Evaluation of the Green Alga *Chlorella pyrenoidosa* for Management of Diabetes

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ABSTRACT

The inhibitory effects of the green alga *Chlorella pyrenoidosa* against key enzymes relevant for type-2 diabetes were reported for the first time in the present work. The alga exerted its anti-diabetic actions by scavenging free radicals and/or chelating transition metals but not by suppressing reactive carbonyl species. The findings strongly supported the benefits of microalgae as functional foods in the prevention and alleviation of diabetes.

Key words: Green alga, Amylase, Glucosidase, Diabetes, Free radical scavenger

INTRODUCTION

Diabetes is a significant public health threat and responsible for various long-term complications such as retinopathy, nephropathy and neuropathy, leading to severe socio-economic problems throughout the world. Asia is the major region of increasing prevalence of diabetes; the latest study in 2010 shows that China has overtaken India as the new global epicenter of diabetes[(1)]. Previously, we had hypothesized the anti-diabetic potential of microalgae and successfully obtained some positive results[(2-4)]. For example, a green alga *Chlorella pyrenoidosa* was preliminarily found to possess the inhibitory activities against the formation of advanced glycation endproducts (AGEs), a major pathogenic factor in diabetes[(2)]. To support and extend our earlier findings, in the present study, *C.pyrenoidosa* was further evaluated for its effects on the activity of key enzymes relevant for type-2 diabetes. The underlying mechanism was also investigated.

MATERIALS AND METHODS

Ⅰ. *Reagents, Chemicals and Algal Samples*

All reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless otherwise stated. All analytical and HPLC grade solvents used were obtained from BDH Laboratory Supplies (Poole, UK). The green alga *C. pyrenoidosa* 15 - 2070 was purchased from Carolina Biological Supply Company (Burlington, NC, USA) and grown in the Kuhl medium[(5)]. The cultural condition was described in our previous report [(6)]. The preparation of algal samples was carried out according to Li *et al*.[(7)] with slight modifications.

Ⅱ. *Enzyme Inhibition Assay*

To evaluate the activity of α-amylase, porcine pancreatic α-amylase (EC 3.2.1.1) solution (500 μg/mL) was prepared by adding α-amylase into 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride). The solution (495 μL) was then mixed with 5 μL of the algal extract and incubated at 25°C for 10 min. After pre-incubation, 500 μL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added. The mixtures were further incubated at 25°C for 10 min and the reaction was stopped with 1 mL of dinitrosalicylic acid color reagent. The test tubes were subsequently put in a boiling water bath for 5 min and cooled to room temperature. After appropriate dilution, the absorbance of solution was measured at 540 nm. To evaluate the activity of α-glucosidase (EC 3.2.1.20), 25 μL of the algal extract was mixed with 250 μL of α-glucosidase solution (0.04 units/mL) and 250 μL of *p*-nitrophenyl-β-D-glucopyranoside (0.5 mM). The mixture was quantified to 1 mL with phosphate buffer (0.1 M, pH 7.0). After incubation at 37°C for 30 min, the reaction was stopped by addition of 1 mL of sodium carbonate (0.2 M). The absorbance of solution was measured at 400 nm. The percentage of inhibition was calculated according to the equation: % inhibition = [1-(absorbance of the solution with algal extract /absorbance of the solution without algal extract)] ×100.

Ⅲ. *Detection of Hydroxyl Radicals from Sugar Autoxidation*

Sodium benzoate (1 mM), glucose (500 mM) and

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$CuSO_4$ (0.1 mM) were dissolved in potassium phosphate buffer (100 mM) and incubated at 37°C for 4 days in the absence or presence of the algal extract. The decrease in benzoate hydroxylation resulted from the scavenging of hydroxyl radical production was measured by the fluorescence intensity (excitation maxima: 308 nm; emission maxima: 410 nm) using a Hitachi F-2500 fluorescent spectrometer (Hitachi Corporation, Tokyo, Japan).

Ⅳ. *Methyglyoxal (MG) Suppression Assay*

MG solution (0.25 mL, 5 mM) was mixed with equal volume of PBS (blank), algal extract (1000 μg/mL) or aminoguanidine solution (5 mM) for 50 min at 37°C using water bath. Samples were taken at 10-min intervals whilst 0.125 mL of derivatization agent (1, 2-phenylenediamine, 20 mM) and 0.125 mL of internal standard (2, 3-dimethylquinoxaline, 5 mM) were added. The mixture was shaken by vortex for 5 s. After 30 min that the derivatization reaction was completed, the derivatized product 1-methylquinoxaline (MQ) was formed. Based on the amount of MQ, the residual MG in each sample can be quantified by HPLC. Separation was carried out on a Luna Phenyl-hexyl column (150 × 4.6 mm, 5 μm, Phenomenex Inc., Torrance, CA, USA). The flow rate was 1.0 mL/min, and the injection volume was 15 μL. Isocratic elution was applied using H_2O /MeOH (50 : 50, v/v) as the mobile phase. The total running time was 10 min, and chromatograms were recorded at 315 nm. Percentage decrease in MG was calculated using the following equation, % inhibition = [1- (amounts of MG in samples with testing agents /amount of MG in control)] × 100.

RESULTS

Ⅰ. *Inhibition of Enzyme Activity*

As shown in Figure 1, the *C. pyrenoidosa* extract exhibited moderate α-amylase inhibitory capacity (less than 50% inhibition percentage). At a concentration of 200 μg/mL, there was no inhibitory activity observed. When the concentration increased to 1000 μg/mL, the inhibitory rate was 26.55%. In contrast, the algal extract exhibited much stronger effects in the α-glucosidase inhibitory assay. At concentrations of 200 - 1000 μg/mL, *C. pyrenoidosa* reduced the activity of α-glucosidase by 22.15 - 68.28%.

Ⅱ. *Inhibition of Benzoate Hydroxylation*

Hydroxyl radicals can be generated from glucose autoxidation under the influence of transition metal ions. To evaluate whether *C. pyrenoidosa* could inhibit glucose autoxidation, it was reacted with sodium benzoate with the presence of glucose and Cu^{2+}. The amount of benzoate hydroxylation induced by glucose autoxidation was detected through the measurement of its characteristic fluorescence. As shown in Figure 2, the algal extract exhibited significant inhibitory effects on the formation of benzoate hydroxylation. Increasing the concentration was associated with lower values of hydroxylated benzoate. At concentrations of 200 - 1000 μg/mL, the algal extract suppressed the formation of benzoate hydroxylation by 7.55 - 55.25%.

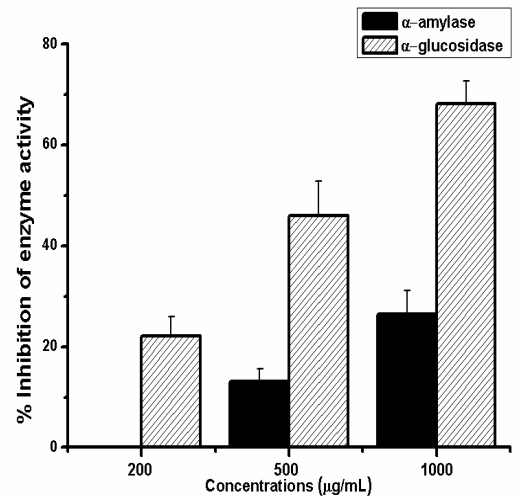

Figure 1. Inhibitory effects of *C. pyrenoidosa* against α-amylase and α-glucosidase. Each value represents the mean ± SD (n = 3).

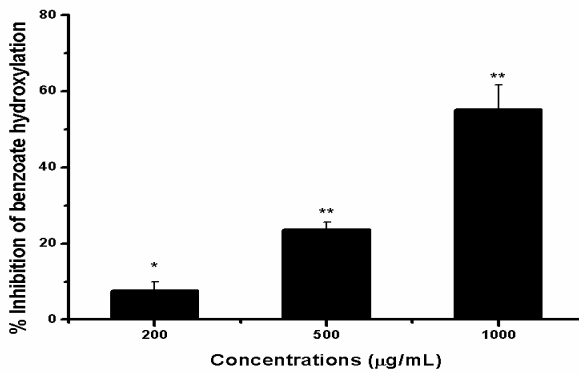

Figure 2. Inhibitory effects of *C. pyrenoidosa* on the generation of benzoate hydroxylation induced by glucose autoxidation. Each value represents the mean ± SD (n = 3). All values statistically different from that of the control. *$p < 0.05$; **$p < 0.01$.

Ⅲ. *Trapping of MG*

The effect of *C. pyrenoidosa* against the generation of MG, a representative reactive carbonyl species (RCS) product was examined. As shown in Figure 3, at a concentration of 1000 μg/mL, the algal extract failed to trap the formation of MG. Its effect was much weaker than aminoguanidine, a typical scavenger for reactive carbonyl compounds.

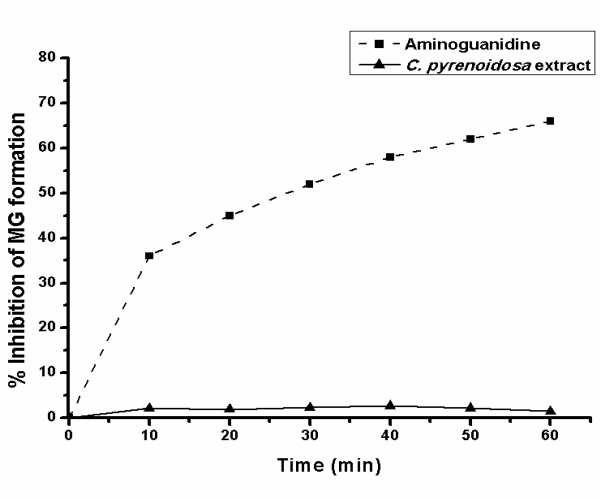

Figure 3. Kinetic study of MG suppression of *C. pyrenoidosa* (1000 μg/mL) and aminoguanidine (5 mM). Results are expressed as mean ± 1/2 range (n = 2).

DISCUSSION

The investigation of anti-diabetic agents from natural sources has attracted more and more attention because the clinical trials of several synthetic anti-diabetic drugs have been terminated due to safety concerns. Such research has made good progress in China as well as in some other Asian countries/regions, where a number of natural products that are safe for human consumption, e.g. tomato paste[8], cinnamon bark[9] and guava[10] have been highlighted for their benefits for diabetic patients. In our laboratory, we have been focused on the anti-diabetic properties of microalgae. In our earlier studies, a green alga *C. pyrenoidosa* was preliminarily found to be able to suppress the formation of AGEs, a key step responsible for the pathogenesis of diabetes[2]. In the present work, this algal strain was further evaluated.

The effect of *C. pyrenoidosa* on the activity of α-amylase and α-glucosidase, which are responsible for starch breakdown and intestinal absorption, was examined. The inhibition of these two enzymes helps to slow the breakdown of ingested carbohydrates and delay the absorption of glucose into bloodstream, therefore acting as an effective strategy in the management of type-2 diabetes, the most common form of diabetes[11]. Results of the present study showed that *C. pyrenoidosa* possessed moderate α-amylase inhibitory activity and strong α-glucosidase inhibitory activity. Such property makes *C. pyrenoidosa* an optimal inhibitor, because a major concern for currently used α-amylase and α-glucosidase inhibitors, such as acarbose, is that they have excessive inhibitory activities of pancreatic α-amylase, which may lead to the abnormal bacterial fermentation of undigested carbohydrates in the colon, resulting in a number of side effects such as flatulence, meteorism and possibly diarrhea[12]. Findings of the present work suggested the capacity of *C. pyrenoidosa* to reduce the abnormal increase of blood glucose levels after a mixed carbohydrate diet. Combining with its AGE inhibitory activity, the potential of *C. pyrenoidosa* in the management of postprandial hyperglycemia-linked type-2 diabetes has been strongly supported.

The underlying mechanism of anti-diabetic property of *C. pyrenoidosa* was further investigated. For most natural agents, their anti-diabetic activities were found to be highly correlated with free radical scavenging capacities. These free radicals, including superoxide anions, hydrogen peroxides and hydroxyl radicals induce the structural modification and functional impairment of key molecules during the glycation process, promoting the development of diabetes[13]. Results of the present study showed that under the presence of Cu^{2+}, the amount of hydroxyl radical production generated from glucose autoxidation was significantly reduced by *C. pyrenoidosa*, suggesting its capacity to scavenge free radicals, or to chelate transition metals, or both.

In addition to free radicals, highly reactive carbonyl species (RCS) like 3-deoxyglucosone (3-DG), glyoxal (GO) and MG act as important intermediates in the glycation process and greatly contribute to the formation of AGEs. Indeed, for some synthetic anti-diabetic agents, suppression of RCS is likely to be a major mechanism of their actions[9]. Thus it would be of interest to evaluate whether *C. pyrenoidosa* is able to sequester RCS in addition to its metal chelating and/or free radical scavenging capacity. In the present work, the effect of *C. pyrenoidosa* on the formation of MG, a representative RCS product was examined, and results showed that its effect was much weaker than aminoguanidine, a typical scavenger for reactive carbonyl compounds. These data suggested that the anti-diabetic property of *C. pyrenoidosa* was not attributed to its reactive RCS quenching ability, as evidenced by its failure to intervene against the glycation reaction through the trapping of MG.

In conclusion, this is the first time that the green alga *C. pyrenoidosa* was reported as an inhibitor of key enzymes relevant for type-2 diabetes. Combing with its AGE blockade effect, the anti-diabetic property of *C. pyrenoidosa* has been strongly supported. Its free radical scavenging and/or metal chelating capacity is a major underlying mechanism. The green alga therefore might be considered as a beneficial functional food for diabetic patients.

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