinase activity was deter-

FIG. 1. Purification of yam tuber proteins. A, proteins obtained by hydrophobic chromatography were fractionated on a HiTrap Q HP column (5 ml) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Elution of proteins was performed by linear NaCl gradient from 0 to 0.3 M at a flow rate of 1 ml/min. Peaks 1–4 were designated as DB1–DB4, respectively. B, DB3 subunits were separated on a HiLoad 16/60 Superdex 200 column (1.6 × 60 cm) equilibrated with 8 M urea in 50 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl at a flow rate of 1 ml/min. DB3L, 66-kDa subunit of DB3; DB3S, 31-kDa subunit of DB3.



mined according to Hou *et al.* (17) using 0.05% deacetylated glycol chitin (G-7753, Sigma) as a substrate. The increased reducing ends were determined by the method of Imoto and Yagishita (18). One unit of chitinase activity was defined as an amount of enzyme capable of releasing reducing ends corresponding to 1 μ g of GlcNAc from deacetyl glycol chitin in 30 min at 20 °C. GlcNAc (5–100 μ g) was used to plot the standard curve. The chitinase activity of tuber proteins was compared with that of lysozyme.

Carbonic anhydrase activity was measured by the hydrolysis of 4-nitrophenyl acetate resulting in an increase of absorbance at 348 nm (19). The activity of tuber proteins was compared with that of carbonic anhydrase from bovine erythrocytes.

Protease inhibitory activity was determined on bovine trypsin at 25 °C in 0.1 M Tris-HCl buffer (pH 8.2), containing 20 mM CaCl_2 with N^α -benzoyl-L-arginine ethyl ester as substrate (100 μ g/ml). After pre-incubation for 10 min, enzyme/inhibitor mixtures (20 μ l) were added to a solution containing substrate in 0.1 M Tris-HCl buffer (pH 8.2), containing 20 mM CaCl_2 to give a final volume of 1 ml. The substrate hydrolysis was monitored by measuring absorbance at 253 nm. The trypsin inhibitory activity of tuber proteins was compared with that of soybean trypsin inhibitor (Wako).

Amylase activity was assayed according to the method of Bernfeld (20) using potato amylose (Sigma) (5 mg/ml) as substrate in a 16 mM sodium acetate buffer (pH 4.8) for β -amylase or 20 mM Tris-HCl buffer (pH 6.9) containing 6.7 mM NaCl and 10 mM CaCl_2 for α -amylase. One unit of activity was defined as the amount of enzyme producing 1 mg of maltose in 3 min at 25 °C. The amylase activities of tuber proteins was compared with that of α -amylase from *Bacillus* spp. and β -amylase from soybean.

RESULTS

Purification and Subunit Structures of Tuber Proteins—Extraction of yam tubers followed by phenyl-Toyopearl 650M chromatography (result not shown) yielded 2.0 g of crude protein from 1 kg of fresh tubers (87% yield) (Table I). The protein fraction, subjected to HiTrap Q HP chromatography gave four major peaks, designated DB1, DB2, DB3, and DB4 (Fig. 1). The yield of the fractions was 0.22, 0.60, 0.27, and 0.13 g, respectively. Approximate ratios of the yields are 20:50:20:10. None of the fractions was glycosylated. DB1, which was not retarded on the latter column, gave two bands corresponding to 20 and 10 kDa on SDS-PAGE under nonreducing conditions, and a single band of 10 kDa under reducing conditions (Fig. 2). MALDI-TOF mass spectrometry revealed molecular masses of 11,784 for monomeric form and 23,428 Da for dimeric form (Fig. 3).

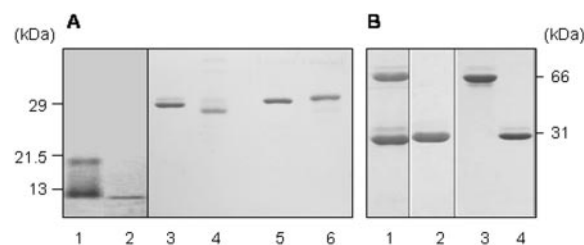


FIG. 2. SDS-PAGE of yam tuber proteins in 15% acrylamide gels. A, lanes 1 and 2 are nonreduced and reduced DB1, respectively. Lanes 3 and 4 are nonreduced DB2 and DB4, and lanes 5 and 6 are reduced DB2 and DB4, respectively. B, lanes 1 and 2 are nonreduced and reduced DB3, respectively. Lanes 3 and 4 are DB3 subunits (DB3L and DB3S, respectively) which were separated on HiLoad 16/60 Superdex 200 column using 8 M urea in 50 mM Tris-HCl (pH 8.0), 0.15 M NaCl. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa), and cytochrome *c* (13 kDa) were used as markers.

DB2 gave a single band of 31 kDa using nonreducing/reducing SDS-PAGE (Fig. 2). The molecular mass was estimated to be 27,788 Da by mass spectrometry, showing a multimeric form (Fig. 3). Gel filtration chromatography showed that DB2 existed as an aggregated form (result not shown).

DB3 yielded two bands of 31 and 66 kDa under nonreducing conditions, and a single band of 31 kDa on SDS-PAGE under reducing conditions (Fig. 2), indicating that the band of 66 kDa was composed of 31-kDa subunits cross-linked by disulfide bonds. DB3 migrated as a single band in native PAGE (data not shown) and eluted as a single peak at 120 kDa by gel filtration on HiLoad 16/60 Superdex 200 or TSKgel 3000G SW. DB3 dissociated into 66- (DB3L) and 31-kDa (DB3S) components by gel filtration in the presence of 8 M urea (Fig. 1B). These results indicated that DB3 was composed of one 66-kDa subunit and two 31-kDa subunits. The subunit structure of DB3 was confirmed by the combined use of chemical cross-linking of subunits and SDS-PAGE. DB3 cross-linked by either DMS or DMA yielded a band of 120 kDa (Fig. 4). The chemical cross-linking of DB3S gave a band of 66 kDa (Fig. 4), suggesting that the subunit formed a noncovalently bound dimer even after dissociating from DB3. Furthermore, DB3 showed a peak at m/z 111,291 as well as a peak corresponding to m/z 27,648 (Fig. 3). No sugars were de-

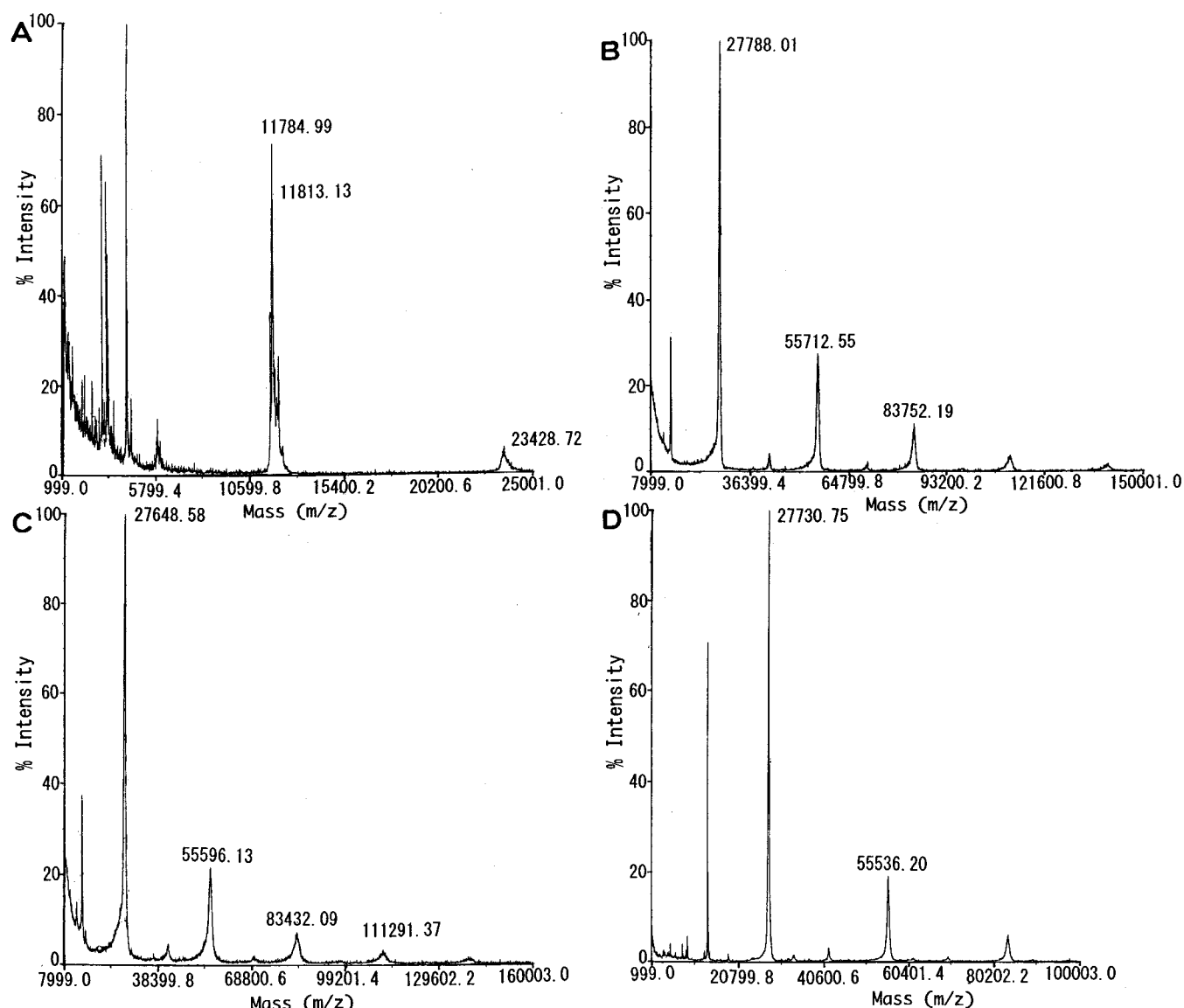


FIG. 3. MALDI-TOF mass spectral analysis of yam tuber proteins. A, DB1. B, DB2. C, DB3. D, DB4.

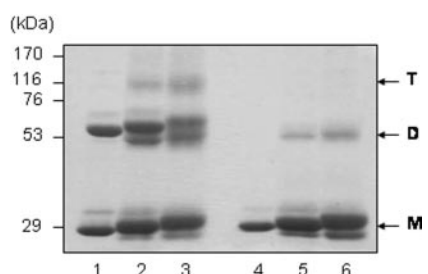


FIG. 4. Determination of molecular mass of DB3. DB3 subunits were cross-linked by either DMS or DMA and subjected to 12% SDS-PAGE. Lane 1, DB3. Lanes 2 and 3, DB3 subunits cross-linked with DMA and DMS, respectively. Lane 4, 31-kDa subunit (DB3S) of DB3. Lanes 5 and 6, DB3S cross-linked with DMA and DMS, respectively. α_2 -Macroglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76 kDa), glutamate dehydrogenase (53 kDa), and carbonic anhydrase (29 kDa) were used as markers. T, tetramer; D, dimer; M, monomer.

tected in DB3 either by Schiff staining or the 4-*N,N*-dimethyl-amino-4'-azobenzenesulfonyl-hydrazide method (21), indicating that DB3 was not a glycoprotein.

DB4 gave a single band of 28 kDa on SDS-PAGE under nonreduced conditions and 31 kDa under reduced conditions (Fig. 2). MALDI-TOF mass spectrometry showed the peak at

m/z 55,536 corresponding to a dimer together with the peak at *m/z* 27,730 corresponding to a monomer (Fig. 3).

CD Spectral Analysis—DB2 and DB3 showed characteristic far-UV CD spectra. Although both DB2 and DB3 showed high α -helix contents, the β -structure contents were quite different with 55% for DB2 and 9% for DB3. By increasing urea concentrations from 0 to 8 M, the spectral changes of DB3 were much larger than those of DB2 in the lower concentration range (Fig. 5).

N-terminal Sequence of DB1—N-terminal amino acid sequences of yam tuber proteins were analyzed by Edman degradation on a gas-phase protein sequencer. DB1 was electroblotted on a polyvinylidene difluoride membrane, and 10- and 20-kDa protein bands were subjected to a protein sequencer for N-terminal sequencing. The first 24 amino acid residues of both proteins identified are as follows: Asp-Phe-Ile-Leu-Tyr-Ser-Gly-Glu-Ser-Leu-Arg-Ser-Gly-Gln-Ala-Leu-Thr-Arg-Gly-Ser-Tyr-Thr-Phe-Ile, which was homologous to the N-terminal amino acid sequence of mannose-binding lectin, named GNA, from *Galanthus nivalis* (22) (58% sequence identity).

Amino Acid Sequence of DB3—The 66-kDa subunit (DB3L) and the 31-kDa subunit of DB3 (DB3S) were reduced, *S*-carboxamidomethylated, and subjected to N-terminal sequencing

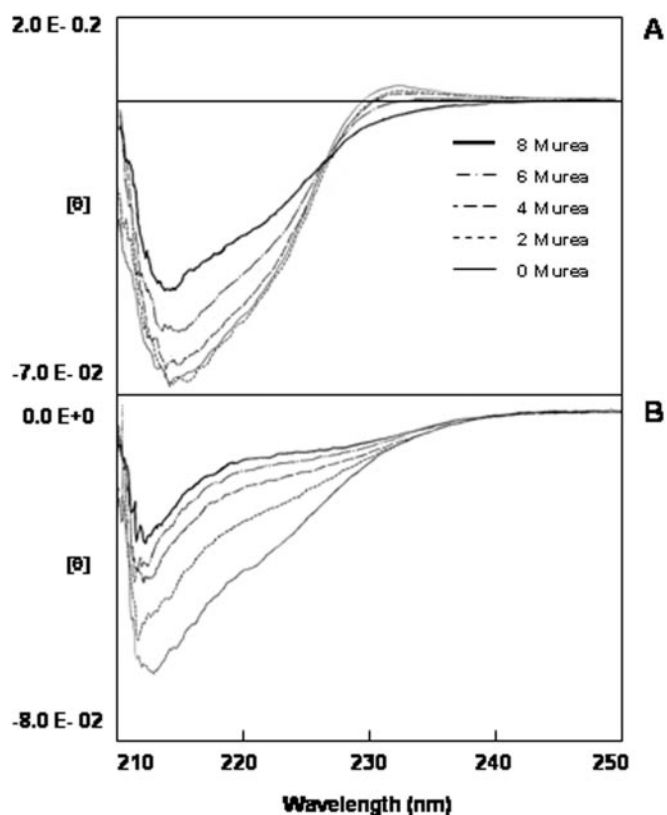


FIG. 5. Circular dichroism spectra of DB2 and DB3 in the far-UV region in the presence of various concentrations of urea. A, DB2. B, DB3.

(Fig. 6). The N-terminal sequence of DB3S was the same as that of DB2 (Fig. 6B). The total amino acid sequences of DB3L and DB3S were determined by both Edman degradation and cDNA sequencing as summarized in Fig. 6. The amino acid sequences of proteins were determined by analysis of peptides generated by digestion of CAM proteins with various proteases. DB3S was composed of 241 amino acid residues with a molecular mass calculated to be 27,599 Da, which is in good agreement with the value (27,611 Da) obtained from MALDI-TOF mass spectrometry. The nucleotide sequence was analyzed by using a method of rapid amplification of cDNA ends. The cDNA of DB3S included 962 nucleotides with an open reading frame of 804 nucleotides encoding for a mature protein of 246 amino acid residues and a signal sequence of 22 residues. The stop codon at nucleotide position 805 was followed by a polyadenylation signal AATAAA, starting at position 905 (Fig. 6B). It should be noted that the C-terminal amino acid sequence, Lys-Val-Cys-Ala-Ile, which was deduced from the cDNA sequence, could not be detected in any digest prepared with various proteases.

CAM-DB3L was composed of 242 amino acid residues with three half-cysteine residues. The molecular masses of DB3L before and after *S*-carboxamidomethylation were calculated to be 55,379 and 27,689 Da, respectively, which are in agreement with the values, 55,879 and 27,852 Da, respectively, obtained from MALDI-TOF mass spectrometry. The cDNA of DB3L included 964 nucleotides with an open reading frame of 816 nucleotides encoding for a mature protein of 247 amino acid residues and a signal sequence of 25 residues (Fig. 6A). The stop codon at nucleotide position 817 was followed by a polyadenylation signal AATAAA, starting at position 917. As in the case of DB3S, the C-terminal amino acid sequence, Asn-His-Asp-Ala-Ile, which was deduced from the cDNA sequence, was lacking when the protein fragments were sequenced.

Amino Acid Sequence of DB2—DB2 was subjected to amino

acid sequencing as described for DB3. The amino acid sequence of DB2 was identical with that of DB3S. N-terminal amino acid sequences of DB2 and DB3 were highly homologous to the amino acid sequences deduced from the dioscorin cDNAs of *Dioscorea cayenensis* (23, 24) and *Dioscorea alata*² (Fig. 7). DB2 and DB3S were classified to be dioscorin A (93% identity), and DB3L was dioscorin B (90% identity).

Localization of Disulfide Bonds of DB3—The amino acid sequences of DB3S and DB3L included two and three half-cysteine residues, respectively. Free cysteine residue could not be detected in either of the proteins. DB3L gave two fragments (C1 and C2) containing disulfide bonds upon cyanogen bromide cleavage. The Edman degradation of C2 yielded two amino acid sequences corresponding to Asp¹–Met³² and Gly¹⁸¹–Met²¹⁰. The result indicates that Cys²⁸ and Cys¹⁸⁸ formed an intra-chain disulfide bond. The amino acid composition and the molecular mass of C2 supported the localization, *m/z* 6,673 (calculated as 6,687). On the other hand, C1 yielded a single sequence corresponding to Glu³³–Met⁷². However, the molecular mass was measured to be *m/z* 9,080, which was double that of the calculated value (4,545) for the sequence, allowing us to locate one interchain disulfide bond at Cys⁴⁰–Cys⁴⁰.

N-terminal Amino Acid Sequence of DB4—The N-terminal sequence analysis of DB4 yielded no N-terminal amino acids, indicating that the N terminus of this protein was blocked. Therefore, the protein was treated with pyroglutamyl aminopeptidase and subjected to sequencing. The N-terminal sequence was identified as follows: Asn-Cys-Gln-Cys-Asp-Thr-Thr-Ile-Thr-Cys-Cys-Ser-Gln-His-Gly-Tyr-Cys-Gly-Asn-Ser-Tyr-Asp-Tyr-Cys-Gly-Pro-, revealing that DB4 was highly homologous to acidic endochitinase (EC 3.2.1.14) from *D. japonica* with a calculated molecular mass of 27,890 Da (8). Half of the amino acid sequence of DB4, which was determined with the peptide fragments derived from endoproteinase Lys-C digestion, showed 87% identity with acidic endochitinase (data not shown).

Hemagglutinating Activities and Carbohydrate Binding Specificity—DBs were examined for hemagglutinating activity using rabbit erythrocytes. DB1 and DB3 agglutinated rabbit erythrocytes at 2.7 and 3.9 μ g/ml, respectively. DB3 lost its agglutination activity after EDTA treatment. Agglutinating activity was fully recovered by the addition of 5 mM Ca²⁺ ions, whereas Mg²⁺ and Mn²⁺ had only a marginal effect on recovery of the agglutinating activity of DB3. Neither DB2 nor DB4 showed hemagglutinating activity.

The best inhibitor for DB1 was mannose- α 1,3-D-mannose- α -OMe (IC₅₀ 0.2 mM), followed by methyl α -D-mannopyranoside, whereas both fructose and GlcNAc were about 16 times less potent than mannose (Table II). Type I mucin inhibited hemagglutination at a concentration of 8 μ g/ml, whereas other glycoproteins were moderately inhibitory. DB1 was not stable against heat treatment because its activity of DB1 rapidly decreased to 40% by incubating at 50 °C for 30 min. DB1 was unstable below pH 7 and above pH 9 at room temperature (Fig. 8).

DB3 was exclusively inhibited by maltose and its derivatives (Table III). The best inhibitors for DB3 were maltopentaose and maltohexaose (IC₅₀ 0.4 mM), followed by maltose and isomaltose. Although starch showed some reactivity at 125 μ g/ml, no affinity was detected toward glucose. Most interesting, none of the glycoproteins tested was inhibitory. The activity of DB3 was stable at 50 °C up to 40 min but decreased to 50% after 80 min. DB3 kept its full activity after a 3-h incubation at pH 3–9 at room temperature.

² W. C. Hou, H. J. Chen, and Y. H. Lin, DDBJ/GenBank™/EBI Data Bank accession number Q9M501.

A

B

1	ATAGGTTTCATCCACCCCTTTTGCATCTCTTCTCCTCTCGCTCCCTCCTCTTCTCTTGCTTA	61
-25	M S S S T L F H L F L L S S L L F S C L	-6
61	TCAAATGCAAGCGTAGGAGTACTTTAGCTACATTGAAGGAAGTCTAATGGTCCT	121
-5	S N A R P D E D D F S Y I E G S P N G P	15
121	GAAAACCTGGGGAATCTTAATCGGAGTGGAAAGACTTGTGGCAATTGGCATGGAGCAGTCA	181
16	E N W G N L N P E W K T C G N G M E Q S	35
181	CCCATTAAATTTGTGTGATGADAGVTAITTCAGACTCCAGCTTTGGGGAGKCTGAGAACA	241
36	P I N L C D D T A G I T Q T P A L G K L R T	51
241	AGTTATCAGGCTGCTCGTGCAACACTGAAGAACATGGACATGATATAATGGTGAACTTT	301
56	S Y Q A A R A T L K N N G H D I M V N F	75
301	AAAAGTGATGCTGGTTTCACAATTTCATCAATCAAGTTAGGTACCAACTCAAGCGAATTCAT	361
76	K S D A G S Q F I N Q V Q Y Q L K R I H	95
361	TTTCACTGCCCATCAGAACATGTACTAAATGGTGAAAGGTTTGACCTTGAGGTCCAGATG	421
96	F H S P S E H V L N G E R F D L E V Q M	115
421	GTCCATGAGAGSCCAAGATCAAGAGGAGAGCACTAAGTCTATTCTGTTCAGATTGGACGT	481
116	V H E S Q D Q R R A V T A I L F R F G R	135
481	TCGACCATTCTCTCAGACCTTTGAAGACTTTATCAGCCAGATAAGCAAGAGTGAGAAG	541
136	S D P F L S D L E D F I S Q I S K S E K	155
541	AATGAAGTAGATGCTGGAGTTGTGGATCCAAGGCAATTTGTACAGTTTGATGACCCGTGA	601
156	N E V D A G V V D P R Q L L Q F D D P A	175
601	TATTATAGATACATGGGCTCATTCACAGCTCCACCTTGCCTGAGATATTACATGGACC	661
176	Y Y R Y M G S F T A P P C T E D I T W T	195
661	GTTATTAAAGACTTTGGAAGCTGTTTACCACCAAGGCAAGTACTGATGTTGAAGCAAGCAGTG	721
196	V I K K L G T V S P R Q V L M L K Q A V	215
721	AATGAAAAATGCTATAAACAATGCAAGGCCACTTCAACCACTGAAATTTTCGACAAATTTTT	781
216	N E N A I N N A R P L Q P L K F R T I F	235
781	TTCTATCCGCGTCAGAAATCGAATCATGATGCCATAATATAATCAAGCTCGTAGTAGT	841
236	F Y P R Q K S N H D A I *	
841	TATGAGTGTAAATGTGTATGGTTAAATAAAAAAGGTGGAGTTCTCCNCTCTGCATGTAC	901
901	CCGGCCCCATCATATAATAATAATGGGAAATTTAAAGT poly(A)	

1 ATGAGTTCATCCACCCCTTCCTCATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGCGTT 61
-22 M S S S T L L H L L L L S S L L F S C L -3

61 GCAAAATGTAGAGGATGAGTTTAGCTACATTGAAGAAATCTTAATGGTCCTGAAAAC TGG 121
-2 A N V E D E F S Y I E G N P N G P E N W 18

121 GGAATCTTAAACCGGAGTGGGAGACTGTGGCAAAGGCATGGAGCAGTCAACCATTAG 181
19 G N L K P E W E T C G K G M E Q S P I Q 38

181 TTGCGTGTACAAGCAGTGATATTTCGATCAAACTTTGGGGAGGTTGAGAAGAAATACAGA 241
239 L R D N R V I F D Q T L G R L R N Y R 248

241 GCCGTGTAGCAAGATTAAAGCAAGCTGGACATGATGTATTGGTGGAAATTTAAGGGTAAT 301
59 A V D A R L R N S G H D V L V E F K G N 78

301 GCTGGTCTACTATCAATCAATCGAGTTGCATACCAACTCAAGCGAATTCATTTTCACTCC 361
79 A G S L S I N R V A Y Q L K R I H F H S 98

361 CTTTCAGAGCATGAATGAATGGCGAAAGGTTTGACCTTGAGGCACAGCTGGTTCATGAG 421
99 P S E H E M N G E R F D L E A Q L V H E 118

421 AGCCAAGATCAAAGAGAGCAGTGGTTTCTATTCTTTTCATATTTGGACGTGCTGACCCA 481
119 S Q D Q K R A V V V S I L F I F G R A D P 138

481 TTCCTCTCAGATCTTGAAGACTTTATCAAGCAGTTTACAGTAGCCAGAGAATGAATA 541
139 F L S D L E D F I K Q F S S S Q K N E I 158

541 AATGCAGGAGTGTGGATCCCAATCAATTACAGATTGATGACCTGCATATTATAGATAC 601
159 N A G V V D P N Q L Q I D D S A Y Y R Y 178

601 ATGGGCTCATTCACAGCTCCACCTTGCTCACTGAAGGTATTTTCATGGACCGTCATGAGGAAG 661
179 M G S F T A P P C T E G I S W T V M R K 198

661 GTTGCACCTGTTTCACCAAGACAACTACTGCTGTTGAAGCAGGCAGTGAATGAAAATGCT 721
199 V A T V S P R Q V L L L K Q A V N E N A 218

721 ATAAACAATGAAGACCACTTCAACCAACCAATTTCCGCTCCGTTTTTTTACTTTGAACAG 781
219 I N N A R P L Q P T N F R S V F Y F E Q 238

781 CTGAAATCGAAGGTTTGTGCCATATAATAATAAGCTCATAATATGATGAGTGTAAATA 841
239 L K S K V C A I *
.....

841 TGGTGATGGTTAAATAAAGAGGGTGGAGTGTCCACTCTTCATGTACTATCATCCCATG 901

901 TAATAATAAATAATGTGCTTTTTCAGT poly(A)

FIG. 6. **Nucleotide and amino acid sequences of DB3 subunits.** A, 66-kDa subunit of DB3 (DB3L). B, 31-kDa subunit of DB3 (DB3S). Nucleotides and amino acid residues are numbered on the sides. *Dotted underlines* represent amino acid sequences determined by analysis of the peptide fragments derived from various enzymatic digestions.

Enzymatic Activity Measurements—The enzymatic activities such as carbonic anhydrase, trypsin inhibitor, chitinase, and amylase activities of yam tuber proteins were examined as shown in Table IV. Only chitinase activity was detected with DB4, and the activity was comparable with that of lysozyme. Other DBs showed no enzymatic activity tested in this study.

DISCUSSION

We isolated and characterized four major proteins designated DB1, DB2, DB3, and DB4 from the *D. batatas* tubers. DB1 was a mannose-binding lectin and accounted for 20% of the total protein. DB1 gave two bands on SDS-PAGE under nonreducing conditions revealing the same N-terminal sequence and converted to a single band of 10 kDa under reducing conditions. Structural determination of DB1 revealed that it consisted of two isoforms.³

Most of the currently known plant lectins can be classified into seven families of structurally and evolutionary related proteins (26). These seven families are the legume lectins, chitin-binding lectins, type 2 ribosome-inactivating proteins, monocot mannose-binding lectins, amaranthin group of lectins, Cucurbitaceae phloem lectins and jacalin-related lectins. Undoubtedly, DB1 can be classified into the monocot mannose-binding lectin family. The occurrence of the family is confined to a subgroup of the monocotyledonous plants comprising the families Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Iri-

TABLE II
Inhibition of hemagglutination activity of DB1 by saccharides

Sugar/glycoprotein	IC ₅₀	
	<i>mM^a</i>	<i>μg/ml</i>
Mannose	3.1	
Me-α-Man	1.5	
α1,2-Mannobiose	6.2	
Man-α1,3-D-Man-α-OMe	0.2	
Fructose	50	
GlcNAc	50	
Sucrose	50	
Mucin (type I)		8
Asialomucin		16
Fetuin		31
Asialofetuin		31
Others ^b	>200	

^a Minimum concentration required to give a 50% inhibition of the agglutination of rabbit erythrocytes.

The inhibitors used are as follows: D-glucose, Me- α -Glc, D-glucosamine, maltose, maltopentaose, maltohexaose, maltoheptaose, melibiose, trehalose, cellobiose, D-galactose, D-*N*-acetylgalactosamine, D-xylose, L-fucose, lactose, L-arabinose, L-rhamnose, muramic acid, chitin, chitosan, chitotriose, *N*-acetyl-chitotriose, *N*-acetyl-chitobiose, starch, and pectin.

daceae, and Orchidaceae. The GNA is the first lectin that was isolated in this family (27). The three-dimensional structure of GNA has been resolved, and the structural basis for the carbohydrate recognition has been determined (28). GNA interferes with the growth and development of insects when added to artificial diets (29). Thus, mannose-binding lectins are consid-

³ M. Gaidamashvili, Y. Ohizumi, S. Iijima, T. Takayama, T. Ogawa, and K. Muramoto, unpublished results.

FIG. 7. Comparison of amino acid sequences of *Dioscorea* tuber proteins and homologous proteins. Amino acid sequences were aligned to maximize similarity. The identical amino acids are boxed. DB3L, 66-kDa subunit of DB3 from *D. batata*; DioB, dioscorin B from *D. cayenensis* (23); DB3S, 31-kDa subunit of DB3 from *D. batata*; Stpr, dioscorin A from *D. alata* (25); DioA, dioscorin A from *D. alata* (25); Carb, putative carbonic anhydrase from *A. thaliana* (X. Lin, DDBJ/GenBankTM/EBI Data Bank accession number Q9SL34).

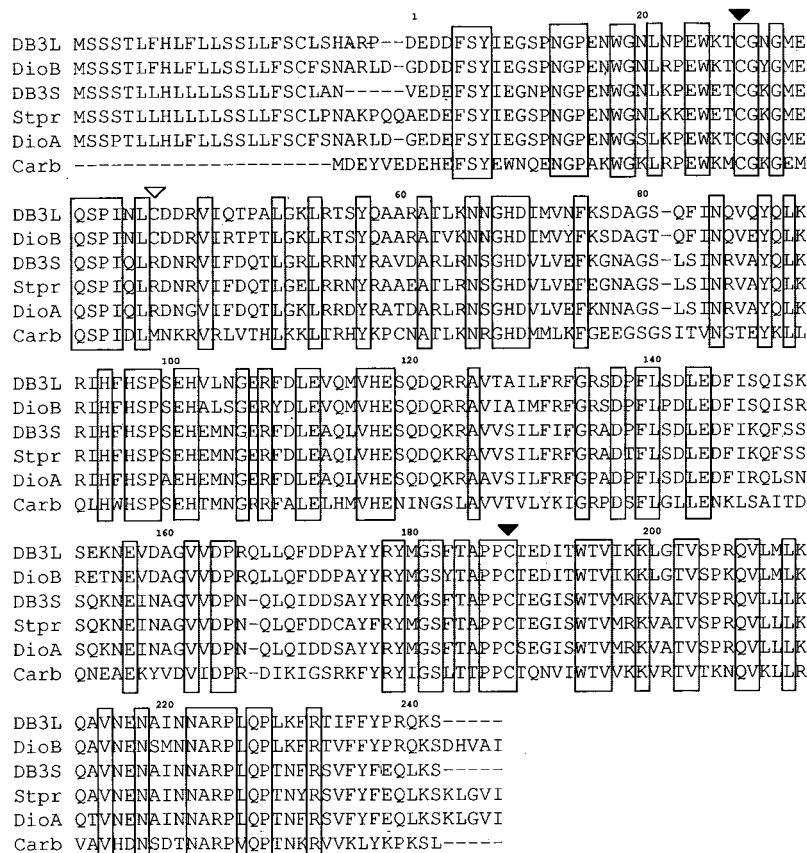


FIG. 8. Effect of pH and temperature on the hemagglutinating activity of DB1 and DB3. A, DB1 and DB3 were incubated for 3 h at various pH values. After adjusting the pH to 7.0, their hemagglutinating activities were measured. Activity was expressed as relative percentage. Buffers used are as follows: 0.1 M HCl-KCl (pH 1.0–2.0); 0.1 M sodium acetate (pH 3.0–6.0); 0.1 M Tris-HCl (pH 7.0–9.0); 0.1 M sodium borate (pH 10.0); 0.1 M NaHCO₃-NaOH (pH 11.0); and 0.1 M NaOH-KCl (pH 12.0). B, DB1 and DB3 were incubated at 50 °C for various periods. ▲, DB1; △, DB3.

TABLE III
Inhibition of hemagglutination activity of DB3 by saccharides

Sugar/glycoprotein	IC ₅₀	
	mm ^a	μg/ml
Maltose	1.5	
Maltotriose	0.8	
Maltopentaose	0.4	
Maltohexaose	0.4	
Isomaltose	25	
Amylose		2.0
Starch		125
Others ^b	>200	

^a Concentration required to give a 50% inhibition of the agglutination of rabbit erythrocytes.

^b The inhibitors used are as follows: D-mannose, Me-α-Man, α1,2-mannobiose, Man-α1,3-D-Man-α-Ome, fructose, D-glucose, N-acetyl-D-glucosamine, Me-α-Glc, D-glucosamine, sucrose, melibiose, trehalose, cellobiose, D-galactose, D-N-acetyl-galactosamine, D-xylose, L-fucose, lactose, L-arabinose, L-rhamnose, muramic acid, chitin, chitosan, chitotriose, N-acetyl-chitotriose, N-acetyl-chitobiose, and pectin.

TABLE IV
Enzymatic activities of *D. batatas* tuber proteins

Proteins	Carbonic anhydrase	Trypsin inhibitor	Chitinase ^a	Amylase
DB1	NT ^b	NT	NT	ND ^b
DB2	ND	ND	NT	ND
DB3	ND	ND	NT	ND
DB4	NT	NT	2.0	ND

^a Chitinase activity is defined as the amount of enzyme (μg) producing 1 μg of GlcNAc from deacetyl glycol chitin in 30 min.

^b NT, not tested; ND, no activity detected.

is rather surprising that the yam tuber storage proteins contain GNA-like protein as a major component. DB1 exhibits the same enhanced specificity toward the α(1–3) linkage as does GNA.

DB2 accounting for 50% of the total protein was an aggregate of a single protein of 31 kDa, with no interchain disulfide bonds. DB2 was classified as a storage protein, commonly called the dioscorins (2, 23). Dioscorins from various yam species have been characterized previously (1) and usually consist of a number of isoforms of 31 kDa and are deposited in the vacuoles of tuber cells (24). The storage proteins from *D. cayenensis* consist of the 31-kDa proteins, which is a mixture of

ered to be a promising plant resource for generating genetically modified plants. In fact, transgenic plants expressing the GNA gene exhibit increased resistance against sucking insects (30). It

two classes of dioscorins, referred as a class A and B (24).

DB3 was identified as a maltose-binding lectin, having an apparent molecular mass of 120 kDa, and was composed of one 66-kDa subunit (DB3L) and two 31-kDa subunits (DB3S). Both subunits are homologous to dioscorins; DB3L is classified to be class B and DB3S and DB2 to be class A. This is the first report to describe the isolation of lectins from the monocotyledonous family of Dioscoreaceae and the lectin activity for a dioscorin-like protein. Although a number of plant lectins have been characterized, to our knowledge, no lectin has a binding specificity exclusively to maltose. DB3L itself expressed the hemagglutinating activity, and no activity was observed with purified DB3S or DB2. Nevertheless, the latter two showed moderate binding affinity to an immobilized maltose column (data not shown). At present, we do not know how DB3L has acquired its hemagglutinating activity.

In contrast to DB1, DB3 cannot be classified into any of the known lectin families in terms of both structure and sugar specificity (31). The jacalin family consists of the galactose-specific and mannose-specific subgroups. A jacalin-related lectin, which has been isolated from *Calystegia sepium* (hedge bindweed), is inhibited not only by maltose but also by mannose, whereas DB3, which is inhibited by maltose but not by mannose or glucose at all, is very unique among plant lectins.

A significant degree of sequence similarity was found between dioscorins and α -carbonic anhydrase from animals despite the alteration of active sites (32). Hou *et al.* (33, 34) claimed that dioscorins from *D. batatas* showed both carbonic anhydrase and trypsin inhibitory activities by activity staining on SDS-PAGE. However, it should be mentioned that the activities could be detected even after 2-mercaptoethanol treatment and the carbonic anhydrase activity was zinc-independent. Furthermore, dioscorin showed only a weak trypsin inhibitor activity, with 1.9 μ g of trypsin inhibited per 100 μ g of the proteins. They also reported dehydroascorbate reductase (35) and antioxidative (7) activity for the dioscorins. The dioscorins showed two bands (28 and 82 kDa) in nonreducing SDS-PAGE and only one band (32 kDa) in reducing ones. The first 21 amino acids in the N-terminal region of 28-kDa dioscorin are homologous to those of DB2 except at positions 7, 10, and 11. We could not detect carbonic anhydrase or trypsin inhibitory activity associated with DB2 and DB3 in the present study. Although we have no explanation for this discrepancy, the hemagglutinating activity seems to be predominant for DB3.

The biological role of the lectin activity found with DB3 remains unanswered. It has been proposed that lectins found in the legumin families might be engaged in an ordered deposition of storage proteins in the protein bodies during development (36). The storage proteins are intracellularly located within vacuoles as protein aggregates and also in the cytoplasm in *D. rotundata* (2). It may be possible that the agglutinating properties of DB3 participate in the interactions between the protein bodies and the starch grains in the tubers. With no sequence homology between DB3 and amylase protein families, the exclusive specificity toward maltose and its high derivatives raised the possibility for speculation of amylase-like activity in DB3. However, neither α - nor β -amylase activities were found associated with purified DBs.

We detected chitinase activity with DB4, which accounted for 10% of total storage protein content. DB4 showed about 90%

homology to acidic chitinase from *D. japonica* (8). The high pathogen resistance in tubers may occur as a result of the contribution of the chitinase to a considerable extent.

In Japan, the yam tubers of *D. batatas* are stored for a year after harvesting, despite its high water content, and are generally consumed without cooking. The good preservability may be correlated with the presence of defense-related proteins in the yam. Moreover, the presence of lectins in foodstuffs is important from a nutritional point of view, because they may affect the entire digestive tract and its bacterial population, body metabolism, and health (11). The yam lectins may be stable enough at their pH range passing through the digestive tract. This is not well known although the yam tubers of the Dioscoreaceae are known as herbal medicines in East Asia (25, 37).

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