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Enhancement of water soluble wheat bran polyphenolic compounds using different steviol glucosides prepared by thermostable β-galactosidase

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ABSTRACT:

Background: Production of wheat bran (WB) for human consumption is estimated to be about 90 million tons per year. WB contains an abundant source of dietary fiber, minerals, vitamins, and bioactive compounds. WB is a by-product of milling and contains an abundant source of carbohydrate (60%), protein (12%), fat (0.5%), minerals (2%), and bioactive compounds such as phenolic acids, arabinoxylans, flavonoids, caroteinoids alkylresorcinol and phytosterols. These are known for health promoting properties such as controlling glycemic index, reducing plasma cholesterol level, antioxidant, anti-inflammatory, and anticarcinogenic activities. Several terpene glycosides such as mogroside V, paenoiflorin, geniposide, rubusoside (Ru), stevioside (Ste), rebaudioside A (RebA), steviol monoside, and stevioside glucoside have been discovered to enhance the solubility of a number of pharmaceutically and medically important compounds that normally show poor solubility in water.

Context and purpose of this study: In this study, in order to increase soluble extraction of polyphenol compounds of WB using Ru, the expression of β-galactosidase from *Thermus thermophilus* (*T. thermophilus*) was optimized using different *E. coli* hosts and a different concentration of lactose inducer rather than of isopropyl-1-thio-β-D-galactopyranoside (IPTG) for industrial production. Additionally, the effect of different steviol glucosides (Ru, Ste, RebA, and SG) on the enhancement of polyphenol compounds extraction from wheat bran was studied.

Results: β-galactosidase from *T. thermophilus* was used for the specific conversion of stevioside (Ste) to rubusoside (Ru) with 92% productivity. The enzyme was optimized to be expressed in *E. coli.* With 7 mM lactose, the β-galactosidase activity expressed was 34.3, 14.2, or 34.4 ± 0.5 U/mL in *E. coli* BL21(DE3)pLysS, Rosetta(DE3)pLysS, or BL21(DE3) at 37°C, and 9.8 ± 0.2, 7.0 ± 0.5, or 7.4 ± 0.2 U/mL at 28°C respectively. The expression of β-galactosidase was dependent on the lactose concentration and the highest activity was obtained with the conditions of 5 mM lactose in *E. coli* Rosetta(DE3)pLysS, 53.3 ± 1.5 U/mL. 78% of the mesophilic proteins was eliminated by heating at 70°C for 15 min with 89% β-galactosidase activity recovery. The total polyphenol content of WB extracted by water, Ru, Ste, rebaudioside A (RebA), and steviol glucosides (SG) were 533.8 ± 9.6 μg/mL, 633.3 ± 1.25 μg/mL, 604.4 ± 10.1 μg/mL, 654.8 ± 26.5 μg/mL, and 601.2 ± 33.4 μg/mL, respectively. The DPPH radical scavenging activity prepared by water, Ru, Ste, RebA, and SG extraction were 8.76 ± 0.3 mg/mL, 4.87 ± 0.3 mg/mL, 5.34 ± 0.22 mg/mL, 7.27 ± 0.1 mg/mL, and 7.82 ± 0.02 mg/mL respectively.

Conclusions: To increase soluble extraction of polyphenol compounds of WB using Ru, the expression of β-galactosidase from *T. thermophilus* was optimized using different *E. coli* hosts and a different concentration of lactose inducer rather than isopropyl-1-thio-β-D-galactopyranoside (IPTG) for industrial production of the enzyme. The highest antioxidant activity was shown in WB extracted by Ru. The number of glucosyl units attached to steviol can possibly affect the efficiency of antioxidant activity of WB extracted by steviol glucosides.

Keywords: Rubusoside, β-galactosidase, lactose induction, immobilized enzyme, wheat bran, steviol glucosides

INTRODUCTION:

Wheat (*Triticum aestivum*) is a leading cereal crop primarily used for human consumption and livestock feed. A wheat kernel is comprised of three principal fractions: bran, germ, and endosperm, which makes up about 14-19% of the whole wheat kernel [1]. Wheat bran (WB) is a by-product of milling and contains abundant source carbohydrate (60%), protein (12%), fat (0.5%), minerals (2%), and bioactive compounds such as phenolic acids, arabinoxylans, flavonoids, caroteinoids alkylresorcinol and phytosterols [1-3]. They are known for health promoting properties including controlling glycemic index, reducing plasma cholesterol level, and reducing the human colon cancer cell growth antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic activities [4-7].

Several terpene glycosides such as mogroside V, paenoiflorin, geniposide, rubusoside (Ru), stevioside (Ste), rebaudioside A (RebA), steviol monoside, and stevioside glucoside have been

discovered to enhance the solubility of a number of pharmaceutically and medically important compounds which normally show poor solubility in water [8-10]. Steviol glucosides such as Ru, Ste, and RebA are the main sweet components of *Rubus suavissimus* S. Lee (*Rosaceae*) and *Stevia rebaudiana* Bertoni leaves [11]. Ste and RebA are isolated and extracted from the *Stevia rebaudiana* (bertoni) plant commercially cultivated in Japan, Singapore, Taiwan, South Korea, China, Israel, India, Brazil, Australia, and Paraguay [11]. Although Ru is not commercially available, Nguyen et al have developed a facile enzymatic process for preparing Ru from Ste by using β-galactosidase from *T. thermophilus* [12].

In this study, to increase soluble extraction of polyphenol compounds of WB using Ru, the expression of β-galactosidase from *T. thermophilus* was optimized with different *E. coli* hosts and with different concentrations of lactose inducer rather than isopropyl-1-thio-β-D-galactopyranoside (IPTG) for industrial production of the enzyme. Additionally, the effect of different steviol glucosides (Ru, Ste, RebA, and SG) on the enhancement of polyphenol compounds extraction from wheat bran was studied.

MATERIALS AND METHODS:

Preparation of ß-galactosidase: The β-galactosidase gene, β-glypi, was ligated into the *XhoI/EcoRI*-digested pRSETB (pRSETB-β-GLYPI) vector as described in the previous study [12]. pRSETB-β-GLYPI was transformed and expressed in *E. coli* BL21(DE3)pLysS, *E. coli* Rosetta(DE3)pLysS, and *E. coli* BL21(DE3). Cells were grown in 100 mL LB media containing 0.5% (w/v) yeast extract, 1.0% (w/v) tryptone, 1.0% (w/v) NaCl, and supplemented with ampicilin (50 μg/mL) in 500 mL flasks at 37°C. The cells were induced with 7 mM lactose when the optical density reached (OD_{600}) 0.5 with 15 hrs of shaking. The cells were collected by centrifugation (8000 x g for 30 min at 4°C), resuspended in 50 mM Tris-HCl pH 7.0, and then lysed via sonication (Ultrasonic processor 250, Sonics and Materials, Inc., Newtown, CT, USA; output 30, for 1 min, 4 repeat on ice). After centrifugation (12,000 x g for 30 min), the clarified cell lysate was checked for enzyme activity using lactose as the substrate. The reaction mixture containing 200 mM lactose and enzyme in 50 mM Tris-HCl pH 7.0 was reacted at 70°C for 20 min. The mixture was analyzed by thin layer chromatography (TLC) using precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany) developed in a solvent system consisting of acetonitrile:water [85:15 (v/v)]. The plates were visualized by dipping into a solvent mixture of 0.5% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol and heating at 120°C for 10 min. The concentration of glucose released by β-galactosidase was analyzed using the integrated density values (IDV) derived by the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA) with glucose standard. One unit (U) of galactosidase activity is defined as the amount of enzyme required to release 1 μmol glucose per minute under the above reaction conditions. *E. coli* BL21(DE3)pLysS was selected to determine the effect of lactose concentration for enzyme expression. The induction was carried out as described above with varying concentrations of lactose for induction (1 ~ 7 mM) at 37°C for 15 hrs.

Large scale production of β-galactosidase was carried out in 19 L fermenter (Bioengineering AG., Riki, Switzerland) that contained 14 L LB media. The fermentation conditions were 37°C, 250 rpm, and an aeration rate of 2 vvm. Cells were grown at 37°C until OD_{600} reached 0.6, at which point 5 mM lactose was added; the cells were harvested after 15 hrs. The cells were collected by

centrifugation (7,871 x g for 30 min at 4°C), re-suspended in 500 mL of 50 mM Tris-HCl pH 7.0, and disrupted by sonication. The clarified cell lysate was obtained after centrifugation for 30 min at 12,000 x g and partially purified β-galactosidase was heat treated at 70°C for 15 min. Heat-denatured proteins were removed by centrifugation at 8,000 x g for 15 min. The supernatant was freeze dried for further study. The protein concentration was determined by the Bradford method with crystalline bovine serum albumin as the standard.

Production of rubusoside using double jacket columns: The β-galactosidase bead was prepared as described in the previous study [12] by mixing 300 U/mL of β-galactosidase with 2.5% (w/v) sodium alginate solution. The double jacket column reactors [Condenser, Liebig, with joint, 30 x 3 (cm) in diameter] were filled with β-galactosidase beads. The reaction temperature was controlled using a heat circulator (EYELA, NCB-1200, Tokyo, Japan) at 70°C. The reaction mixture containing 0.5% of Ste solution was pumped into the columns using a mini pump at the speed of 0.01 mL per min for 45 g Ste per day. The reaction mixture passed through columns and the fractions were collected into Eppendorf tubes. The Ru mixture was analyzed by TLC measuring integrated density values (IDV) derived from the AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA) with purified Ru as the standard. Ru was purified as previously reported [12] by using Reveleris® Amino 120 g Flash Cartridges (Grace Discovery Science, Shanghai, China) with an evaporative light scattering detector (ELSD, Grace Discovery Science, Shanghai, China). A mixture of acetonitrile and water was used as an eluent with the gradient from 99:1 (v/v) to 50:50 (v/v) of acetonitrile: water at a flow rate of 60 mL/min at room temperature. The purified Ru was collected and freeze dried for further study.

Extraction of wheat bran using different steviol glucosides: Wheat bran was kindly supplied by the Sajo Donga One Corporation (Seoul, Korea). RebA and SG with α-1-4 linkages were provided by Daepyung Co., Ltd (Gyeonggi-do, Korea). WB-Ru (wheat bran powder treated with Ru to solubilize polyphenol), WB-Ste (wheat bran powder treated with Ste to solubilize polyphenol), WB-Reb (wheat bran powder treated with RebA to solubilize polyphenol), and WB-SG (wheat bran powder treated with SG to solubilize polyphenol) solutions were prepared as previously reported [10, 12] with some modifications. Each Ste, RebA, or SG at 10% (w/v) and Ru at 2% (w/v) was mixed with 20% (w/v) of WB powder, followed by the addition of ethanol. The mixture in ethanol solution was vortexed for 15 min and centrifuged at 12,000 x g for 10 min. The supernatant was transferred to a new tube and ethanol was evaporated [10, 12]. The resulting powders were dissolved in water, centrifuged at 12,000 x g for 10 min, and filtered through 0.20 μm membrane (Agilent, Santa clara, CA, USA). TLC with an ascent of acetonitrile/water 85:15 (v/v) was used for the analysis of compounds. Polyphenol compounds on silica gel $60F_{254}$ TLC plate (Merck, Darmstadt, Germany) were visualized under UV_{254nm} and by dipping the TLC plate into a solvent mixture of 0.5 (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 121^{o}C for 10 min. The WB extracted using the same method but without the addition of steviol glucosides served as the control.

Determination of total phenols content: The total polyphenols content in each extraction sample was determined by using Folin-Ciocalteu reagent with gallic acid as the standard (Sigma) [13]. 120 µL of samples and gallic acid (1 ~ 50 µg/mL) were added into the 96 well plate and 15 µL Follin-Ciocalteu reagent (Sigma) was mixed for 3 min. 15 µL of 10 % (w/v) Na_2CO_3 was then added and reacted with the samples for 30 min at dark condition. The phenol content was determined using SpectraMax M3 (Molecular Devices, Sunnyvale, CA, USA) at 760 nm.

Antioxidant activity: The antioxidant activities of water soluble polyphenol of WBs prepared with different steviol glucosides were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [14]. Samples were dissolved in water and mixed with 100 µM (DPPH) in methanol solution to give a final concentration of 0.2 to 12 mg WB extracted powder by Ste, Ru, Reb, or SG /mL. After 30 min of incubation at 25°C in total darkness, the absorbance of each mixture was measured at 517 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). A mixture containing all reagents except the test sample was used as a negative control for this test. DPPH radical-scavenging activity was converted to percentage of antioxidant activity using the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{(Absorbance\ of\ control - Absorbance\ of\ test\ sample)}{Absorbance\ of\ control}\ x\ 100$$

A linear regression curve was established in order to determine the SC_{50} value which is the amount of sample necessary to decrease 50% of the DPPH absorbance. All analyses were carried out in triplicates. Results were expressed as mean ± standard deviation (SD). Statistical significance was considered when $p<0.05$.

RESULTS AND DISCUSSION:

Optimization for the expression of β-galactosidase: Table 1 shows the β-galactosidase activity from *T. thermophilus* expressed in *E. coli* BL21(DE3)pLysS, *E. coli* Rosetta(DE3)pLysS, and *E. coli* BL21(DE3) at 28°C or 37°C. The expressed β-galactosidase activity was 34.3, 14.2, or 34.4 ± 0.5 U/mL in *E. coli* BL21(DE3)pLysS, *E. coli* Rosetta(DE3)pLysS, or *E. coli* BL21(DE3) at 37°C, and 9.8 ± 0.2, 7.0 ± 0.5, or 7.4 ± 0.2 U/mL at 28°C, respectively. The β-galactosidase activity at 37°C induction was 3.5 fold greater in *E. coli* BL21(DE3)pLysS expression, 2.0 fold greater in *E. coli Rosetta*(DE3)pLysS expression, and 4.7 fold greater in *E. coli* BL21(DE3) expression than those at 28°C induction. The *E. coli* BL21(DE3)pLysS was selected for the investigation of the lactose concentration effect of the β-galactosidase expression (Table 2) based on the similarity in β-galactosidase activity expressed in *E. coli* BL21(DE3)pLysS and *E. coli* BL21(DE3). β-galactosidase activity was increased from 19.1 ± 0.7 U/mL to 53.3 ± 1.5 U/mL when the concentration of lactose inducer was increased from 1 to 5 mM. For lactose induction concentrations above 5 mM, the β-galactosidase activity expressed was decreased to 48.4 ± 1.7 mM. Hence, the selected lactose concentration for induction was 5 mM.

Table 1. *β*-galactosidase activity expressed from different *E. coli* at 7 mM lactose induction

Temperature	*E. coli* BL21 (DE3)pLysS (U/mL)	*E. coli* *Rosetta*(DE3)pLysS (U/mL)	*E. coli* BL21 (DE3) (U/mL)
28°C	9.8 ± 0.2	7.0 ± 0.5	7.4 ± 0.2
37°C	34.3 ± 0.0	14.2 ± 0.3	34.4 ± 0.5

One unit (U) of β-galactosidase activity is defined as the amount of enzyme required to release 1 μmol glucose per min under the above reaction conditions.

Table 2. *β*-galactosidase activity expressed from *E. coli* BL21(DE3)pLysS with lactose induction

Lactose concentration (mM)	Activity (U/mL)
1	19. 1 ± 0.7
2	22.3 ± 2.2
3	46.6 ± 0.0
5	53.3 ± 1.5
7	48.4 ± 1.7

The reaction mixture containing 200 mM lactose, enzyme in 50 mM Tris-HCl pH 7.0 was reacted at 70°C for 20 min. One unit (U) of β-galactosidase activity is defined as the amount of enzyme required to release 1 μmol glucose per min under the above reaction conditions.

Compared to mesophilic enzymes, thermostable enzymes exhibiting maximal activity at temperatures ranging from 70 to 90°C have significant advantages, including greater reaction velocity, reduced risk of microbial contamination, and longer enzyme half-lives under operational conditions [15, 16]. After the heating of crude β-galactosidase solution, 78% of the mesophilic proteins were eliminated with 89% β-galactosidase activity recovery (Table 3). The partially purified β-galactosidase demonstrated a specific activity of 23 U/mg corresponding to a 4-fold purification.

Table 3. Partial purification of ß-galactosidase

	Total volume (mL)	Total protein (mg)	Total unit (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	2,000	35,814.9	204,000	5.7	1	100
After Heat treatment	1,650	7,885.0	181,500	23.0	4	89.0

Partially purified β-galactosidase was heat treated at 70°C for 15 min. Heat-denatured proteins were removed by centrifugation at 8,000 x g for 15 min. The reaction mixture containing 200 mM lactose, enzyme in 50 mM Tris-HCl pH 7.0 was reacted at 70°C for 20 min. One unit (U) of β-

galactosidase activity is defined as the amount of enzyme required to release 1 μmol glucose per min under the above reaction conditions.

Production of rubusoside using immobilized *β*-galactosidase on double jacket columns: The free enzyme reaction was carried out by mixing 0.5% (w/v) Ste and 300 U/mL in 50 mM Tris-HCl pH 7.0 at 70°C in a water bath for 12 hrs. By using free enzyme, the amount of Ru produced was 3.3 g/L and the Ste conversion was 74.3%. For the double jacket column, the reaction was carried for 31 days. The amount of Ru released from Ste is shown in Table 4. The total amount of Ru produced was 738.67 g for 31 days. Immobilized enzyme demonstrated 77% activity up to 21 days and then the activity gradually reduced in the span of 31 days, while still maintaining 51.4% β-galactosidase activity (Table 4).

Total polyphenol content of wheat bran extract: Figure1 shows the total polyphenol content of the WB extracts measured using the Folin-Ciocalteu method. Total polyphenol values were obtained from the calibration curve, y = 0.0256 x – 0.0133 with R^2= 0.9993 where x is the absorbance and y is the concentration of gallic acid (GAE) solution (μg/mL). The total polyphenol values of the WB extracts were 533.8 ± 9.6 μg/mL for WB extracted by ethanol, 633.3 ± 1.25 μg/mL for WB extracted by Ru, 604.4 ± 10.1 μg/mL for WB extracted by Ste, 654.8 ± 26.5 μg/mL for WB extracted by RebA, and 601.2 ± 33.4 μg/mL for WB extracted by SG. The total polyphenol values of WB extracted by Ru, Ste, RebA, and SG were 1.19, 1.13, 1.23, and 1.13 fold greater than that of WB extracted by water. Interestingly, while 2% (w/w) of Ru was used for the extraction of polyphenol from WB powder and 10% (w/w) of Ste, RebA, and SG were used, the total polyphenol content of WB extracted by Ru (633.3 ± 1.25 μg/mL) was greater than those of WBs extracted by Ste (604.4 ± 10.1 μg/mL) and SG (601.2 ± 33.4 μg/mL) but lower than WB extracted by RebA (654.8 ± 26.5 μg/mL). These results suggest that Ru, Ste, RebA and SG could be used for enhancing the solubility of polyphenol compounds from WB extracted in water.

Table 4. Stability of the immobilized enzyme during double jacket columns reaction

Day*	The amount of Ru production** (g/ day)	Immobilized enzyme stability (%)
6	31.3 ± 5.3	100
8	26.7 ± 8.6	85.1
11	28.9 ± 3.5	92.3
12	26.1 ± 6.1	83.3
14	24.4 ± 6.0	77.9
16	25.5 ± 0.1	81.4
19	27.4 ± 3.1	87.3
21	24.4 ± 3.0	78.0
22	18.1 ± 0.9	57.9
28	16.9 ± 3.2	54.0
29	17.1 ± 0.1	54.5
31	16.1 ± 1.4	51.4

*The reaction was carried on for 1 month.

**45 g of Ste was totally used per day. The theoretical maximum amount of Ru production was 36 g per day.

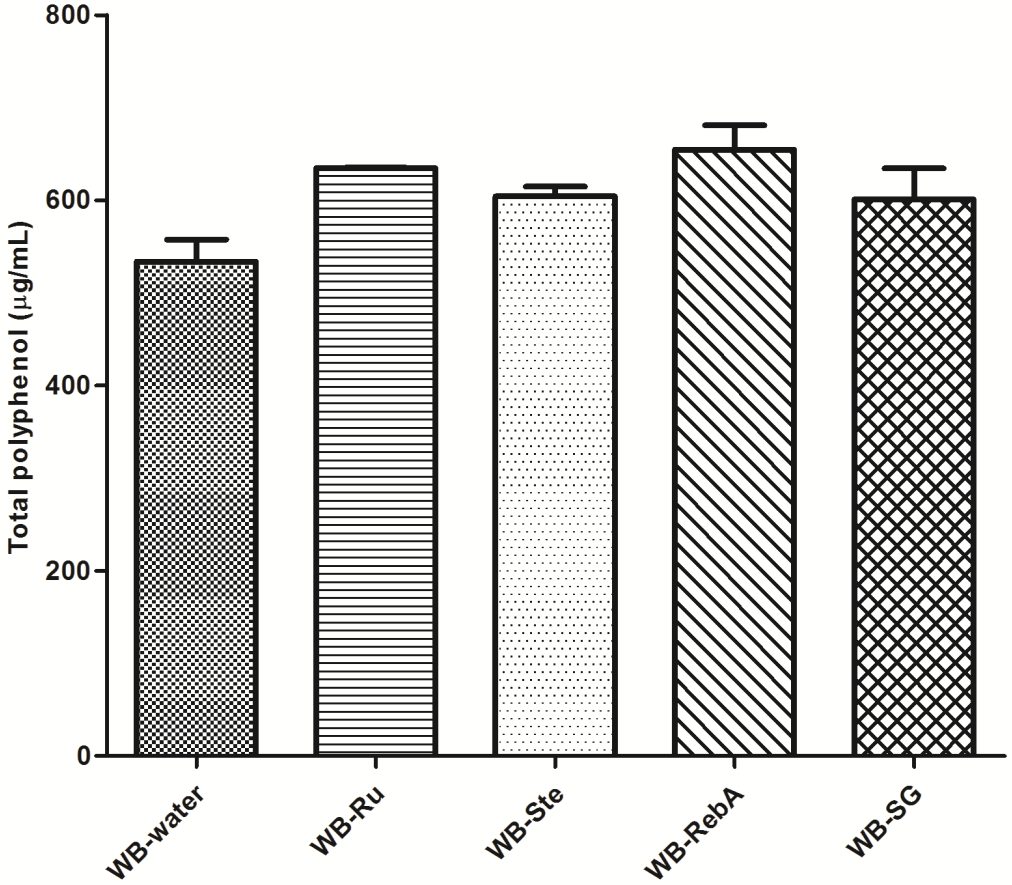

Fig. 1. The amount of water soluble polyphenol obtained from wheat bran powder by using different steviol glucosides.

Antioxidant activity: Antioxidant activity of water soluble WB extracted using different steviol glucosides was compared using a free radical-scavenging method of DPPH with SC_{50} values representing antioxidant activity. The SC_{50} values of water soluble WB prepared with water, Ru, Ste, RebA, and SG were 8.76 ± 0.3 mg/mL, 4.87 ± 0.3 mg/mL, 5.34 ± 0.22 mg/mL, 7.27 ± 0.1 mg/mL, and 7.82 ± 0.02 mg/mL, respectively (Figure. 2). The WB extracted by Ru, Ste, RebA, and SG showed higher antioxidant activity compared to WB extracted by water. The highest antioxidant activity was shown in WB extracted by Ru. The antioxidant activity order of WB extracted by steviol glucosides is as follows: WB-Ru > WB-Ste > WB-RebA > WB-SG. Ste, RebA, and SG are glycosides on aglycone's carbon skeleton of steviol. Ste, RebA, and SG have a D-glucose group affixed at C_{19}. Additionally, Ste has a di-glucosyl while RebA has a tri-glucosyl sugar moiety affixed at C_{13}. SG is glycoside mixture of steviol. An inverse relationship was demonstrated between the glucosyl unit and antioxidant activity. Thus, the number of glucosyl units attached to steviol can possibly affect the efficiency of antioxidant activity of WB extracted by steviol glucosides.

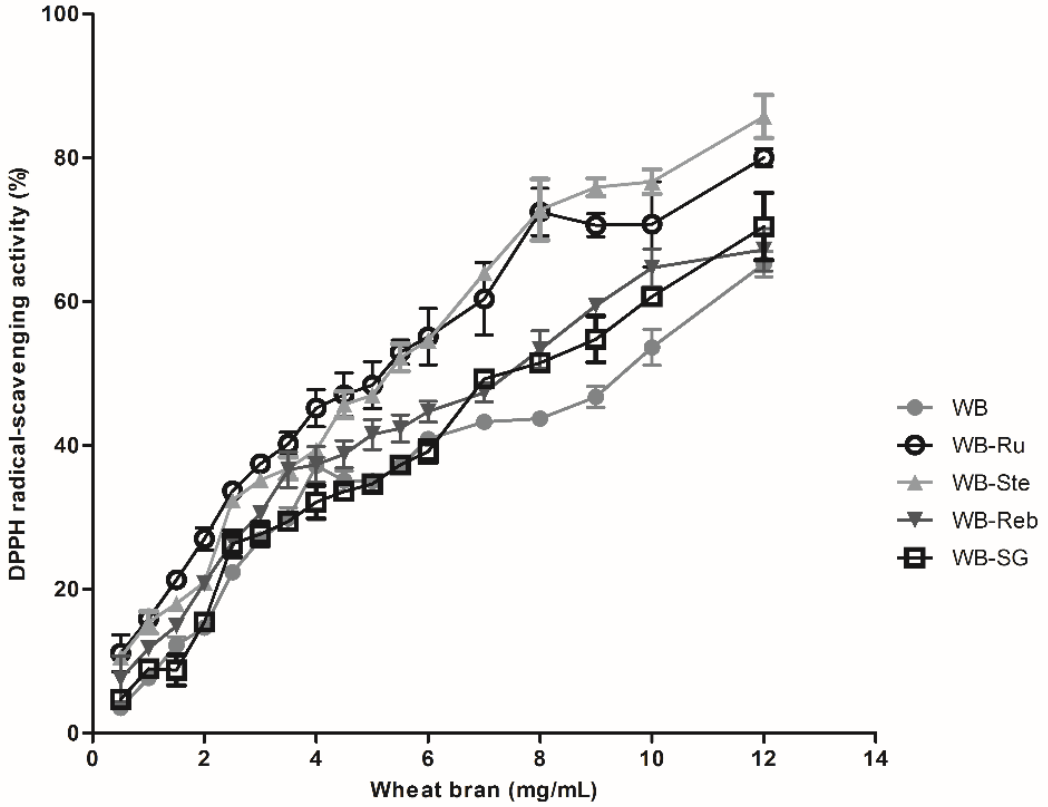

Fig. 2 The antioxidant activity of water soluble WB extracts prepared by using different steviol glucosides.

CONCLUSIONS: To increase soluble extraction of polyphenol compounds of WB using Ru, the expression of β-galactosidase from *T. thermophilus* was optimized using different *E. coli* hosts and a different concentration of lactose inducer rather than isopropyl-1-thio-β-D-galactopyranoside (IPTG) for industrial production of the enzyme. The highest antioxidant activity was shown in WB extracted by Ru. The number of glucosyl units attached to steviol can possibly affect the efficiency of antioxidant activity of WB extracted by steviol glucosides.

Abbreviation: WB, wheat bran; DMSO, dimethyl-sulfoxide; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; Reb A, rebaudioside A; Ste, stevioside; Ru, rubusoside; SG, steviol glucosides; SC, DPPH radical-scavenging activity; WB-Ru, wheat bran powder treated with Ru to solubilize polyphenol; WB-Ste, wheat bran powder treated with Ste to solubilize polyphenol; WB-Reb, wheat bran powder treated with RebA to solubilize polyphenol; WB-SG, wheat bran powder treated with SG to solubilize polyphenol

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