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CHANGES IN SAPONIN COMPOSITION OF *Dioscorea zingiberensis* C. H. WIGHT TUBERS UNDER DIFFERENT STORAGE CONDITIONS

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Efficient storage of large amounts of fresh *Dioscorea zingiberensis C. H. Wright* (DZW) tubers with the minimum loss of useful components is important in diosgenin production. In this study, the components changes, in particular the saponins change of DZW tubers during storage in four different conditions were investigated. Saponins were preliminarily identified through liquid chromatograph-mass spectrometer (LC-MS). Results revealed that plant endogenous enzymes, compared with the other factors concerned most significantly affected diosgenin yield by modifying the composition and content of steroidal saponins. Glycosyl transferase, a plant endogenous enzyme, converted furostanol glycoside to its spirostanol type. Some glycosidases further participated in the biodegradation of polysaccharides at the C-3 position to their corresponding secondary glycosides. Moreover, the environmental microbes also played an important role in DZW tuber storage by secreting enzymes with similar functions. It exhibited some synergistic effect with the plant endogenous enzymes in affecting the components of DZW tubers during storage. This work, to the best of our knowledge, presented the first relatively systematic study of the effects of plant endogenous enzymes and environmental microbes on the storage of DZW tubers, and could provide a preliminary theoretical guidance to the volume storage of fresh DZW tubers in industry.
Keywords: Plant endogenous enzymes, microorganisms, transformation, saponins, storage.

INTRODUCTION

Dioscorea zingiberensis C. H. Wright (DZW) is an important plant cultivated particularly for diosgenin production in China (Liu *et al.*, 2010). The DZW tuber consists of 1% to 5% saponins, 45 to 50% starch, and 40 to 50% cellulose in dry weight. Diosgenin (25R-Spriost-5-en-3β-OH), mainly existing as ligands of steroidal saponins in DZW tubers (Zhu *et al.*, 2010), is an important steroidal sapogenin in the steroid pharmaceutical industry; it is mainly used as a starting material for partial synthesis of oral contraceptives, sex hormones, and other steroids (Zhang *et al.*, 2009).

Fresh DZW tubers are harvested from October to May in China not only because of the high saponin content during that period, but also for convenient harvest and transportation during the dry season (Wei *et al.*, 2013). In Shiyan of Hubei province, available DZW tubers exist in two forms: sun-dried tubers with approximately 10 wt% moisture content and fresh tubers with approximately 75 wt% moisture content. About 120 tons to 150 tons of fresh DZW tubers are required to produce 1 ton of diosgenin (Huang *et al.*, 2008). In this regard, companies that produce diosgenin purchase large amounts of fresh tubers to meet their processing requirements. However, it is inconvenient for them to store the large amounts of fresh DZW tubers in practical processing because of their high water content and large volume. *Dioscorea zingiberensis* C. H. Wright tubers often become rotten and hollow during volume storage, which may cause serious loss of useful ingredients, such as starch, saponins, and diosgenin, leading to a great economic loss, waste of resources, and unstable diosgenin productivity. Thus, it is of great importance to investigate the factors that affect the components of DZW tubers during storage, and to find out the optimum storage conditions so as to lower the loss to the minimum in diosgenin production.

It is well known that a series of factors can affect the components of DZW tubers during storage, such as oxygen, acid, among others, of which the enzymes both from plants and from environmental microbes usually play the biggest role and are of special concern.

Previous studies revealed that some plant endogenous enzymes can affect DZW tuber components. Endogenous glucosidase in yam could alter saponin structures during drying processes. Crude furostanol glycoside 26-O-β-glucosidase prepared from yam can also remove and change

glucose at the C-26 position of the furostanol glycoside structure to its corresponding spirostanol glycoside (Yang *et al.*, 2009). α-Glucosidase inhibitors from fresh tuberous rhizomes of *Dioscorea opposita* Thunb. were also evaluated against yeast α-glucosidase to determine their active principal components for treatment of diabetes (Zhang *et al.*, 2011). Another study also evaluated the effect of the activity of α- and β-amylases from *Dioscorea dumetorum* tubers on its starch component during storage (Afoakwa and Sefa-Dedeh, 2002).

Environmental microbes can also affect the DZW tuber components during storage. They can degrade and transform steroidal saponins into diosgenin and other saponin forms by secreting relevant enzymes (Wang *et al*., 2014; Dong *et al*., 2015). β-Glucosidase called AfG, which was purified from *Aspergillus fumigates*, effectively released diosgenin from spirostanosides of DZW tubers (Lei *et al*., 2008). Glucoamylase from *Curvularia lunata* also exhibited steroidal saponin–rhamnosidase activities to yield secondary spirostanosides from the substrates (Feng *et al.*, 2007).

Our previous work also demonstrated that plant endogenous enzymes and environmental microbes can affect DZW tuber components, such as reducing sugar, saponins, and diosgenin (Wei, 2014). Although the influences of plant endogenous enzymes and environmental microbes on the storage of DZW tubers have been widely admitted, few reports systematically studied the underlying mechanisms and the metabolism of DZW tuber components, and their comprehensive effects on the storage of DZW tubers. In this work, we aimed to study the components change, in particular the saponins change of DZW tubers during storage in different conditions, with emphasis on the effects of plant endogenous enzymes and environmental microbes and their action mechanisms. It is believed that the findings of this work will facilitate the exploration of the optimum storage conditions for fresh DZW tubers, and contribute some theoretical guidance to the volume storage of fresh DZW tubers in industry.

MATERIALS AND METHODS

***Materials and chemicals*:** Fresh DZW tubers were obtained from the Zhuxi county of Hubei province, China. Diosgenin (98%) was purchased from Sigma, USA, and dioscin was obtained from Chinese Pharmaceutical and Biological Products, Ltd. All other chemicals were of analytical grade.

Storage conditions

Dioscorea zingiberensis C. H. Wright tubers were cut into pieces before heating on a stove at 40 °C. The samples were crushed and then divided into 45 parts. From each part, 4 g of samples were weighed and mixed with water (1:1 w/v) under stirring. The suspensions were then categorized into the following groups stored under different conditions: A-sterilized without enzymes: high-temperature sterilization at 121°C for 1 h; B-unsterilized without enzymes: high-temperature water bath at 100°C for 1 h to inactivate the enzyme and then stored at room temperature; C-sterilized with enzymes: UV irradiation for 30 min, treatment with antibacterial and antifungal agents (i.e., cephalosporins, sulfa drugs and nystatin), and then placed in a sterile storage flask under the laminar flow hood; and D-unsterilized with enzymes: stored at room temperature.

Diosgenin sample preparation: The suspensions stored under different conditions were first treated with the low-pressure steam expansion pretreatment (LSEP). Then, the consequent DZW slurry was treated with microorganisms to release saponins from intracellular environment. The mixtures can be converted to diosgenin via acid hydrolysis (Wei *et al.*, 2013).

Analysis methods:

***Quantitative analysis of reducing sugar content*:** Reducing sugar content was determined using DNS method (Miller, 1959).

***Diosgenin analysis*:** Diosgenin was analyzed through HPLC on an Agilent 1200 system equipped with a C18 column (4.6 mm × 250 mm, 5 μm). The solvent system consisted of methanol with a flow rate of 1 mL/min within 15 min at 30°C. Diosgenin was detected with a UV detector at 204 nm (Wei *et al.*, 2013). Diosgenin yield was presented as gram of product/100 g of dried DZW tubers. The degree of release of saponins under different treatment conditions was expressed as the ratio of diosgenin content in the liquid phase to that in DZW powder.

***Steroidal saponin analysis*:** Plant material extracts were analyzed using an HPLC-MS/MS system to determine the chromatographic behavior of steroidal saponins. Analysis was performed on an Agilent 1100 system equipped with an auto sampler, quaternary pump system, photodiode array, multiple wavelength detector, thermostated column compartment, and degasser with a C18 column (4.6 mm × 250 mm, 5 μm). Solvent A was 0.1% acetic acid in water, and solvent B was acetonitrile. The gradient was 0 min to 20 min with 48% B at a flow rate of 1.0 mL/min. The column was equilibrated under starting conditions for 10 min prior to injection of 10 μL of the sample. Steroidal saponin was identified through electrospray ionization mass spectrometry. Spray voltage was fixed at 3.5 kV, and heat capillary was maintained at 350°C. The scan range was set from 100 m/z to 1500 m/z. The zoomscan and tandem MS (MS/MS) functions were performed in a data-dependent mode, and LC-MS was applied in the positive ion mode (Zhang *et al.*, 2014).

Analyses were conducted in triplicates, and mean values were determined. Data were also subjected to analysis of variance and least significant difference test to determine significant differences between means at $p < 0.05$.

RESULTS AND DISCUSION

Changes in reducing sugar content of DZW tubers under four different storage conditions **:**

Changes in the reducing sugar content of DZW tubers under different storage conditions are shown in Figure 1. The reducing sugar content was constant under the two sterile storage conditions (A and C) but continuously decreased with time under B and D conditions. Microorganisms secreted enzymes in the environment to degrade starch of DZW tubers, and the degradation products were completely utilized as energy supplements for their metabolism. Microorganisms can also secrete enzymes including cellulase, pectinase, α- amylase, xylanase, and glycosidase, which can disintegrate plant composition as well as degrade or transform steroidal saponins into diosgenin or other saponin forms (Heerd *et al.*, 2012). These findings showed that microorganisms considerably influenced the content of starch, saponins and other components in DZW tubers.

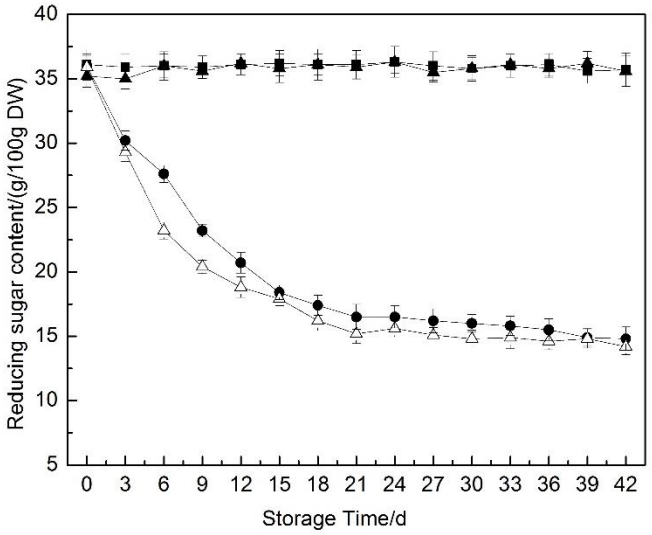

Figure 1. The changes of reducing sugar content in DZW tubers under four different storage conditions. (■) A-sterilized without enzymes group (●) B-unsterilized without enzymes group (▲) C-sterilized with enzymes group (△) D-unsterilized with enzymes group.

Changes in diosgenin yield in DZW tubers under four different storage conditions **:**

The diosgenin yield in the sterilized group without enzymes did not change during storage (Fig. 2). Saponin content was also constant in the absence of endogenous enzymes and microorganisms. These results indicated that saponins were minimally affected by chemistry effects, including oxidation and chemical reactions among substances in the samples during storage (Wang *et al.*, 2009). After 6 d, the diosgenin yield increased under all conditions, except in the sterilized group without enzymes (A). The dynamic changes in diosgenin yield were irregular and decreased compared with the original values under B, C, and D conditions before 18 d. The results obtained with unsterilized material without enzyme activities are incomprehensible since the diosgenin content is lowest at day 15, but increases to similar levels reached at the other storage conditions. This ascribed to the effect of microorganisms which can secrete degradation enzymes to degrade DZW tubers, resulting in some loss of saponins under B condition before 15 days. After that, microbes may begin to secrete glycosyltransferases to convert furostanol saponins into spirostanol type, which can improve the yield of diosgenin (Pang *et al.*, 2015). Enzyme activity in the fermentation broth indicated the existence of glycosyltransferases were detected after 15 days (data not shown). After 24 days, the conversion was substantially completed, and the yield of diosgenin basically stabilized.

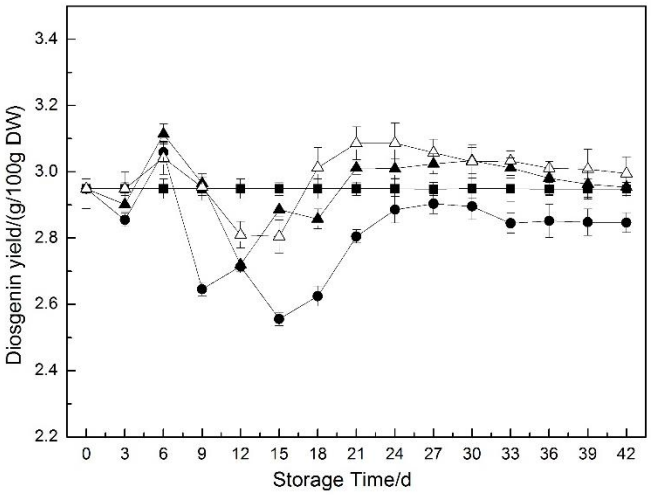

Figure 2. The changes of diosgenin yield in DZW under four different storage conditions. (■) A-sterilized without enzymes group (●) B-unsterilized without enzymes group (▲) C-sterilized with enzymes group (△) D-unsterilized with enzymes group.

The diosgenin yield was also highest in the unsterilized group with enzymes (D), followed by the sterilized group with enzymes (C), sterilized group without enzymes (A), and unsterilized group without enzymes (B). It was suggested that day 24 was the best time to use the raw material in this experiment. The dynamic changes in diosgenin yield increased first and then decreased from day 6 to the lowest at day 15 and reached to the highest at day 24 under D conditions. The reasons of the dynamic changes are the same with B conditions before 15 days. After that,

microbes may begin to secrete glycosyltransferases to convert furostanol saponins into spirostanol type, meanwhile the plant endogenous enzymes can also improve the yield of diosgenin, which reached the highest level at day 24. After 24 days, the conversion was substantially completed, and the yield of diosgenin basically stabilized which was still higher than the initial content. It was not conducive to diosgenin production when use the raw materials during the first 6-21 days. The diosgenin yield was influenced by the composition and content of saponins because it was obtained by hydrolyzing total saponins in DZW tubers. The increase in diosgenin yield was mainly ascribed to the synergistic effect of plant endogenous enzymes and environmental microorganisms (Peng *et al.*, 2010). Experimental data further showed that effect of endogenous enzymes was greater and more stable than that of microorganisms (Yang *et al*., 2009).

Changes in saponin composition under four different storage conditions **:** Steroidal saponins are oligoglycosides connected by spirostan compounds and glycosylations. The genus *Dioscorea* contains up to 68 types of steroidal saponins, including 25 steroidal sapogenins and 17 sugar chains connected with C-3 (Chen and Zhang, 2007; Lu, 2008), hence, *Dioscorea* plants are an important source of steroidal saponins (Liu *et al.,* 2010). In the present study, total saponins were extracted and analyzed through LC-MS under four different storage conditions to determine the factors that influence the changes in steroidal saponins during storage. The changes in saponin composition after 24 days were shown in Fig. 3 and Table 1.

The chemical composition of saponins significantly differed under four storage conditions (Fig. 3). Eleven substances were detected in the sterilized group without enzymes (A) after 5 min, and an additional column representing No. 1 substance was determined in the unsterilized group without enzymes (B) and unsterilized group with enzymes (D). The total ion chromatogram within 5.8 min showed that the molecular weight of the substance was 1049.6, 1070.5, 625, or 544.6.

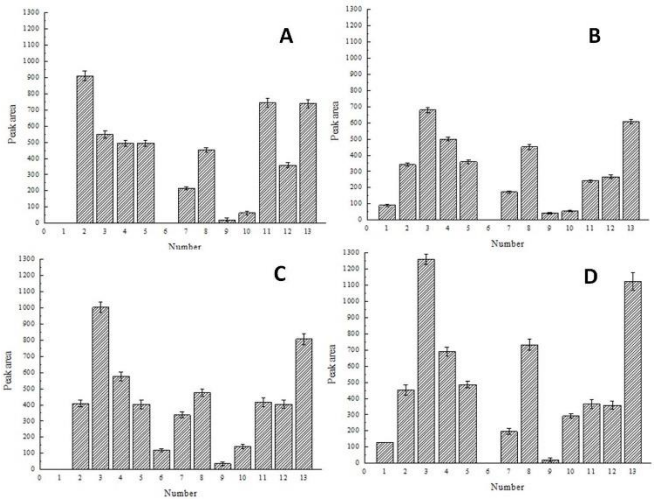

Figure 3. The variations in the compositions of saponins under four different storage conditions after 24 days. (A) sterilized without enzymes group, (B)

Table 1. The chemical compositions of saponins under four different storage conditions.

Peak No.	RT/min	ESI-MS		Proposed fragmentation Pathway	Molecular weight	Proposed molecular formula	Possible type of aglycone
		Molecular ion peaks	Secondary ion fragment peaks				
1	5.820	1049.6	1032.4: 885.7: 577.4: 415.3	$[M+H\text{-}Rha\text{-}Glu\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Rha\text{-}Glu\text{-}Rha\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	1030.2	$C_{51}H_{82}O_{21}$	Furostanol
2	7.047	1069.6	923.7; 761.7: 544.6	$[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}Glu\text{-}H_2O\text{-}C_8H_{16}O_2\text{-}H_2O]^+$	1046.2	$C_{51}H_{82}O_{22}$	Furostanol
3	7.580	1069.7	923.5: 761.5: 543.3: 467.1	$[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	1046.2	$C_{51}H_{82}O_{22}$	Spirostanol
4	8.627	907.5: 885.7	761.5: 723.4: 577.4: 415.2: 397.1: 271.0	$[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	884	$C_{45}H_{72}O_{17}$	Spirostanol
5	9.750	907.5: 884.9	723.4: 577.3: 577.3: 415.2	$[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	884	$C_{45}H_{72}O_{17}$	Furostanol
6	10.431	577.7	433.1: 271.0	$[M+H\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$Or $[M+H\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]$	577	$C_{33}H_{52}O_8$	Furostanol
7	10.785	907.5: 885.6	723.4: 577.4: 415.2	$[M+H\text{-}Glu\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Glu\text{-}Rha\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	884	$C_{45}H_{72}O_{17}$	Furostanol
8	11.108	907.5: 885.5: 508.5	723.3: 577.2: 415.2	$[M+H\text{-}Glu\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Glu\text{-}Rha\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	884	$C_{45}H_{72}O_{17}$	Spirostanol
9	12.334	869.6	725.3: 577.3: 415.2: 271.0	$[M+H\text{-}Rha\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ Or $[M+H\text{-}Rha\text{-}Rha\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$	868	$C_{45}H_{72}O_{16}$	Furostanol
10	13.187	923.5	577.5: 470.4: 415.4	$[M+H\text{-}Glu\text{-}Glu\text{-}Glu\text{-}H_2O\text{-}C_8H_{16}O_2\text{-}H_2O]^+$	902	$C_{45}H_{74}O_{18}$	Spirostanol
11	14.374	723.6	577.5: 415.2: 253.0	$[M+H\text{-}Rha\text{-}Glu\text{-}H_2O]^+$	722	$C_{39}H_{62}O_{12}$	Daucosterol
12	18.833	761.3: 745.3: 723.5	599.3	$[M+H\text{-}Glu\text{-}Glu\text{-}H_2O]^+$	738	$C_{39}H_{62}O_{12}$	Daucosterol
13	19.507	761.8: 745.6: 723.5: 577.5	559.3: 433.1: 271.0	$[M+H\text{-}Glu\text{-}H_2O]^+$	577	$C_{35}H_{60}O_6$	Daucosterol

unsterilized without enzymes group, (C) sterilized with enzymes group, (D) unsterilized with enzymes group.

Note: The numbers in abscissa represent the substance in Table 1, respectively, the vertical axis denotes the relative concentration and content of each substance in Table 1.

The combination of the size and concentration of the molecular ion peak in the MS spectrum revealed that the molecular weight of the substance should be 1030.2; moreover, 1049.6 and 1070.5 were assigned to $[M\text{-}H_2O]^+$ and $[M\text{-}K]^+$, respectively. Secondary ion fragment peaks, such as 1,032.4, 885.7, 577.4, and 415.3, indicated that the substance may contain fragments of $[M+H\text{-}Rha\text{-}Glu\text{-}Rha\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ or $[M+H\text{-}Rha\text{-}Glu\text{-}Rha\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$. Enzymes secreted by environmental microorganisms were important to transform and synthesize No. 1 substance, regardless of the presence or absence of endogenous enzymes.

Mass spectrometry data showed that Nos. 2 and 3 substances were isomers and exhibited a molecular weight of 1046.2. No. 2 substance contained fragments of $[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}Glu\text{-}H_2O\text{-}C_8H_{16}O_2\text{-}H_2O]^+$, and No. 3 substance comprised fragments of $[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}H_2O]^+$ or $[M+H\text{-}Rha\text{-}Glu\text{-}Glu\text{-}C_8H_{16}O_2\text{-}Glu\text{-}H_2O]^+$. The steroidal sapogenins of No. 2 and 3 substances were furostanol and spirostanol, respectively (Kite *et al.*, 2007). The content of No. 2 substance was also higher than that of No. 3 in the sterilized group without enzymes (A) but lower under the three other conditions. In addition, the content of No. 3 substance was higher in the sterilized group with enzymes (C) than that in the unsterilized group without enzymes (B). These results could be attributed to the conversion of glycosyltransferases from furostanol glycoside to the respective spirostanol type (Fig. 4); in this process, plant endogenous enzymes play a significant role, which was consistent with previously reported results(Yang *et al.*, 2009; Pang *et al*. 2015). Nos. 4 and 5 substances were also isomers with a molecular formula of $C_{45}H_{72}O_{17}$. The obtained composition of mass fragment peaks and that in the literature revealed that No. 4 substance was spirostanol glycoside and No. 5 was furostanol. The content of No. 4 substance increased in different degrees, whereas that of No. 5 substance decreased under B, C, and D conditions compared with that under A condition. These results further suggested that plant endogenous enzymes play a significant role in converting furostanol glycoside to the respective spirostanol type. Glycoside bonds can be easily broken to obtain diosgenin from spirostanol glycoside (Yang *et al.*, 2009). As a result, the highest diosgenin yield was obtained in the unsterilized group with enzymes (D) (Fig. 2).

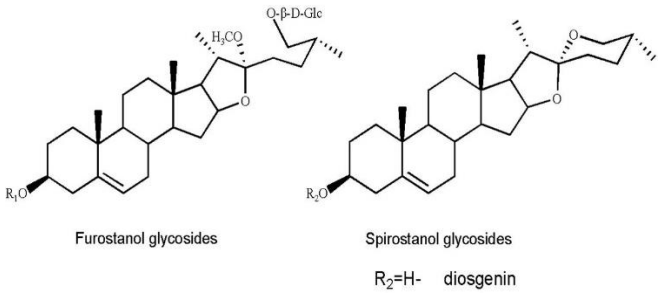

Figure 4. Structures of furostanol glycosides, spirostanol glycosides and diosgenin.

No. 6 substance could be obtained from Nos. 1, 2, 5, and 7 substances, as well as from other polysaccharide furostanol glycosides cut by one to three carbohydrate ligands. Glucosidases from plant endogenous enzymes can cut the multi-carbohydrate ligands connected with the C-3 bond. The content of No. 10 substance increased in different degrees under B, C, and D conditions compared with that under A condition. The content of this substance under different conditions were in the following order: unsterilized group with enzymes (D) > unsterilized group without enzymes (B) > sterilized group with enzymes (C) > sterilized group without enzymes (A) (Fig. 3). This result could be attributed to the presence of glucosidases from plant endogenous enzymes and environmental microorganisms; glucosidases from the latter can cut rhamanopyranosyl (-Rha) that existed at the end of No. 10 substance, whereas those from the former played a main role in enzymolysis. The content of No. 11 substance was highest under A condition compared with that under the other conditions. No. 11 substance can be degraded or converted into other compounds because of the presence of plant endogenous enzymes and microorganisms. Unfortunately, it could not to be determined what the other compounds were based on the present data. Plant endogenous enzymes and microorganisms did not significantly affect No. 12 substance, while promoting the accumulation of No. 13 substance.

Enzymes play a key role in the biosynthesis of steroidal saponins; these enzymes include geranyl diphosphate synthase, farnesyl diphosphate synthase, squalene synthase, squalene epoxidase, epoxidized squalene cyclase, hydroxylase, glycosyltransferase (steroidal glycosyltransferase), and β- glucosidase (26-O-β-glucosidase) (Chen and Zhang, 2007). These enzymes can be obtained from plants or secreted by microorganisms in the environment (Feng *et al.*, 2010; Pang *et al.*, 2015). Steroidal saponins are relatively similar between these enzymes, and their isomers can be easily transformed by glycosyltransferases, glycosidases, and other enzymes. Therefore, enzymes endogenous in plants and secreted by environmental microorganisms can considerably affect the

composition of steroidal saponins in DZW tubers during storage. Steroidal saponin transformation was also related to enzymatic metabolic reactions.

***Conclusion*:** This study investigated the effects of plant endogenous enzymes and environmental microbes on the components of DZW tubers during storage, particularly steroidal saponin composition. It was found that plant endogenous enzymes had the most significant effects on the subsequent diosgenin production by affecting saponin content and composition during DZW tubers storage. Glycosyl transferase converted spirostanol glycoside to its furostanol type. Some glycosidases further participated in the biodegradation of polysaccharides at the C-3 position to their corresponding secondary glycosides. In addition, their isoenzymes secreted by environmental microorganisms also played an important role in this process, which exhibited a synergistic effect with the plant endogenous enzymes. It is foreseeable that a relatively optimized set of storage conditions for DZW tubers can be formed by adjusting the enzymes endogenous in plants and secreted by environmental microbes. As far as we know, this is the first study comprehensively describing the effects of plant endogenous enzymes and environmental microbes on the storage of DZW tubers, which may enlighten the volume storage of fresh DZW tubers in industry to some extent.

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