Physicochemical, enzymatic and molecular characterisation of the storage protein of aerial tuber, *Dioscorea bulbifera* Linn.

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**Abstract**

**Background:** The storage protein of the aerial tuber of *Dioscorea bulbifera* was purified and its physicochemical, enzymatic and molecular properties determined with a view to comparing its functionality and genetic relatedness with other storage proteins.

**Results:** The purified protein had molecular weight of 21 kDa. The protein showed carbonic anhydrase, trypsin inhibitory, dehydroascorbate reductase and monodehydroascorbate reductase activities. Amplifications with polymerase chain reactions resulted in the detection of two genes encoding the storage protein. The deduced amino acid sequence of the shorter and larger genes had homologies with the storage proteins of members of the *Dioscorea* family.

**Conclusion:** The study concluded that the storage protein of the aerial tuber of *D. bulbifera* had similar properties with those of other *Dioscorea* species and may be suitable for development as functional food.

**Keywords:** Aerial potato, Aerial yam, Bulb, Dioscorin, Storage protein, Protein purification

**Introduction**

Yam, a dioecious plant belonging to the *Dioscorea* genus, is an important staple crop in many areas of the tropics and sub-tropics [1]. There are 8 genera and 880 species of yam plant. They produce edible tubers, bulbils, corms or rhizomes [2] that are basically made up of carbohydrates and are important sources of proteins and micronutrients [3]. Yam tubers are widely utilized as food due to their compositions [4]. Yam tubers also contain functional components such as mucin, dioscin, diosgenin, allantoin, choline and polyphenol oxidases [5] and, in addition, minerals and vitamins such as calcium, zinc, phosphorus, copper, iron, sodium, potassium, β-carotene, thiamine, riboflavin and niacin [6]. About 80% of the proteins in yam are storage proteins [7], which are usually affected by factors such as cultural practices, climate, soil fertility, maturity at harvest and length of storage time [8].

Plants accumulate storage substances such as starch, lipids and proteins in certain phases of development. The major role of storage proteins is to act as stores of nitrogen, sulphur and carbon, which are accumulated in both vegetative and reproductive tissues. Thus, they serve as a reservoir for later stages of plant development [9, 10]. Storage proteins provide nutrients to support the growth of new plants as seedlings (from seeds) or shoots (from tubers). They are localized in specific organs, cell types and subcellular compartments in discrete deposits (protein bodies) where they facilitate high-level accumulation without any adverse effects on other cellular functions. They also allow plants to survive periods of adverse conditions between growing season [11]. Plant storage proteins are grouped into two classes: seed storage proteins that accumulate to high levels in seeds during the late stages of seed development, and vegetative
storage proteins, which accumulate in vegetative tissues such as leaves, stems and tubers [12].

Dioscorins, yam storage proteins, isolated from different Dioscorea species have been shown to have various biological activities, which include enzymatic (α-carbonic anhydrase, trypsin inhibitory), antioxidant, antihypertensive and immunomodulatory activities. They are thus worth developing as healthy or functional foods. Dioscorea bulbifera belongs to the family Dioscoreaceae assigned to the order Dioscoreales. It is commonly known as air potato, potato yam, air yam, or bulbil-bearing yam. It is native to Africa and Asia but widely grown and consumed in the tropics [13], the Caribbean Islands, South East Asia, South Pacific and West Indies. The uncultivated form is bitter, not edible and may be poisonous.

Preparation of crude extracts
The crude extract of the aerial tubers of Dioscorea bulbifera was prepared at different pH and varying temperature, in order to ascertain conditions at which most of the proteins in the aerial tuber are solubilized.

Dioscorea bulbifera aerial tubers were peeled, sliced and homogenized with 4 volumes (w/v) of buffers at different pH: 0.5 M citrate/phosphate buffer (pH 4–6), 0.5 M Tris-HCl buffer (pH 7 and pH 8.3) and glycine-NaOH buffer (pH 9 and 10). The mixtures were stirred for 4 h and centrifuged at 13,500 rpm for 30 min at 4 °C. The supernatants collected were stored as crude extracts.

Also, approximately 100 g portions of yam slices were boiled in 1 L of water at 25, 30, 40, 50, 60, 70, 80, 90 and 100 °C for 10 min. The treated aerial tubers were drained, cooled, weighed and homogenized with 50 mM Tris-HCl (pH 8.3) at 1:4 (w/v). The mixture was stirred for 4 h and centrifuged at 13,500 rpm for 30 min at 4 °C. The supernatants were collected as crude extracts.

Protein content of extracts was determined by Lowry method using 1 mg/mL bovine serum albumin (BSA) as standard.

Purification of protein
The crude extract obtained at pH 8.3 and 25 °C (which had the highest protein concentration) was used for further studies. Purification of the storage protein of Dioscorea bulbifera aerial tuber was carried out following the method of Hou et al. [18] with a little modification.

The crude extract of the aerial tuber of Dioscorea bulbifera was subjected to 70% ammonium sulphate precipitation, stirred and kept overnight at 4 °C. The mixture was centrifuged at 13,500 rpm for 30 min and the precipitate recovered. The precipitate was dissolved in 10 volumes of 50 mM Tris-HCl buffer, pH 8.3 and dialyzed exhaustively against distilled water.

Ion-exchange chromatography on DEAE Sephadex A-25
The dialyzed protein solution (7.5 mg/mL; 2.5 mL) was loaded on DEAE Sephadex (A-25) ion exchange column (1.5 × 20 cm) previously equilibrated with 50 mM Tris-HCl buffer, pH 8.3. Unadsorbed proteins were eluted with 50 mM Tris-HCl buffer, pH 8.3, and adsorbed proteins were eluted stepwise with 150 mM NaCl in 50 mM Tris-HCl buffer, pH 8.3, at a flow rate of 15 mL/h. Fractions of 5 mL each were collected, and elution was monitored at 280 nm. The adsorbed protein fractions, which correspond to the major storage protein of the aerial tuber of D. bulbifera, were pooled and concentrated.

Gel filtration on Sephadex G-75
Adsorbed protein sample (1.5 mg/mL; 5 mL) obtained from ion-exchange chromatography was further purified by gel filtration on Sephadex G-75 column (1.5 × 40 cm)
previously equilibrated with 50 mM Tris-HCl buffer, pH 8.3. The column was eluted with 100 mM Tris-HCl buffer (pH 7.9) containing 100 mM NaCl at a flow rate of 27 mL/h. Fractions of 3.6 mL each were collected. The purified protein was collected, concentrated and stored at –20 °C for further use. Protein concentration was determined after each purification step.

Non-SDS polyacrylamide gel electrophoresis
The protein samples were subjected to polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulphate (SDS) according to the modified method of Shiu et al. [19] to monitor the purity of the protein obtained after each purification step. Electrophoresis was performed on a 10% discontinuous gel system under non-denaturing conditions and stained with Coomassie Brilliant Blue.

**Determination of molecular weight**

The native molecular weight of the protein was determined by gel filtration on a Bio gel P-200 column (1.5 x 63 cm) using the following protein markers: lysozyme (Mr 14,000), α-chymotrypsinogen A (Mr 25,000), egg ovalbumin (Mr 45,000) and bovine serum albumin (Mr 66,000). Each protein (5 mL) was applied on the column and run separately using 10 mM phosphate buffer pH 7.0 as eluant at a flow rate of 10 mL/h. Fractions of 5 mL were collected, and the elution was monitored at 280 nm. The void volume (V_v) of the column was determined using Blue dextran (elution monitored at 620 nm).

The purified storage protein was subjected to SDS-polyacrylamide gel electrophoresis for subunit molecular weight determination following the modified method of Shiu et al. [19] using the following protein markers: ovalbumin (Mr 45,000), carbonic anhydrase (Mr 29,000), trypsinogen (Mr 24,000), trypsin inhibitor (Mr 20,000) and α-lactalbumin (Mr 14,200).

**Detection of protein-bound carbohydrate**

The presence of covalently-bound carbohydrate in the storage protein was investigated by staining the gels with periodic acid-Schiff’s reagent (PAS) after electrophoresis, as described in the Pharmacia Manual of Laboratory Techniques, revised edition. The protein sample was subjected to electrophoresis under non-denaturing conditions using phosphate-buffered system. After electrophoresis, the gel was fixed in 7.5% acetic acid at room temperature for 1 h. The fixed gel was transferred into a beaker containing 0.2% aqueous periodic acid and kept at 4 °C for 45 min. Afterwards, the gel was removed and transferred into a beaker containing Schiff’s reagent, kept at 4 °C for 45 min. The gel was destained in 10% acetic acid. Glycoprotein band (if present) will stain purplish red.

**Amino acid composition of the protein**

The storage protein was subjected to amino acid content analysis using methods described by Ekeanyanwu [20]. The sample was hydrolysed, evaporated in a rotary evaporator and loaded into the Technicon Sequential Multi-Sample Amino Acid Analyzer (TSM).

**Enzymatic activities of storage protein of Dioscorea bulbifera**

**Determination of carbonic anhydrase activity**

Carbonic anhydrase activity of the protein was measured by hydrolysis of 4-nitrophenyl acetate resulting in an increase of absorbance at 348 nm [21]. The activity of the tuber storage protein was compared with that of carbonic anhydrase from bovine erythrocytes. The reaction mixture contained 0.3 mL of freshly prepared 3 mM 4-nitrophenyl acetate in aqueous 3% acetone and 0.7 mL of 15 mM Tris sulphate buffer, pH 7.6. Exactly 10 μL purified protein solution (1 mg/mL) was added, and the catalyzed reaction was monitored by measuring the increase in absorbance at 348 nm for 5 min.

**Determination of dehydroascorbate reductase activity**

Dehydroascorbate (DHA) reductase activity of the protein was carried out according to the method of Hou et al. [18]. In this reaction, 10 μg of DHA was dissolved in 5 mL of 100 mM phosphate buffer of different pH values (pH 6.0, 6.5 and 7.0). The reaction was carried out at 30 °C; 100 μL purified protein solution (1 mg/mL) was added to 0.9 mL DHA solution with or without 4 mM glutathione. Increase in absorbance at 265 nm was recorded for 5 min. Non-enzymatic reduction of DHA in phosphate buffer was measured in a separate cuvette.

**Determination of monodehydroascorbate reductase activity**

Monodehydroascorbate (MDA) reductase activity of the protein was assayed according to the method described by Hou et al. [18] by monitoring the decrease in absorbance at 340 nm due to NADH oxidation. MDA free radicals were generated by ascorbate oxidase in the assay system. The reaction mixture contained 50 mM phosphate buffer (pH 6.0, 6.5 and 7.0); 0.33 mM NADH; 3 mM ascorbate, ascorbate oxidase (0.9 U); and 200 μL purified protein solution (200 μg protein) in a final volume of 1 mL. Distilled water was used to replace protein solution in blank solutions. One unit of MDA reductase is defined as the amount of protein required to oxidize 1 μmol of NADH per min.

**Determination of trypsin inhibitory activity**

Trypsin inhibitory activity of the protein was determined according to the method of Xue et al. [22] by monitoring the inhibition of trypsin-catalyzed hydrolysis of N-benzyol-L-arginine-4-nitroanilide (substrate) in 0.1 M Tris-
HCl buffer (pH 8.2). Different concentrations of the protein were pre-incubated with 20 μM trypsin at room temperature for 15 min. The substrate (100 μg/mL) was added to give a final volume of 1 mL for an additional 20 min. The absorbance at 405 nm was measured. The inhibitory activity is calculated as the percentage decrease in substrate hydrolysis rate, which is directly proportional to increase in absorbance at 405 nm. The result was expressed as micrograms of trypsin inhibited.

Molecular characterization of the storage protein of Dioscorea bulbifera

Genomic DNA extraction

The aerial tuber was peeled, cut into bits and ground into fine powder with a mortar and pestle under liquid nitrogen. Genomic DNA was extracted using QIAGEN DNeasy Plant Mini Kit. DNA concentrations were determined with a Nanodrop spectrophotometer (Beckman Coulter) and adjusted to 25 ng/μL for PCR amplification.

Primer design for polymerase chain reaction

Sequences of some Dioscorin genes from various Dioscorea sp. were obtained from NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). These sequences were inserted into the input window of the web-based polyacrylamide chain reaction (PCR) primer designing program, Primer3 (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). The primer minimum and maximum sizes were set to 100 and 900 nucleotides, respectively. The DNA was subjected to PCR amplifications using the designed Dioscorin-specific primers (5’-CTCCTCTCCTCCCTCCTTT-3’ (forward primer) and 5’-GGGGGTACAATGGAGAAGTG-3’ (reverse primer)) and 4 μL of Reaction Dye Terminator Premix (Qiagen) with standard sequencing conditions. Amplification products were stored at 4 °C before use. One microlitre of 125 mM EDTA, 1 μL 3 M sodium acetate (pH 4.8), 25 μL 100% ethanol (−20 °C) and 50 μL 70% ethanol (−20 °C) were added to the amplification products, mixed and centrifuged for 10 min at 10,000 rpm at 4 °C. DNA pellet was dried at room temperature and re-suspended in 5 μL sterile deionized distilled water. One microlitre of the re-suspended DNA was added to 9 μL Hi formamide, mixed and denatured for 3 min at 94 °C. It was placed inside ABI PRISM 3130 X1 genetic analyser, which carried out the automated sequencing analysis using a standard sequencing module with Performance Optimized Polymer and 50-cm array.

Sequence analysis

The nucleotide sequences of the purified dioscorin genes were subsequently translated to protein sequence using bioinformatic resource tool from CLC Genomics Workbench software (CLC Bio Denmark). The nucleotide and translated protein sequences were further subjected to computer-based homology search with NCBI BLAST program. Phylogenetic analysis was carried out to compare the relationship of the major storage protein of D. bulbifera with the storage proteins of other Dioscorea spp.

Results

Crude extracts

The protein concentration of crude extracts of the aerial tuber of Dioscorea bulbifera extracted at different temperature and varying pH is as shown in Fig. 1. The result showed that maximum protein concentration was obtained at pH 8.3 and at room temperature, 25 °C.
Purification of storage protein

The crude extract of the aerial tuber of *Dioscorea bulbifera* (379.60 mg) when subjected to 70% ammonium sulphate precipitation gave 130.80 mg protein which corresponds to 34.46% yield. The elution profile of the ion-exchange chromatography on DEAE-Sephadex A-25 of the dialyzed protein sample is as presented in Fig. 2a. Two protein peaks were obtained from the DEAE-Sephadex A-25 column, one unadsorbed peak and the adsorbed protein peak which was eluted with 150 mM NaCl. The adsorbed peak which was pooled and further purified by gel filtration on Sephadex G-75 column is as shown in Fig. 2b. At the end of purification, the amount of protein recovered was 13.20 mg, corresponding to 3.48% of the starting material.

Molecular weight of *D. bulbifera* storage protein

The molecular weight of the native storage protein of the aerial tuber of *D. bulbifera* as determined by gel filtration on Bio-gel P-100 was 22,000 Da. The subunit molecular weight, which was determined by SDS-PAGE under denaturing conditions, was estimated to be 21,095 Da (Fig. 3).

Detection of protein-bound carbohydrate

The storage protein of the aerial tuber did not stain purplish-red with Schiff’s reagent suggesting that it has no covalently linked carbohydrate molecule and thus is not a glycoprotein.

Amino acid composition

The amino acid composition of the storage protein of the aerial tuber of *D. bulbifera* is presented in Table 1. The amino acid composition is characterized by an abundance of neutral and charged polar amino acids, especially tyrosine, arginine, glutamate and cysteine, which constituted about 58% of the total concentration amino acids of the protein (g/100 g protein). Among the non-polar amino acids, proline and phenylalanine were present in relatively high concentration. Of the sulphur-containing amino acids, concentration of cysteine was higher when compared with methionine. Tryptophan, which was probably destroyed during acid hydrolysis of the protein, was not detected.
Carbonic anhydrase activity
The protein had low carbonic anhydrase activity (0.202 units/mg) as compared with standard carbonic anhydrase from bovine erythrocytes.

Dehydroascorbate reductase activity
The storage protein of the aerial tuber of *Dioscorea bulbifera* exhibited dehydroascorbate reductase activity. The protein was able to regenerate ascorbate from dehydroascorbate in the presence and absence of glutathione as shown in Fig. 4a, b. In the presence of glutathione, the specific activities of dehydroascorbate reductase for the protein were 4.14 and 6.01 μmol ascorbic acid produced/min/mg protein at pH 6.5 and pH 7.0, respectively. In the absence of glutathione, the specific activities were 2.07 and 2.76 μmol ascorbic acid produced/min/mg protein at pH 6.5 and pH 7.0, respectively. No activity was observed at pH 6.0.

Monodehydroascorbate reductase activity
The storage protein from the aerial tuber of *Dioscorea bulbifera* showed monodehydroascorbate reductase activity. The protein reduced monodehydroascorbate to ascorbate coupled with NADH oxidation. At pH 6.0, the activity was 0.0017 units/mg which implies that the amount of protein required to oxidize 1 μmol of NADH per min at pH 6.0 was 0.0017 units/mg. At pH 6.5 and pH 7.0, the activity was 0.00038 units/mg and 0.00051 units/mg respectively. Monodehydroascorbate reductase activity was higher at pH 6.0 than at other pH as shown in Fig. 4c.

Trypsin inhibitory activity
Different amounts of the protein were used to determine trypsin inhibitory activity, and the activity was expressed as micrograms of trypsin inhibited as shown in Fig. 5. A positive correlation ($r^2 = 0.9752$) was found between trypsin inhibitory activity and amounts of storage protein from the aerial tuber of *Dioscorea bulbifera*. The storage protein of the aerial tuber of *Dioscorea bulbifera* exhibited low trypsin inhibitory activity with an average of 0.94 μg trypsin inhibited per 100 μg of the protein.

Presence of dioscorin genes
Screening of the primer sets designed for the study with the genomic DNA samples revealed some of the specific primers were able to detect the dioscorin gene in the
genomic DNA sample of Dioscorea bulbifera. Target sequences are readily obtained by polymerase chain reaction if the flanking sequences of the target sequences are known. The presence of dioscorin gene was thus established in the genomic DNA extracted from the aerial tuber of Dioscorea bulbifera. Sequence analysis of dioscorin gene DNA marker produced two DNA fragments and nucleotide sequence sizes which were DBSPOOA1-556 bp and DBSPOOA2-913 bp, respectively (Fig. 6a).

**Homologous similarities of the genes and sequence alignments**

BLAST homology search using nucleotide sequence of DBSPOOA1-556 gave significant alignments of 100% nucleotide identity with Dioscorin B from Dioscorea alata (dioB-1), 96% nucleotide identity with Dioscorea oppositifolia microsatellite Dios23 sequence and 88% nucleotide identity with Arabidopsis thaliana chromosome 3. DBSPOOA2-913 gave significant alignments of 91% nucleotide alignment with D. alata voucher GZY109 ribulose-1,5-bisphosphate carboxylase large subunit (rbcL) gene and 81% nucleotide identity with D. japonica voucher Hsu 231 ribulose-1,5-bisphosphate carboxylase large subunit (rbcL) gene (Table 2).

BLAST homology search using translated amino acid sequences from the nucleotide sequences of DBSPOOA1-556 and DBSPOOA2-913 also produced sequence homology with known protein sequences from Dioscorea spp. DBSPOOA1-556 conceptual amino acid sequence has 75% amino acid sequence identity with Dioscorin B from D. alata, 73% identity with the predicted S-type anion channel SLAH1-like from Solanum tuberosum (potato), 71% identity with the hypothetical protein Os1_01095 of Oryza sativa Japonica (rice) group, 68% identity with S-type anion channel SLAH1 of A. thaliana and 60% amino acid sequence identity with the storage protein of Dioscorea cayenensis. The minimum molecular weight calculated from translated sequence of DBSPOOA1-556 using Protparam online server (https://web.expasy.org/protparam/) is 20,456.38 Da, which is similar to what was obtained for the subunit molecular weight of the storage protein by SDS-PAGE (21,095 Da). DBSPOOA2-913 conceptual amino acid sequence also showed homology with other known proteins, such as ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit of D. bulbifera, putative carbonic anhydrase of Neosartorya fischeri and putative Dioscorin from O. sativa Japonica group (Table 3).

Relationship among the storage protein gene from the aerial tuber of D. bulbifera (DBSPOOA1-556 and DBSPOOA2-913) obtained in this study and the storage protein genes from other Dioscorea spp. was revealed by CLCBio homology nucleotide sequence alignment unweighted pair-group method arithmetic (UPGMA)

**Table 1** Amino acid composition of the storage protein of Dioscorea bulbifera

| Amino acid   | Concentration (g/100 g protein) |
|--------------|----------------------------------|
| Lysine       | 3.50                             |
| Histidine    | 4.43                             |
| Arginine     | 11.87                            |
| Aspartic acid| 4.41                             |
| Threonine    | 3.97                             |
| Serine       | 4.10                             |
| Glutamic acid| 9.92                             |
| Proline      | 16.20                            |
| Glycine      | 4.39                             |
| Alanine      | 5.79                             |
| Cysteine     | 9.66                             |
| Valine       | 4.25                             |
| Methionine   | 3.75                             |
| Isoleucine   | 4.56                             |
| Leucine      | 4.09                             |
| Tyrosine     | 23.11                            |
| Phenylalanine| 12.29                            |
| Tryptophan   | ND                               |
analysis. The analysis revealed that DBSPOOA1-556 and DBSPOOA2-913 are distinctly different. However, DBSPOOA1-556 formed a cluster with other Dioscorin genes from different *Dioscorea* spp. from Asian countries, but DBSPOOA2-913 was distinctly different from all known Dioscorin genes as shown in Fig. 6b.

**Discussion**

The crude extracts of fresh aerial tuber of *D. bulbifera* prepared at 25°C and at pH 8.3 showed optimum protein concentration, which is similar to the results obtained from previous reports [23, 24] on the effect of heating temperature and pH on the major storage protein of various yam species. There was reduction in protein concentration of *Dioscorea alata* L. var. *purpurea* at increasing temperature, and at temperature above 90°C, there was complete denaturation of the protein. Protein concentrations of *D. alata* L. var. Tainung No. 2 and *D. japonica* Thunb. var. *pseudojaponica* showed similar trend with *D. alata* L. var. *purpurea* with increasing heating temperatures except that the storage proteins were not extractable at temperatures above 80°C. Protein concentrations of the yam storage proteins were not changed after heating at temperatures between 30 and 40°C [23]. Protein concentration of *D. bulbifera* storage protein was highest at pH 8.3 which compares reasonably with what was obtained for the major storage proteins of other yam tubers [18, 24–26].

**Fig. 4** Dehydroascorbate reductase activity of storage protein of the aerial tuber of *D. bulbifera* at pHs 6.5 and 7 with (a) or without (b) 4 mM glutathione and (c) monodehydroascorbate reductase activity of the storage protein of the aerial tuber of *D. bulbifera* at pHs 6, 6.5 and 7.
At acidic medium, low protein concentrations were observed for *D. alata* L. var. *purpurea*, *D. alata* L. var. Tainung No. 2 and *D. japonica* Thunb. Var. *pseudojaponica* [24]. The major storage protein of the aerial tuber of *D. bulbifera* obtained was about 87% of the total protein of the aerial tuber which is similar to the percentage concentration of the major storage proteins from other yam tubers [27]. DB2, the major storage protein of *Dioscorea batatas*, accounted for 50% of the total protein of the tuber [21]. The methods of purification of the storage proteins follow similar trends of ammonium sulphate precipitation followed by ion-exchange chromatography and hydrophobic or gel filtration chromatography, or a combination of any two of these steps [21, 23]. However, some researchers purified the major storage proteins from the yam tubers using a one-step purification protocol either by ion exchange (most especially on DE-52 column) or gel filtration on Sephadex G-75 [28, 29].

The native molecular weight of *D. bulbifera* major storage protein was estimated to be 22,000 Da while the subunit molecular weight was 21,000 Da, suggestive of a monomeric structure for the protein. This result is in contrast with those obtained for other underground yam tuber storage proteins. Dioscorins purified from other yam tubers showed a number of isoforms of about 31,000 and 32,000 Da [11, 30]. The storage protein isolated from the tuber mucilage of *D. batatas* had molecular weight above 250,000 Da while that from *D. cayenensis* was 31,000 Da [29]. The dioscorins isolated from *D. batatas* showed two bands (28,000 and 82,000 Da) on non-reducing SDS-PAGE and only one band (32,000 Da) under reducing condition [25]. On the other hand, *D. alata* was reported to have four subunits with molecular weight of 32,000 Da [31], while the storage protein of *Dioscorea opposita* was a monomeric protein with molecular weight of 32,000 Da [32]. Wang et al. [33] also purified a 32,000-Da storage protein from *D. purpurea*. Different yam cultivars have therefore been reported to behave differently in protein composition and structure [34].

*D. bulbifera* storage protein is not glycosylated as shown by periodic acid Schiff’s reagent (PAS) staining technique. Storage proteins from *D. batatas* and *Dioscorea rotundata* were also reported not to be glycosylated with PAS staining method [21]. On the contrary, the yam storage proteins from *D. batatas*, *D. alata* cv. Tainong No. 1 [27] and *D. japonica* [22] were reported to be glycosylated using conA-peroxidase staining method. The protein from *D. opposita* was also shown to be glycosylated with PAS staining [32].

Amino acid composition analysis of *D. bulbifera* storage protein revealed that it is characterized by high content of tyrosine, proline, phenylalanine, cysteine, glutamic acid and arginine. The high content of cysteine residues showed some similarity with the dioscorins from *D. batatas* and *D. japonica* [22, 25, 34] with high half-cystine content. In contrast, dioscorins from four cultivars of *D. alata* (Tainung No. 1, Tainung No. 2, Dasan and Chanhon) had only trace amounts of cysteine [35]. Cysteine, a sulphur-containing amino acid, even though non-essential is required in the diet to meet the body’s requirement. Sulphur is an important element necessary for normal growth and metabolism. Cysteine has been implicated in anti-ageing, promoting healthy hair and skin and also boosts the immune system. Cysteine
residues are also very important especially in crosslinking proteins, increasing the rigidity of proteins and also conferring proteolytic resistance. The storage protein of *D. bulbifera* contains high amounts of essential amino acids, approximately 40.9% of the total concentration of amino acids (g/100 g protein), especially phenylalanine and arginine.

The 22 kDa storage protein of the aerial tuber of *D. bulbifera* exhibited carbonic anhydrase activity, albeit low. Carbonic anhydrases are zinc metalloenzymes that catalyze the simple interconversion of CO₂ and HCO₃⁻. They are pH regulatory and metabolic enzymes found in almost all organisms. In higher plants, carbonic anhydrases play a vital role in CO₂ fixation during photosynthesis [36]. In mammals, they are involved in respiration [37]. Carbonic anhydrases are found in many tissue where they participate in many biological processes such as acid-base regulation, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and electrolyte secretion. Thus, they are important therapeutic targets for treatments of derangements such as edema, glaucoma, obesity, cancer and epilepsy [38]. Six genetically distinct carbonic anhydrases gene families have been identified (α-, β-, γ-, δ-, z- and η-carbonic anhydrases) [39, 40]. Hou et al. [25] showed that the major storage protein of *D. batatas* had carbonic anhydrase activity, which could not be detected in another study by Gaidamashvili et al. [21]. The discrepancies in the two reports, albeit, in the same yam species could not be explained. Also, carbonic anhydrase activity was detected in the major yam storage proteins from different species of *Dioscorea*, *D. alata* (var. Tainong 1, var. Tainong 2, var. Zhongguochang) and *D. pseudojaponica* var. Keelung [27]. Xue et al. [41] also

Fig. 6a PCR amplification of *D. bulbifera* Dioscorin genes (DBSPOOA) with Dioscorin specific primer set (5′-CTCCTCTCCTCCCTCTCTT-3′ and 5′-GGGGTACAATGGAGAAGTG-3′). PCR products were resolved on agarose gels and stained with ethidium bromide. The last lane shows DNA ladder containing DNA fragments of defined length for sizing the bands in the experimental PCRs. The bands outlined in green and red represent DBSPOOA1 and DBSPOOA2, respectively. b Phylogenetic relationship between DBSPOOA genes and other Dioscorin genes.
Ascorbic acid (vitamin C) is a plant secondary metabolite involved in a number of physiological processes. The main role of ascorbic acid is to neutralize free radicals and prevent against oxidative damage [42]. Ascorbic acid also functions as a cell signalling modulator in cell division, growth regulation and senescence in plants [43, 44]. Because of the deleterious effects of reactive oxygen species (mostly as a result of salt imbalance), plants usually have well-developed enzymatic and non-enzymatic antioxidant defense system [45]. In plants, enzymes involved in the ascorbate-glutathione pathway (ascorbic acid-specific peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) assist in peroxides (formed as by-products of normal metabolism) or as a result of environmental stresses) detoxification [46]. In its role as an antioxidant, ascorbic acid is univalently oxidized to monodehydroascorbate, an endogenous index of oxidative stress, which in turn rapidly dissociates to form ascorbic acid and dehydroascorbate in a reaction catalysed by monodehydroascorbate reductase [46]. Thus, monodehydroascorbate reductase and dehydroascorbate reductase are important in the regulating ascorbic acid level and its redox status during oxidative stress [47]. The major storage protein of the aerial tuber of D. bulbifera was shown to have both dehydroascorbate reductase and monodehydroascorbate reductase activities. The dehydroascorbate reductase activity was higher in the presence of glutathione. Dehydroascorbate reductase activity was also detected without glutathione but was lower when compared to the activity in the presence of glutathione. The activity was also found to be pH dependent. At pH 6.0, there was no activity detected without glutathione. This is similar to the report of the yam storage proteins, dioscorins, of D. bulbifera which displayed both dehydroascorbate reductase and monodehydroascorbate reductase activities with and without glutathione [18]. These activities might represent an important defense in the cytoplasm of yam cells in response to environmental oxidative stress [18].

Most storage proteins have been reported to play protective roles against environmental stresses, such as acting as protease inhibitors [48]. Protease inhibitors in plants are usually termed as anti-nutritional compounds because of their ability to inhibit digestive enzymes. However, their presence in plants is often as a result of an evolutionary adaptation which allows plants to survive under natural conditions [49]. In plants, protease inhibitors may be important in regulating and controlling endogenous proteinases, serving as storage proteins, and acting as protective agents against insect and microbial proteases. Protease inhibitors have also been classified under potential cancer-protective micro-components, by controlling misfunctioning of certain proteases in cancer progression [49]. The N-terminal amino acid sequences...
of storage proteins purified from yam bean (Pachyrhizus erosus), YGB1 and YGB2, showed high homology to cysteine protease, but both of them exhibited low protease activities using azocasein as substrates [50]. The storage protein from the aerial tuber of D. bulbifera had low trypsin inhibitory activity as compared with those from sweet potato roots [48]. However, just like the result obtained from this study, dioscorin from D. batatas showed only a weak trypsin inhibitory activity, with 1.9 μg of trypsin inhibited per 100 μg of the protein [25]. Large amounts of these storage proteins could provide a significant protective role in the aerial tuber even with this low trypsin inhibitory activity.

Two dioscorin gene DNA markers were amplified from the total genomic DNA of the aerial tuber of D. bulbifera, DBSPOOA1-556 and DBSPOOA2-913 using primers designed with NCBI primer BLAST tool and Primer 3. In a study done by Barman et al. [51], dioscorin gene was also amplified from RNA extracted from the aerial tuber of D. bulbifera. In a study done by Barman et al. [51], dioscorin gene was also amplified from RNA extracted from the aerial tuber of D. bulbifera. In a study done by Barman et al. [51], dioscorin gene was also amplified from RNA extracted from the aerial tuber of D. bulbifera. In a study done by Barman et al. [51], dioscorin gene was also amplified from RNA extracted from the aerial tuber of D. bulbifera.

Table 3: Dioscorin gene DNA marker (DBSPOOA2-913) nucleotide sequence and deduced amino acid sequence alignments and homologous details

| Nucleotide sequence (913 bp) | Translated amino acid sequence (304 amino acids) |
|-----------------------------|-------------------------------------------------|
| ACCTCTATGGGGTCCGGTGAGGAAGTTGACCCGGCGGAGATTGCTGAGGACAAATCTACCCTCCATCTCTCTCAGGGAGAAGGAGGAGGAGTCCAGGGAGA | TLMGTVGEKQTRORSLSFADGRVQGQDRLRARYCELRGLDFTKODDADEGEKHGRVSHGWLANRLTPSFHGSSRSCS\nGKGYDQAQQRCCDGQFSSFGPELVDFVYEEDRYREKETHKNERRRLIRKSLKEQERIPCEIVHADVRLG\nAMVHELAPAEPEEEEGEGEEYS5ALPI8CDLSLPNPSLYGNCSTCCYSINHLITYLSLYCSTHLRKSWSFSFLHJPT\nWIFMVFLUKY IFPPPEPFCDFPSWNFPFGHALLYHFICACFLFPTP |
| 1. Dioscorea japonica voucher Hsu 231 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast; 89% identity; Accession (JQ733767.1) | 1. Putative Dioscorin [Oryza sativa Japonica Group]; 41% identity; Accession (BAC99799.1) |
| 2. Dioscorea alata voucher GYZ109 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast; 91% identity; Accession (JX139767.1) | 2. Carboxic anhydrase, putative [Neosartorya fischeri NRRL 181]; 45% identity; Accession (XP_001267068.1) |
| 3. Dioscorea nitens voucher YSL 2628 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast; 89% identity; Accession (JQ733810.1) | 4. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Treubia simplicia]; 94% identity; Accession (AUS0559.1) |

| Nucleotide sequence (913 bp) | Translated amino acid sequence (304 amino acids) |
|-----------------------------|-------------------------------------------------|
| ACCTCTATGGGGTCCGGTGAGGAAGTTGACCCGGCGGAGATTGCTGAGGACAAATCTACCCTCCATCTCTCTCAGGGAGAAGGAGGAGGAGTCCAGGGAGA | TLMGTVGEKQTRORSLSFADGRVQGQDRLRARYCELRGLDFTKODDADEGEKHGRVSHGWLANRLTPSFHGSSRSCS\nGKGYDQAQQRCCDGQFSSFGPELVDFVYEEDRYREKETHKNERRRLIRKSLKEQERIPCEIVHADVRLG\nAMVHELAPAEPEEEEGEGEEYS5ALPI8CDLSLPNPSLYGNCSTCCYSINHLITYLSLYCSTHLRKSWSFSFLHJPT\nWIFMVFLUKY IFPPPEPFCDFPSWNFPFGHALLYHFICACFLFPTP |
| 1. Dioscorea japonica voucher Hsu 231 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast; 89% identity; Accession (JQ733767.1) | 1. Putative Dioscorin [Oryza sativa Japonica Group]; 41% identity; Accession (BAC99799.1) |
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D. bulbifera, DBSPOOA1-556 formed a cluster with other dioscorin genes from different Dioscorea spp. from Asian countries, but DBSPOOA2-913 was distinctly different from all known dioscorin genes. This could be because it is an aerial tuber and not an underground tuber. It could also be because of the different geographical locations in which the yam species are cultivated. Several factors such as root-crop species, local climate and fertilization pattern have been reported to directly influence the composition of root crops [35]. The yam species used in this study was cultivated in Africa in contrast to various reported studies of other yam species, D. alata, D. cayenensis, and D. japonica which are cultivated in Asian countries.

Conclusion
In conclusion, a storage protein was isolated from the aerial tuber of Dioscorea bulbifera for the first time. The storage protein has similar functional properties and structural homology with the storage proteins of other Dioscorea species. The storage protein is heat stable and exhibited carbonic anhydrase, dehydroascorbate reductase and trypsin inhibitory activities. It is also a good source of essential amino acids; thus, the protein may be suitable for development as functional food.

Authors’ contributions
AK designed, developed and conceptualised the study. OAA carried out all research project under the supervision of AK and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interest.

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