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Effects of sources of carbon and nitrogen on production of α-glucosidase inhibitor by a newly isolated strain of *Bacillus subtilis* B2

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Abstract

This study examined production of α-glucosidase inhibitors by *Bacillus subtilis* B2 in Luria-Bertani (LB) fermentation with okara, soy powder, starch or pectin as additional source of carbon and nitrogen. All the fermentation broths of *B. subtilis* B2 exhibited gradual increase in α-glucosidase inhibitory activity during the fermentation process with or without supplemented source of carbon or nitrogen. Addition of okara into the LB medium greatly enhanced the strength (nearly twice as much of that without okara supplement) of α-glucosidase inhibitory activity of fermentation broth. The α-glucosidase inhibitory activity of *B. subtilis* B2 fermentation broth was positively correlated ($p < 0.05$) with the bacterial populations grown in LB medium containing okara. Glucose and sucrose were not detected in LB medium during the entire fermentation process and were both reduced drastically in media containing okara, soy powder, starch or pectin after 6 days of fermentation. The fermented LB medium containing okara by *B. subtilis* B2 possessed very strong α-glucosidase inhibitory activity and contained little glucose and sucrose, suggesting that fermentation of *B. subtilis* B2 in LB added with okara might be considered as a strategy for preparing functional foods for diabetic patients.

Keywords: α-Glucosidase inhibitor; *Bacillus subtilis* B2; Okara; Fermentation

1. Introduction

It is well known that α-glucosidases (EC 3.2.1.20, 3.2.1.10, 3.2.1.48 and 3.2.1.106) are exo-acting carbohydrases, which catalyze release of α-D-glucopyranose from the non-reducing ends of various carbohydrate substrates (Frandsen & Svensson, 1998). These enzymes play an important role in the biochemical processes of glycoproteins and glycolipids (Bertozzi & Kiessling, 2001). Presence of α-glucosidase inhibitor in diets can inhibit the activity of α-glucosidase and reduce absorption of dietary carbohydrates. Therefore, it has been proposed that α-glucosidase inhibitors might be useful in development of treatments for carbohydrate-mediated diseases, such as diabetes, certain forms of hyperlipoproteinemia and obesity (Baron, 1998; de Melo, da Silveira Gomes, & Carvalho, 2006). There has been increased interest in the past few years in identifying α-glucosidase inhibitors that can be used as an important tool to understand the biochemical processes and as prospective therapeutic agents (Faridmoayer & Scaman, 2005; Liu, Ma, Chen, Wang, & Xu, 2007; Markad, Karanjule, Sharma, Sabharwal, & Khavale, 2006).

Generally, the α-glucosidase inhibitors can be isolated naturally from plants or food products. However, they can also be synthesized chemically or produced by microorganisms. Several chemical synthetic compounds, such as

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sulfonamide, xanthone derivatives, and deoxy salacious, have been reported to exhibit inhibitory effects against α-glucosidase's activity (Liu et al., 2007; Muraoka et al., 2006; Seo et al., 2005). In addition, the salacinol extracted from *Salacia reticulata* (Yoshikawa, Morikawa, & Matsuda, 2002), the natural compound extracted from *Punica granatum* flower (Li et al., 2005) and the water extract from Chinese traditional food douchi (Chen, Cheng, Yamaki, & Li, 2007) also exhibited α-glucosidase inhibitory activity. Nevertheless, these natural α-glucosidase inhibitors are not easily produced in large scale (Chen et al., 2007; Fujita, Yamagami, & Ohshima, 2003; Li et al., 2005) while chemically synthesized α-glucosidase inhibitors normally cause hepatic disorders and other negative gastrointestinal symptoms due to their strong inhibitory effect (Murai et al., 2002).

Comparatively, synthesis by microorganisms is an effective strategy to produce cost-effective and productive α-glucosidase inhibitors. It has been reported that some microorganisms, including species of *Streptomyces* (Iwasa, Yamamoto, & Shibata, 1970), *Actinoplanes* (Schmidt et al., 1977) and *Flavobacterium saccharophilium* (Kameda, Asano, Teranishi, & Matsui, 1980), were able to synthesize α-glucosidase inhibitors. For example, acarbose, a pseudotetrasaccharide isolated from the fermentation broth of *Actinoplanes* spp. SE-50 (Schmidt et al., 1977), is a very popular α-glucosidase inhibitor and has been utilized as a medicine for treatment of type II insulin-independent diabetes (Brunkhorst, Wehmeier, Piepersberg, & Schneider, 2005). Due to microorganism's fast-growing characteristic, there has been increased interest in identifying α-glucosidase inhibitors from broth of certain microorganisms (Zheng, Xue, & Shen, 2006). However, little has been reported on production of α-glucosidase inhibitor by species of *Bacillus*.

In a previous study, we isolated *Bacillus subtilis* B2 capable of enhancing the antioxidant activity of fermented okara (Zhu, Cheng, Wang, Fan, & Li, in press; Zhu, Fan, Cheng, & Li, 2008). Recently, we found that *B. subtilis* B2 could also produce α-glucosidase inhibitors. However, the factors affecting the inhibitory activity are not yet clear. To this end, this study was conducted to determine factors that influence the activity of α-glucosidase inhibitor produced by *B. subtilis* B2 under various fermentation conditions. Activity of α-glucosidase inhibitor produced by *B. subtilis* B2 in different media broth after fermentation was analyzed. Meanwhile, the content of glucose and sucrose in the fermentation broth was investigated in order to know if they influence α-glucosidase inhibitory activity.

2. Materials and methods

2.1. Microorganism

B. subtilis B2 was isolated from Meitauza (a Chinese traditional fermented okara) in a previous study and identified by the Institute of Microbiology of Chinese Academy of Sciences (Beijing, China) using 16S rDNA PCR-RFLP. The bacteria culture was maintained on a nutrient agar slant (Oxoid) and stored at 4 °C. The culture was inoculated into 30 ml of Luria-Bertani (LB) medium (Oxoid) and allowed to grow at 40 °C for 16 h. The enriched culture was diluted with sterile distilled water containing 0.9% NaCl and 0.1% peptone to prepare a culture suspension of approximately 10^6 colony forming units (cfu)/ml.

2.2. Preparation of growth medium

Okara (4%), soy powder (4%), soluble starch (4%) or pectin (4%) was added to the LB medium to prepare four different growth media each containing different source of carbon and nitrogen for bacterial fermentation. LB medium without supplement was used as a control. The okara (24.3% of protein, 9.1% of fat, 61.5% of crude fiber and 5.1% of others) was prepared from soybeans (containing 40.4% of protein, 16.3% of fat, 40% of cellulose and 3.3% of others) harvested in 2005 at the Center of Soybean Research, Agricultural Academy of Jilin Province (Jilin, China). Soluble starch and pectin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The growth medium (30 ml) was placed in a 150 ml Erlenmeyer flask and sterilized at 121 °C for 20 min in an autoclave.

2.3. Production of α-glucosidase inhibitor by B. subtilis B2

Sterilized growth medium was inoculated with 1% (v/v) of the *B. subtilis* culture suspension and incubated at 40 °C with shaking at 150 rpm for 6 days. Growth of *B. subtilis* and production of α-glucosidase inhibitor in culture broth were analyzed daily. Four milliliters of the culture broth were taken from the flask. One half of the test sample was used for microbiological analysis and the other half was used for analysis of α-glucosidase inhibitor activity.

2.4. Determination of α-glucosidase inhibitory activity

For analysis of α-glucosidase inhibitor activity, the broth culture was centrifuged at 3000*g* for 15 min at 4 °C and the supernatant was filtered through a 0.45 μm membrane under vacuum (Millex-HX, Millipore, Yonezawa, Japan). The filtrate was collected as fermentation broth and analyzed for α-glucosidase inhibitory activity. The inhibitory activity of the fermentation broth against α-glucosidase was determined by reaction between α-glucosidase and 4-nitrophenyl α-D-glucopyranoside (4-PNP, Sigma Chemical Co., St. Louis, MO, USA) according to the protocol using a microtiter plate (0.4 ml × 96 wells flat bottom, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) as described by Yamaki and Mori (2006). The fermentation broth was serially diluted with an equal volume of distilled water and dispensed into wells of the microtiter plates (20 μl per well) followed by addition of 50 μl of rat α-glucosidase (25 mg/mL) (Sigma Chemical Co., St. Louis, MO, USA), 50 μl of 4-NPG (0.91 mg/mL) and 120 μl of 0.5 M phosphate buffer (pH 6.7). The mixture was incubated

at 37 °C for 45 min to allow reaction between the α-glucosidase with 4-NPG and produce 4-nitrophenol. The reaction was terminated with addition of sodium carbonate (50 μl, 0.67 M). Formation of 4-nitrophenol in each well was measured by the intensity of absorbance at 405 nm using a microplate reader (Model 550, BIO-RAD Lab., Tokyo, Japan). The α-glucosidase inhibitory activity of fermentation broth was then computed as the slope value from the curve of absorbance versus concentration of the fermentation broth. A higher slope value means stronger α-glucosidase inhibitory activity of the fermentation broth.

2.5. Determination of growth of *B. subtilis*

The culture broth taken during the fermentation process was serially diluted (1:10) with sterile distilled water and 1 ml of each dilution was mixed with 15 ml of molten (45 °C) nutrient agar and poured into a petri dish. After solidification, the Petri dish was incubated at 37 °C for 24 h. Colonies formed on the plate after incubation were counted and results were recorded as cfu/ml.

2.6. Determination of glucose and sucrose

The content of glucose and sucrose in the fermentation broth was assessed using the method of sucrose/D-glucose test-combination by ultraviolet (UV) with a commercial kit (R-Biopharm AG, Darmstadt, Germany).

2.7. Statistical analysis

All tests and analyses were run in triplicate and results were means of triplicate determinations. Correlation analysis and its significance ($p = 0.05$) were carried out using SAS (Version 8.0; SAS Inst., Cary, NC, USA.).

3. Results and discussion

3.1. The α-glucosidase inhibitory activity

Although LB is suitable for the growth of *B. subtilis* B2, production of α-glucosidase inhibitor by the strain may be affected by sources of carbon or nitrogen. This study examined the effects of sources of carbon (starch and pectin) and nitrogen (soy powder and okara) on α-glucosidase inhibitor production by *B. subtilis* B2 in LB medium. The α-glucosidase inhibitory activity of fermentation broth determined for each growth medium was shown in Fig. 1. In general, α-glucosidase inhibitory activity of *B. subtilis* B2 fermentation broth increased during the fermentation regardless of the source of carbon or nitrogen. After the first day of fermentation, all fermentation broths (except the one obtained from LB medium supplemented with pectin) showed drastic increases in α-glucosidase inhibitory activity. The α-glucosidase inhibitory activity of the fermentation broths obtained from LB medium with starch, soy powder or okara continued to increase until the third day with a slope maintained

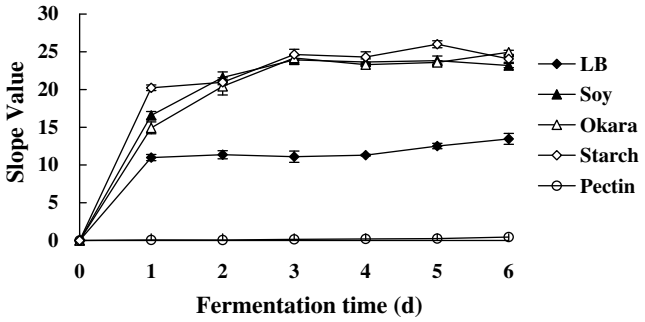

Fig. 1. The α-glucosidase inhibitory activity of the fermentation broth in different media. Values represent the means + standard deviation (SD) of $n = 3$ duplicate assays.

at over 23 until the end of fermentation. However, the α-glucosidase inhibitory activity of the fermentation broth obtained from LB medium without supplement only increased slightly until it reached its maximum slope of 13 after 6 days. The α-glucosidase inhibitory activity of *B. subtilis* B2 in the fermentation broth from the growth medium with okara increased to a slope of 24.9 after 6 days, which is nearly twice that in LB medium. On the other hand, the α-glucosidase inhibitory activity of *B. subtilis* B2 in fermentation broth obtained from the growth medium containing pectin was very low with a slope of 0.774, which was significantly lower ($p < 0.05$) than those detected in other media.

It has been suggested that different carbon and nitrogen sources may play an important role in the synthesis of α-glucosidase inhibitor because they may affect the synthesis of some enzymes related to the α-glucosidase inhibitor. In this study, as shown in Fig. 1, sources of carbon and nitrogen both had effects on the synthesis of α-glucosidase inhibitor by *B. subtilis* B2, consistent with a previous report by Zheng et al. (2006) who reported that different sources of nitrogen could affect yields of valienamine produced from *Stenotrophomonas maltrophilia*. All of the supplements added to LB, with the exception of pectin, conspicuously enhanced the production of α-glucosidase inhibitor as evidenced by strong α-glucosidase inhibitory activity of the fermentation broth. Among them, okara appears to be the best candidate because of the relatively low cost and its effectiveness in enhancing production of α-glucosidase inhibitor (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004; Ma, Liu, Kwok, & Kwok, 1997; O'Toole, 1997).

In a previous study, the aqueous extract of Chinese traditionally fermented soybean product (douchi) was found to possess strong anti-α-glucosidase activity with a slope of 13.9 (Chen et al., 2007). In our study we found that the α-glucosidase inhibitory activity of the fermentation broth obtained from growth of *B. subtilis* B2 in LB exhibited a similar strength (13.1) to that of the extract of commercial douchi. Moreover, the strength of the activity was enhanced to higher than 23 when okara, soy or starch was added to LB medium for fermentation. In the production of commercial douchi by fermentation using *Aspergillus oryzae*, *Actinomucor elegans* and *Rhizopus arrhizus*, very

low activity of α-glucosidase inhibitor was found during the first 48 h of fermentation. However, high activity of α-glucosidase inhibitor was observed in fermentation broth after growth of *B. subtilis* B2 in LB medium supplemented with starch, soy powder or okara. These results suggested that microorganisms played a key role in α-glucosidase inhibitor production. In addition, production of the inhibitor by microorganisms could be affected by growth substrates as well as sources of carbon and nitrogen.

3.2. Growth of B. subtilis B2 during fermentation process

In order to examine whether the α-glucosidase inhibitor comes from the media or is a metabolic product of *B. subtilis* B2, growth of *B. subtilis* B2 during fermentation was determined and analyzed for correlation with α-glucosidase inhibitory activity. *B. subtilis* B2 grew rapidly in all media except the one supplemented with pectin during the first 2 days and the populations all increased to $>10^9$ cfu/mL after 3 days of fermentation before entering a declining stage (Fig. 1). Populations of *B. subtilis* B2 in LB medium supplemented with pectin decreased considerably in the first day and increased slowly in the following 2 days and maintained at a similar level after that, indicating that pectin seems not to be a suitable carbon source for growth of *B. subtilis* B2. The high viscosity of the pectin culture might be the reason for the retarded growth of *B. subtilis* B2. From Fig. 2, starch seems to be a good supplemental carbon source while the soy and okara may be a good supplemental nitrogen source for the growth of *B. subtilis* B2.

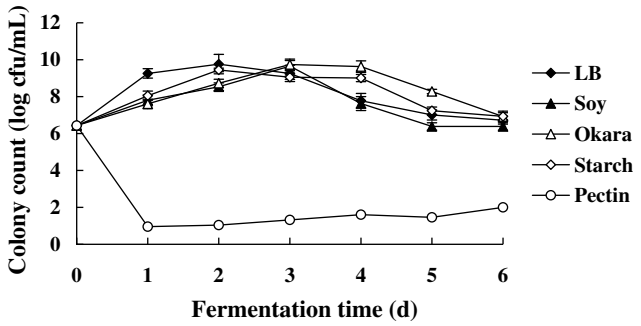

Fig. 2. The colony amount (cuf/mL) of the fermentation broth in the different media. Values represent the means + standard deviation (SD) of $n = 3$ duplicate assays.

Table 1
The correlation between microbial amount and the α-glucosidase inhibitory activity

Media type	LB	Soy	Okara	Starch	Pectin
R square	0.993	0.878	0.996	0.836	0.262
Significant factor	0.003	0.062	0.049	0.085	0.488

Analysis of growth of bacteria and α-glucosidase inhibitory activity of the fermentation broth showed that the α-glucosidase inhibitory activity was positively correlated with the growth of the bacteria in all media except the pectin-supplemented one (Table 1). The lack of correlation between the growth of bacteria and the α-glucosidase inhibitory activity in the media with pectin is due to the negative growth of *B. subtilis* B2 in the pectin medium (Fig. 2). These results suggested that α-glucosidase inhibitors appeared to be extracellular metabolic products of the *B. subtilis* B2 or enzymatic products of ingredients of the growth media formed by activities of enzymes excreted by *B. subtilis* B2. It has been reported that acarbose, α-glucosidase inhibitors, was the secondary metabolite of *Actinoplanes* spp. SE-50 (Schmidt et al., 1977; Wehmeier & Piepersberg, 2004). However, the actual mechanism of the formation of α-glucosidase inhibitors during *B. subtilis* B2 fermentation remains unclear.

3.3. Contents of glucose and sucrose

Results of analysis of glucose and sucrose in the fermentation broth were reported in Table 2. Glucose or sucrose was not detected in LB broth during the entire fermentation process. In the initial media (zero fermentation point) with supplemental carbon or nitrogen sources, the glucose content was over 40 mg/L and the sucrose content in soy and okara reached 1989.7 mg/L and 1043.5 mg/L, respectively. The concentrations of glucose and sucrose decreased with extension of the fermentation, suggesting they were utilized by the *B. subtilis* B2 as carbon sources.

In this study, most of the glucose and sucrose in the medium were depleted by *B. subtilis* B2 at the end of fermentation. However, the fermentation broth still exhibited strong α-glucosidase inhibitory activity which indicated

Table 2
The glucose and sucrose content in the fermentation broth

Media type	Glucose (mg/L)			Sucrose (mg/L)		
	0 day	3 days	6 days	0 day	3 days	6 days
LB	ND	ND	ND	ND	ND	ND
Soy	52.34 ± 1.18	16.39 ± 1.27	2.61 ± 0.21	1989.71 ± 2.13	13.79 ± 0.65	ND
Okara	56.10 ± 0.91	6.74 ± 0.52	1.96 ± 0.08	1043.52 ± 1.96	5.74 ± 0.21	ND
Starch	40.51 ± 0.88	22.28 ± 0.83	3.45 ± 0.53	14.28 ± 0.26	ND	ND
Pectin	82.31 ± 1.33	84.12 ± 1.67	73.70 ± 1.07	53.83 ± 1.03	22.16 ± 0.42	5.90 ± 0.11

ND means not determined.
Values represent the means + standard deviation (SD) of $n = 3$ duplicate assays.

that neither glucose nor sucrose in growth media contributed to α-glucosidase inhibitory activity. As we know, glucose and sucrose are usually limited in the diet of diabetic patients (Lormeau et al., 2005). Thus, the development of functional foods containing no glucose or sucrose would benefit patients with diabetes. In this study, we found that the fermentation broth of LB plus okara using *B. subtilis* B2 possessed strong α-glucosidase inhibitory activity with little glucose and sucrose. Therefore, fermentation of LB plus okara by *B. subtilis* B2 might be used in preparation of a functional food for diabetic patients.

Several α-glucosidase inhibitors have been isolated from the fermentation broth of certain microorganisms. For example, validamycin A is the main component isolated from growth of *Streptomyces hygroscopicus* var. *limoneus* and is a pseudotrisaccharide containing a valienamine moiety (Iwasa et al., 1970). Valienamine could also be produced from validamycin A by microorganisms such as *Pseudomonas denitrificans* (Kameda, Horri, & Yamino, 1975), *Flavobacterium saccharophilium* (Kameda et al., 1980), *Pseudomonas* spp. (Zheng, Zhang, & Shen, 2005), and *Stenotrophomonas maltrophilia* (Zheng et al., 2006). Other compounds such as adiposin, trestatin B, cyclophellitol and CKD-711 have been also isolated from growth of *Streptomyces calvus* TM-521, *Streptomyces dimorphogenes*, and *Phellinus* spp. However, it is noted that all these α-glucosidase inhibitory compounds were isolated from the growth of fungi, whereas very limited information is available on production of α-glucosidase inhibitors by *Bacillus* spp. The strong α-glucosidase inhibitory activity of the fermentation broth of *B. subtilis* B2 observed in this study indicates *B. sublitis* B2 can be used for production of α-glucosidase inhibitors.

As a matter of fact, most of the effective α-glucosidase inhibitors, such as validamycin A, acarbose and validamine, isolated from growth of microorganisms are carbasugars and pseudoaminosugars inhibitors (de Melo et al., 2006; Iwasa et al., 1970; Wehmeier & Piepersberg, 2004; Zheng et al., 2006). In addition, certain peptidic α-glucosidase inhibitor (Tyr-Tyr-Pro-Leu) derived from sardine muscle hydrolyzate and the synthetic analogue peptide (Tyr-Pro-Gly) have been also reported (Matsui, Oki, & Osajima, 1999). In this study, although there is not enough evidence to determine which kind of materials the inhibitor belongs to, further work is in progress to identify the α-glucosidase inhibitor produced by *B. subtilis* B2 and to analyze the mechanism of formation.

4. Conclusions

The α-glucosidase inhibitor could be produced by *B. subtilis* B2 and its production by the bacteria could be affected by sources of carbon and nitrogen. Addition of okara to the growth medium greatly enhanced the production of α-glucosidase inhibitor by *B. subtilis* B2. Fermentation of LB plus okara by *B. subtilis* B2 might be used for production of α-glucosidase inhibitors and the fermented broth containing low glucose and sucrose could be used for preparation of functional foods for diabetic patients.

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