Original paper

Chinese Purple Yam (*Dioscorea alata* L.) Extracts Inhibit Diabetes-Related Enzymes and Protect HepG2 Cells Against Oxidative Stress and Insulin Resistance Induced by FFA

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Received March 12, 2015 ; Accepted May 12, 2015

The present study demonstrated the anti-diabetic activities of Chinese purple yam flesh and peel extracts. Results showed that the ethyl acetate fraction of purple yam extracts could efficiently inhibit the activities of two key diabetes-related enzymes, α -amylase and α -glucosidase, and the non-enzymatic glycation which might lead to the pathogenesis of diabetic complications. On the other hand, the extracts exhibited excellent ability in alleviating free fatty acid-induced oxidative stress and insulin resistance in HepG2 cells. In addition, purple yam peel exerted better effects than the flesh, and more attention should be paid for its value in the prevention and treatment of diabetes and related diseases.

Keywords: Type II diabetes, Chinese purple yam, digestive enzymes, insulin resistance, oxidative stress

Introduction

Type II diabetes (T2D) is a chronic metabolic disorder of glucose, carbohydrate, lipid, and protein associated with hyperglycemia, and is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action (Birkenfeld and Shulman, 2014). Dietary control based on the glycemic index of foods is regarded as a relatively safe and complementary treatment for diabetes (Wolever *et al.*, 1994). However, due to its deficiency of limiting the types of food consumed, another effective approach to decrease blood sugar level was proposed which was to interrupt the digestion of dietary starch by inhibiting α -amylase and α -glucosidase (Geng and Bai, 2008; Shobana *et al.*, 2009). So far, acarbose and voglibose isolated from microorganisms are used either alone or in combination with insulin to lower postprandial blood glucose level (Modak *et al.*, 2007). However, these

compounds have certain side effects, including liver disorders, flatulence, abdominal pain, renal tumours and hepatic injury (Fujisawa *et al.*, 2005; Shobana *et al.*, 2009). Therefore, attempts have been made to search for safer α -amylase and α -glucosidase inhibitors from natural sources to serve as an alternative drug with lesser adverse effects than the existing synthetic drugs.

During chronic hyperglycemia, the non-enzymatic glycation which is characterized by the chemical reaction between the aldehydic group of sugars and the amino group of proteins, can lead to the pathogenesis of diabetic complications (Peyrou and Sternberg, 2006). Amadori products are formed in the early stage of glycation process, and are then converted to reactive dicarbonyl species including glucosones, glyoxal, and methylglyoxal, followed by the formation of cross-linking fluorimetric advanced glycation end products (AGEs) (Peyrou and Sternberg, 2006). The glycation

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process not only impairs functions of modified proteins in living organisms, but also induces overproduction of free radicals. Therefore, it's urgent to search for anti-glycation agents from natural sources to offer promising therapeutic approaches for T2D.

The current epidemic of diabetes is being driven by the obesity epidemic, which is characterized by a state of fat overload in tissues (DeFronzo, 2010). The association between lipids and insulin resistance is widely accepted. It has been reported that increased plasma free fatty acid (FFA) and toxic lipid metabolites play a role in the pathogenesis of muscle/liver insulin resistance and T2D in obese individuals (DeFronzo, 2010; Samuel *et al.*, 2010). One of the postulated mechanisms underlying obesity-associated insulin resistance and T2D is oxidative stress, which is defined as an excessive production of reactive oxygen species (ROS) (Matsuda and Shimomura, 2013). Accumulation of lipid within cells is always accompanied with over-production of ROS (Furukawa *et al.*, 2004; Pereira *et al.*, 2014). Treating obese mice with antioxidant agents could attenuate the development of diabetes (Kaneto *et al.*, 1999). Thus targeting FFA-induced oxidative stress might serve as an effective way to ameliorate obesity-associated insulin resistance and T2D.

Current treatments for T2D which were mainly monotherapies are not fully effective due to the complex regulatory networks involved in T2D (Liu *et al.*, 2010). Therefore, multi-component natural extracts and plant products acting on multi-target may be more effective than single-target based pharmaceuticals. Chinese yam has a long cultivation history in China for both edible and medical purpose. In this study, we used Chinese purple yam (*Dioscorea alata* L.) which is rich in anthocyanin, to evaluate its protective and therapeutic effects on T2D, including the potent α -amylase and α -glucosidase inhibitory effects, anti-glycation activity, and the abilities to alleviate FFA-induced oxidative stress and insulin resistance in HepG2 cells.

Materials and Methods

Chemicals and reagents Purple yams were obtained from Zhejiang Province, China, bovine serum albumin (BSA), α -glucosidase (from *S. cerevisiae*), 4-nitrophenyl- α -D-glucopyranoside (PNPG), acarbose, potato starch, α -amylase (from porcine pancreas), 3,5-dinitrosalicylic acid (DNS), oleic acid, palmitic acid, 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), PBS, Hank's balanced salt solution (HBSS), Fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO Co. (BRL Life Technologies, Grand Island, NY, USA). All other chemical reagents were of analytical grade and purchased from Beijing, China. Assay kits for glucose, glycogen, malondialdehyde (MDA) and glutathione (GSH) were obtained from Nanjing Jiancheng Biological Engineering Research Institute, China. BCA protein assay kit was obtained from Beyotime Institute of Biotechnology, China.

Preparation of Chinese purple yam extracts of peel and flesh

The freeze-dried and 60-mesh-powdered peel and flesh materials from purple yam were ultrasonically extracted by three times with 80% ethanol for 30 min at room temperature. After concentrated using a rotary evaporator, the crude extracts (CE), with yields of 4.417 g/100 g dry weight for flesh and 4.135 g/100 g dry weight for peel, were suspended in water and partitioned sequentially using n-hexane (HX), ethyl acetate (EA), n-butanol (BT), and the remaining water (RW) fraction. The HX, EA, BT fractions were concentrated under reduced pressure to remove solvents and the RW fraction was freeze-dried. The yields for each fraction were 0.869, 0.530, 0.691, 2.327 g/100 g dry weight for flesh and 0.833, 0.676, 0.971, 1.654 g/100 g dry weight for peel, respectively. All fractions were maintained in -80°C until use.

α -Glucosidase assay The α -glucosidase inhibition assay was performed according to the method of Liu *et al.* (2004). The reaction mixture contained 40 μL of 1.6 U/mL α -glucosidase, 20 μL of the samples and 20 μL of substrate PNPG. After incubated at 37°C for 15 min, the reaction was stopped by adding 80 μL of 0.2 M sodium carbonate and the absorbance at 400 nm were recorded with a microplate reader (SpectraMax M2^e, USA).

α -Amylase assay The α -amylase inhibition assay was adapted from the method of Liu *et al.* (2004). 40 μL of 2 U/mL porcine pancreatic α -amylase was mixed with 80 μL diluted sample, and left for 10 min. After which 40 μL of 1% (w/v) starch solution was added and incubated at 37°C for 10 min followed by the addition of 80 μL of DNS solution. After incubated in boiling water for 10 min, the mixture was recorded for the absorbance at 540 nm.

In vitro glycation of proteins The glycation system contained 10 mg/mL BSA, 0.5 M glucose and 3 mM sodium azide in 0.1 M sodium phosphate buffer (pH 7.4) with different concentrations of purple yam extracts, and incubated at 37°C for 14 d (Duraismy *et al.*, 2003). The formation of Amadori products were detected using the method of Baker *et al.* (1994). α -Dicarbonyl compounds were measured by Girard-T assay described by Wells-Knecht *et al.* (1995). The detection of total AGEs can be generally followed by measuring the characteristic fluorescence of the glycated materials using the excitation and emission maxima of 370 and 440 nm, respectively.

Cell culture and MTT assay The HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA), maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution, and grown at 37°C in the presence of 5% CO_2 . The MTT assay was adapted from the method of Mosmann (1983). Briefly, HepG2 cells were plated into 96-well plates at a density of 1×10^4 cells/well. After 24 h, extracts with different concentrations were incubated with HepG2 cells for another 24 h. Then the cells were incubated with 100 μL of 0.05% MTT for 4 h. The absorbance of each well at 570 nm was measured after discarding supernatant and adding 150 μL of DMSO to solubilise the formed formazan crystals.

FFA treatments and evaluation of insulin resistance and oxidative stress in HepG2 cells FFA solution (1 mM) was prepared with oleic and palmitic acid (molar ratio = 2:1) in incomplete DMEM containing 1% BSA. In brief, HepG2 cells were plated into 96-well or six-well plates at a density of 2.5×10^5 cells/mL. After 24 h, supernatant was discarded and the cells treated with 1 mM FFA with or without purple yam extracts for 24 h. 1% BSA in incomplete DMEM was selected as control. For the determination of insulin-induced glucose absorption and glycogen content in HepG2 cells, the supernatant was replaced with incomplete DMEM containing 10^{-9} M insulin for 24 h. The glucose concentrations in supernatants and glycogen contents within cells were determined respectively by commercially available kits.

DCFH assay was used to measure intracellular ROS levels (Hempel *et al.*, 1999). HepG2 cells were rinsed once with PBS, and treated with 5 μ M DCFH-DA in HBSS at 37°C for 40 min in the dark. Subsequently, cells were rinsed twice with HBSS and the fluorescences were recorded (excitation 485 nm, emission 515 nm) using a microplate reader.

For the measurement of cellular MDA and GSH contents, the treated cells were rinsed twice with PBS, followed by addition of RIPA lysis buffer. The cell lysates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatants were used for determination of MDA and GSH by commercial kits.

Statistical analysis Statistical significance was analyzed by one-way ANOVA using Origin version 8.5, followed by Tukey's tests. A *p* value less than 0.05 was considered to be statistically significant, and a *p* value less than 0.01 was highly significant. The results were expressed as the mean $\pm$ standard deviation (SD) of at least three individual experiments.

Results

Inhibition of purple yam extracts on diabetes related-enzymes

Table 1 showed the inhibitory effects of different fractions of purple yam extracts on α -amylase and α -glucosidase. Comparing between the IC₅₀ values of the five fractions of flesh and peel extracts, we could obtain the potency order for the α -amylase inhibitory activity as ethyl acetate > n-hexane > n-butanol > crude extracts > remaining water, and for the α -glucosidase inhibitory activity, the order became ethyl acetate > crude extracts > n-hexane > n-butanol > remaining water. According to the results, ethyl acetate fraction was the most effective for inhibiting both α -amylase and α -glucosidase, with IC₅₀ values of 75.55 ± 5.83 μ g/mL and 0.76 ± 0.08 mg/mL for flesh extracts, and 53.47 ± 2.78 μ g/mL and 0.73 ± 0.05 mg/mL for peel extracts. However, the inhibitory activities of all fractions were less potent than the positive control acarbose, which possess IC₅₀ values of 13.45 ± 0.77 μ g/mL for α -glucosidase and 19.03 ± 1.20 μ g/mL for α -amylase.

Antiglycation activities of purple yam extracts The

Table 1. Inhibitory effects of different fractions of purple yam extracts on α -glucosidase and α -amylase activities ^a.

Fractions	IC 50 (μ g dried extract/mL)	
	α -amylase	α -glucosidase
F-CE	1093.3 ± 90.3 c	1661.6 ± 137.3 e
F-HX	89.3 ± 5.0 f	2343.3 ± 190.4 d
F-EA	75.6 ± 5.8 f	763.5 ± 83.6 f
F-BT	332.8 ± 32.4 e	2676.9 ± 266.4 d
F-RW	2904.1 ± 195.6 a	7629.8 ± 664.3 b
P-CE	813.8 ± 33.8 d	1519.9 ± 151.0 e
P-HX	68.3 ± 2.5 f	1743.3 ± 145.7 e
P-EA	53.5 ± 2.8 f	727.4 ± 51.6 f
P-BT	279.8 ± 19.7 e	1779.3 ± 188.2 e
P-RW	2632.0 ± 91.1 b	7101.6 ± 200.0 c
Acarbose ^b	19.0 ± 1.2 f	13.5 ± 0.8 a

F: flesh; P: peel; CE: crude extracts; HX: n-hexane fraction; EA: ethyl acetate fraction; BT: n-butanol fraction; RW: remaining water fraction. Values in a column with the same letters are not significantly different (*p* > 0.05).

^a The data represent the means $\pm$ SD of three determinations.

^b Acarbose was used as positive control.

antiglycative activities of different fractions of purple yam extracts were summarized in Table 2. All fractions inhibited the formation of Amadori products, α -dicarbonyl compounds and AGEs dose-dependently. The ethyl acetate fraction exhibited the best antiglycation activity, with AGE-inhibition rates of up to 93.76% and 99.29% for flesh and peel extracts, respectively, at the concentration of 150 μ g/mL. The well-known antiglycation agent, aminoguanidine (AG), exerted no inhibitory effects on formation of Amadori products, consisted with previous reports (Ardestani and Yazdanparast, 2007), and much less potency on inhibiting α -dicarbonyl compounds and AGEs formation compared with ethyl acetate fraction of flesh (F-EA) and peel (P-EA) extracts.

Effect of ethyl acetate fraction of purple yam extracts on FFA-induced HepG2 cell dysfunction Considering the outstanding enzyme inhibition and antiglycation abilities, ethyl acetate fraction was chosen to conduct the cell experiments, and the non-cytotoxicity concentrations for F-EA and P-EA were determined to be lower than 60 and 25 μ g/mL, respectively (Fig. 1).

As shown in Fig. 2, FFA-induced insulin resistance was significantly improved by treating cells with F-EA and P-EA, as well as N-acetylcysteine (NAC), an antioxidant and precursor of GSH, which was indicated by increased insulin-induced glucose absorption (Fig. 2A) and glycogen contents (Fig. 2B). 50 μ g/mL F-EA and 25 μ g/mL P-EA even exerted significantly better effects than the well known hypoglycemic agent metformine, and there were no significant differences with the control regarding to the glucose absorption and glycogen contents.

FFA-induced oxidative stress was evaluated by ROS production, MDA and GSH contents. MDA is a marker of intracellular lipid peroxidation, and GSH is a kind of endogenous

Table 2. Antiglycative effects of different fractions of purple yam extracts ^a.

Fractions	Concentrations ($\mu\text{g/mL}$)	Inhibitory effect (%)		
		Amadori product	α -Dicarbonyl compounds	AGEs
F-CE	50	4.71 ± 2.04 o	10.65 ± 0.57 o	10.12 ± 0.63 q
	100	8.43 ± 1.73 mn	12.96 ± 2.00 no	16.21 ± 1.61 o
	150	17.44 ± 0.47 fgh	17.53 ± 0.26 lm	19.4 ± 2.78 mn
F-HX	50	8.54 ± 0.69 mn	1.25 ± 0.68 q	0.89 ± 0.36 s
	100	15.10 ± 1.11 hijk	1.78 ± 0.73 pq	2.82 ± 1.41 rs
	150	20.63 ± 2.83 cde	5.10 ± 1.59 p	2.91 ± 1.50 rs
F-EA	50	18.05 ± 0.93 efg	40.58 ± 1.81 h	76.74 ± 3.15 d
	100	22.45 ± 1.76 c	47.20 ± 1.44 ef	91.99 ± 1.00 b
	150	30.22 ± 1.02 b	50.17 ± 0.54 de	93.76 ± 0.12 b
F-BT	50	6.87 ± 1.74 no	3.49 ± 1.16 pq	17.11 ± 2.23 no
	100	10.34 ± 2.59 m	10.96 ± 1.59 o	22.14 ± 0.94 m
	150	17.73 ± 2.07 fgh	15.49 ± 2.44 mn	32.21 ± 4.24 l
F-RW	50	8.55 ± 0.72 mn	0.70 ± 1.20 q	1.95 ± 0.87 s
	100	10.56 ± 1.23 m	11.82 ± 2.79 o	2.53 ± 0.88 rs
	150	22.25 ± 1.96 cd	21.20 ± 0.63 k	5.12 ± 2.45 r
P-CE	50	9.94 ± 1.28 m	18.27 ± 2.54 klm	36.68 ± 3.98 j
	100	13.56 ± 1.45 jkl	45.66 ± 2.87 fg	62.90 ± 0.48 g
	150	19.67 ± 2.69 def	51.00 ± 1.83 d	71.90 ± 0.29 ef
P-HX	50	10.87 ± 1.14 lm	12.60 ± 2.02 no	36.41 ± 2.35 jk
	100	14.45 ± 1.70 ijk	34.97 ± 2.12 i	61.12 ± 0.87 g
	150	16.17 ± 1.77 ghij	53.02 ± 3.22 d	74.27 ± 1.17 de
P-EA	50	16.38 ± 1.99 ghi	47.29 ± 2.54 ef	86.38 ± 1.65 c
	100	23.15 ± 0.75 c	61.99 ± 1.29 b	94.63 ± 0.24 b
	150	33.33 ± 0.34 a	70.27 ± 3.15 a	99.29 ± 1.17 a
P-BT	50	13.81 ± 2.72 ijk	17.86 ± 3.89 klm	44.43 ± 1.47 i
	100	15.06 ± 1.82 hijk	39.37 ± 1.95 h	70.58 ± 0.45 f
	150	22.72 ± 1.07 c	58.11 ± 1.76 c	74.27 ± 1.26 de
P-RW	50	9.90 ± 0.85 m	17.62 ± 0.96 lm	12.88 ± 1.74 pq
	100	13.26 ± 1.29 kl	20.64 ± 1.29 kl	14.54 ± 1.58 op
	150	19.52 ± 2.36 ef	33.85 ± 3.89 i	15.56 ± 1.78 op
AG ^b	50	1.03 ± 1.65 p	18.79 ± 2.18 klm	33.69 ± 0.30 kl
	100	0.78 ± 0.74 p	27.44 ± 2.39 j	48.75 ± 0.64 h
	150	0.78 ± 1.40 p	42.77 ± 2.42 gh	60.68 ± 1.84 g

AGEs: advanced glycation end products; F: flesh; P: peel; CE: crude extracts; HX: n-hexane fraction; EA: ethyl acetate fraction; BT: n-butanol fraction; RW: remaining water fraction; AG: aminoguanidine. Values in a column with the same letters are not significantly different ($p > 0.05$).

^a The data represent the means $\pm$ SD of three determinations.

^b AG was used as positive control.

antioxidants, both of which are markers of redox status within cells. As shown in Fig. 3, 1 mM FFA caused overproduction of ROS and increased MDA contents in HepG2 cells, whereas, when co-incubated with F-EA and P-EA, both ROS production and MDA level were significantly reduced in a dose dependent manner (Fig. 3A, B). Interestingly, we demonstrated that GSH, a crucial antioxidant within cells, was elevated after treatment by 1 mM FFA. The level of GSH in model group was nearly twofold than the control, and was further increased after co-incubation with extracts (Fig. 3C).

Discussion

The present study attempted to search for alternative drugs from natural sources with more potency and lesser adverse effects than existing drugs for treating diabetes. One of the strategies adopted to control diabetes involves the inhibition of carbohydrate digesting enzymes such as α -amylase and α -glucosidase, which could diminish glucose absorption in intestine, and thereby lowering postprandial glucose level (Mccue *et al.*, 2005). Different fractions of purple yam extracts exhibited different inhibitory effects on α -amylase and α -glucosidase activities, which might contribute to their different polyphenol contents. Since many

Fig. 1. Cytotoxicity of F-EA (A) and P-EA (B) on HepG2 cells by MTT assay. Data shown were compared with the controls which were considered as the 100% viability value. Results are means $\pm$ SD ($n = 6$). * $p < 0.05$.

Fig. 2. Effect of F-EA and P-EA on insulin resistance in HepG2 cells induced by FFA. (A) glucose absorption; (B) glycogen content. Met (1 mM) and NAC (5 mM) are used as positive controls. Results are means $\pm$ SD ($n = 6$). # $p < 0.05$ vs. control; * $p < 0.05$ vs. model.

Fig. 3. Effect of F-EA and P-EA on oxidative stress in HepG2 cells induced by FFA. (A) ROS production; (B) MDA level; (C) GSH level. NAC (5 mM) is used as positive control. Results are means $\pm$ SD ($n = 6$). # $p < 0.05$ vs. control; * $p < 0.05$ vs. model.

polyphenols were demonstrated to be potential inhibitors of α -amylase and α -glucosidase (Mai *et al.*, 2007; Shobana *et al.*, 2009; Grussu *et al.*, 2011), and this was probably related to their ability to bind with proteins (Ranilla *et al.*, 2009; Shobana *et al.*, 2009). Adedayo *et al.* (2012) demonstrated that water extracts of raw yam possessed good inhibitory effect on α -amylase and α -glucosidase, and their IC 50 values were mostly lower than our

results. We assume that the water extracts in the above mentioned paper contained higher levels of polyphenols. Actually, in our study, those polyphenols were mostly extracted by organic solvents (n-hexane, ethyl acetate and n-butanol) before the yield of water extracts.

Normally, AGEs form with time in healthy body at a constant but slow rate. However, in diabetic patients, increased glycation

resulted from hyperglycaemia was observed with AGEs formation up to four times (Alikhani *et al.*, 2005). Therefore, it's urgent to search for anti-glycation agents from natural sources to offer promising therapeutic approaches for T2D. Interestingly, peel extracts possess better antiglycation activities than flesh under the same concentrations, which may attribute to its better antioxidant activity, since oxidation plays a role in the formation of fluorescence and the molecular crosslinking in the AGEs (Fu *et al.*, 1994). However, it was reported that no oxidation reaction is involved in the early stage of glycation period characterized by formation of Amadori products. Therefore, we proposed that the antioxidant activities were not the unique mechanism for the excellent anti-glycation activity of purple yam extracts, since they possess the ability to inhibit both oxidation and non-oxidation periods of glycation process. The mechanism for inhibiting the non-oxidation period of glycation need to be further investigated.

In T2D patients, there is resistance to the stimulation of glucose uptake and glycogen synthesis by insulin and a reduction of glycogen synthase activity (Thorburn *et al.*, 1991; Henry *et al.*, 1996). It has been proposed that ROS are involved in insulin resistance, and the increase of ROS is an important trigger for insulin resistance in numerous settings (Houstis *et al.*, 2006). Thus we employed NAC, an antioxidant and precursor of GSH, as a positive control. The results demonstrated that both purple yam extracts and NAC could efficiently alleviate FFA-induced insulin resistance in HepG2 cells, indicating that P-EA and F-EA might functioned through their antioxidant activity. As is well known, the cells have antioxidative defense system to protect them from free radical-induced damage. Žegura *et al.* (2006) demonstrated that cellular defense against microcystin-LR-induced DNA damage, which was mediated by ROS, was strengthened in HepG2 cells by the increase of the intracellular GSH level. Similarly, 1 mM FFA caused increase of GSH within cells, indicating that it was the protection mechanism of HepG2 cells to counteract the deleterious effects of ROS. As a kind of natural antioxidant, purple yam extracts could further strengthen the antioxidant defense system in HepG2 cells after treated by FFA.

Antioxidant therapy is regarded as a useful strategy in T2D and other insulin-resistant states (Houstis *et al.*, 2006). Therefore, effective and relatively safer antioxidant agents from natural sources are worthy of investigation in diabetes area. So far, it was reported that polyphenols from various plants including crop, fruits and ornamental plants could be regarded as potential alternative therapy for T2D or as part of a dietary strategy (Ranilla *et al.*, 2009; Shobana *et al.*, 2009; Grussu *et al.*, 2011; Yoshida *et al.*, 2008). Although further experiments are required to identify the active compounds from Chinese purple yam, it has been proposed that sinapic acid and ferulic acid are major phenolic compounds (Fang *et al.*, 2011), which might be responsible for the anti-diabetic activities of Chinese purple yam.

In conclusion, the present study proposed that purple yam

exhibited its anti-diabetic activity through different ways, including inhibiting the activities of digestive enzymes and the non-enzymatic glycation, improving insulin resistance as well as oxidative stress in HepG2 cells induced by FFA. It's worth mentioning that Chinese purple yam peel, a kitchen garbage or by-product of yam processing industry, possessed better anti-diabetic and antioxidant abilities than flesh extracts, and showed value to serve as functional food or alternative medicine. The excellent anti-diabetic and antioxidant abilities might due to the higher contents of bioactive compounds or the synergistic interactions of them in purple yam peel, however, the mechanism need to be further investigated.

Acknowledgments This work is financially supported by the National Key Technology R&D Program in the Twelfth Five-year Guideline of China (Project 2011BAD08B03-01).

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