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Full Length Research Paper

Purification and characterization of *β*-glucosidase from newly isolated *Aspergillus* sp. MT-0204

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***Aspergillus* sp. MT-0204, a novel strain for producing *β*-glucosidase, was isolated from soil in this study. The *β*-glucosidase can hydrolyze glycosides and increase free isoflavones in soy products isoflavones. By examining physiological, biochemical characteristics and comparing its ITS rDNA gene sequence, it was identified as *Aspergillus niger*, and named *A. niger* MT-0204. The *A. niger* *β*-glucosidase was purified in four steps. Some characteristics of purified *β*-glucosidase were also studied. The *β*-glucosidase activity could be assayed at the pH ranging from 3.5 to 10.0, and its maximum activity was obtained between pH 5.0 and 5.5. The enzyme was sensitive to heat, decreasing slowly between 60 and 80°C, and significantly at 85°C. The enzyme activity was activated by K^+, Ca^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+}, while strongly inhibited by Ag^+ and Hg^+, and slightly inhibited by Cu^{2+}, Zn^{2+}, Ba^{2+}, Ni^+, EDTA-Na_2 and fumarate.**

Key words: *Aspergillus niger* MT-0204, *β*-glucosidase, characterization, isoflavones, soybean.

INTRODUCTION

Isoflavones are a group of diphenolic secondary metabolites produced in a very limited distribution of higher plants, most frequently in the Leguminosae (Dewick, 1994). The major types of isoflavones in soybean are daidzein, genistein, and glycitein, which are conjugated with glucose or malonylglucose in soybean seeds (Wang and Murphy, 1996). Moreover, the acetylglucose conjugates are also detected in small amounts in soy products, but it appears that acetyldaidzin and acetylgenistin may be generated from daidzin and genistin during their heat-induced decompositions (Xu et al., 2002).

From the natural food sources, soybeans and soy foods contain the most the highest level of dietary isoflavones, which provide many health benefits. It is reported that soybean isoflavones may have potential benefits for reducing the occurrences of diseases afflicting humans, such as certain types of breast, prostate, and colon cancer (Messina et al., 1994; Hendrich et al., 1994). Moreover, the soybean isoflavones also exhibit antiatherosclerotic, blood glucose lowering, antibacterial (Kwon et al., 1998), and antioxidative (Chung et al., 2000) properties. With the discovery of increased cancer risks associated with estrogen-based hormone replacement therapy, the use of isoflavones as an alternative for menopausal women has received much public and scientific interest (Albertazzi and Purdie, 2002; Barnes, 2003). It is also found that isoflavones may reduce low-density lipoproteins and increase high-density lipoproteins, which help to prevent coronary heart disease (Demonty et al., 2003). Because of these health benefits, there is interest in increasing the amounts of free isoflavones in soy products (Chuankhayan et al., 2007). The *β*-glucosidases from *Saccharopolyspora erythraea* could hydrolyze genistin during fermentation of soy-based media (Hessler et al., 1997), and *β*-glucosidase from *Bifidobacteria* in soy milk was capable of converting glucosides to their aglycones (Tsangalis et al., 2002). Pandjaitan et al. (2000) reported soy protein isolate with almond *β*-glucosidase to convert most of its isoflavone glucosides to their aglycones. However, it has been

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shown that *Escherichia coli* β-glucosidase was more effective than almond emulsion β-glucosidase, and neither of these enzymes could effectively hydrolyze malonylglucosyl isoflavone conjugates, even at high concentrations and extended times (Ismail and Hayes, 2005). Hsieh and Graham (2001) reported that some partially purified and characterized β-glucosidase from soybean that could hydrolyze isoflavone conjugates. Recently, isoflavonoid β-glycosidases have been described from the seeds of *Dalbergia cochinchinensis* Pierre and *D. nigrescens* Kurz (Srisomsap et al., 1996; Svasti et al., 1999; Chuankhayan et al., 2005). These enzymes had high hydrolytic activity on isoflavonoid glycosides from the same seeds. It could also hydrolyze genistin and daidzin, which are isoflavonoid 7-*O*-β-D-glucosides. It is reasoned that it is a promising approach to use β-glycosidases from microorganism origin. In the recent studies, β-glycosidases production by biocatalysis has been attracted a considerable research interest, because of its potential use in the industrial application, such as the improvement the conversion rate, the mild reaction conditions and easier separation of product from reaction broth as well as low cost for commercial production of free isoflavones. Finding the producing strains and investigating the function and application of β-glycosidases for increasing free isoflavones in soy products have become hot issue in this field.

The objective of this study was to isolate and identify a β-glucosidase producing strain *Aspergillus* sp. MT-0204. Physiological, biochemical characteristics, and ITS DNA sequence were employed to identify the strain. Moreover, the β-glucosidase from *Aspergillus* sp. MT-0204 was purified, and some characteristics of purified *Aspergillus* sp. MT-0204 β-glucosidase were also studied.

MATERIALS AND METHODS

Strains and cultivations

Aspergillus sp. MT-0204 was isolated and maintained on potato dextrose agar at 4℃ in our lab. The microorganism grows aerobically at 30℃ for 24 h in 50 mL medium containing soybean isoflavonoid glycosides 1%, bran 1.0%, peptone 2.0%, NaOAc 1%, NaH_2PO_4 0.5%, $MgSO_4$ 0.05%, sodium ascorbate 0.1%, salicin 0.05% and distilled water (pH at 6.0). This medium was autoclaved at 121℃ for 15 min. All the chemicals used in this study were of analytical grade.

Isolation of strains producing β-glucosidase

The plate medium containing soybean isoflavonoid glycosides 1%, bran 1.0%, peptone 2.0%, NaOAc 1%, NaH_2PO_4 0.5%, $MgSO_4$ 0.05%, sodium ascorbate 0.1%, salicin 0.05% (pH at 6.0) was used to isolate the strains producing β-glucosidase. Different strains including *Aspergillus* sp. MT-0204 were isolated based on their morphology from soil samples. After cultivation, the cells were used to produce β-glucosidase.

Identification of *Aspergillus sp.* MT-0204

The identification of the strain was based on standard morphological and physiological properties (Abrusci et al., 2005), and nucleotide sequence analysis of enzymatically amplified ITS rDNA. For the morphological characterization of the strain, observations were made with both an optical microscope and a scanning electron microscope (SEM). For the sequence analysis, chromosomal DNA was isolated according to the method described by Reader and Broda (Reader and Broda, 1985). Amplification was carried out with primer set pITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and pITS4 (5'– TCCTCCGCTTATTGATATGC -3') (White et al., 1990; Liu et al., 2008) in a thermal cycler (Bio-Rad, USA) under the following conditions: 5 min at 95℃, 35 cycles of 40 s at 95℃, 60 s at 55℃, 1 min at 72℃ and one final step of 10 min at 72℃. The PCR products were extracted and purified from the agarose gel using High Pure PCR Product Purification Kit (Roche, Germany). The resulting PCR fragments were ligated with pMD18-T (Takara, Japan) by using the T/A cloning procedure (Sambrook et al., 2001; Liu et al., 2007). The constructed vectors were transformed into the competent *E. coli* JM109 cells according to the method of Chung et al. (Chung et al., 1989), and then spread on the LB plate containing the X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside), IPTG (isopropyl-1-thio-β-D-galactoside) and ampicillin (50 μg mL^{-1}) (Liu and Sun, 2004). Subsequently a positive clone, designated *E. coli* JM109/pMD18-T-EPs, was obtained. DNA was sequenced on both strands with an Applied Biosystems Model 377 Bautomatic DNA sequencer, and a dye-labeled terminator sequencing kit (Applied Biosystems, Foster, CA, USA). The sequences obtained were compiled and compared with sequences in the GenBank databases using BLAST program.

Enzyme purification

The spore concentration was adjusted to 10^6 or 10^7 mL^{-1} and was used as the inoculums. 10% (v/v) inoculums were transferred into the fermentation medium. The culture was grown for 72 h at 28℃ in an orbital shaker (200 rpm). β-glucosidase activity and protein content were determined in samples from each purification step. Unless otherwise specified all steps were conducted at 4℃. The purification process employed a Biologic Duo Flow system (Bio-Rad). The washed cells were suspended in 50 mM Tris-HCl buffer (pH 8.0) and ruptured with sonication for 20 min with ice cooling. The supernatant resulted from centrifugation at 48,000 g for 30 min was used as the crude enzyme, and its activity was taken as 100% for calculation of recovery. Solid ammonium sulfate was added, over ice, into the crude extract to 30% saturation; after centrifugation (10,000 g, 20 min), ammonium sulfate was added to bring the supernatant to 80% saturation. The latter was stored overnight at 4℃ and then centrifuged. The precipitate was redissolved and dialyzed against several changes of 0.01 M phosphate buffer (pH 6.5). The dialyzed enzyme solution was concentrated by ultrafiltration, and applied to an anion ion exchange column (High Q IEX, 20 mL, Bio-Rad), equilibrated with 50 mM Tris-HCl buffer (pH 8.0). β-glucosidase bound to the column was eluted in 50 mL of a NaCl concentration linear gradient (0 - 0.5 M) at a flow rate of 1 mL/min. Solid ammonium sulfate was added, over ice, into the crude extract to 30% saturation. After centrifugation (10,000 g, 20 min), ammonium sulfate was added to bring the supernatant to 80% saturation. The latter was stored overnight and then centrifuged. The precipitate was redissolved and dialyzed against several changes of 0.05 M Tris-HCl buffer (pH 8.5). Active β-glucosidase fractions were applied to a hydrophobic interaction chromatography column (Methyl HIC, 5 mL, Bio-Rad), equilibrated with 50 mM Tris-HCl buffer (1.5 M $(NH_4)_2SO_4$ in the buffer, pH 8.5). The enzyme was eluted with a linear gradient of 1.5 - 0.6 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 1 mL/min. The active fractions were pooled and dialyzed against 10 mM NaOAc buffer (10% glycerol in the buffer, pH 5.4) for 18 h. Then, the enzyme was applied to a cation ion exchange column (High S, IEX, 5 mL, Bio-

Rad), equilibrated with 10 mM NaOAc buffer (10% glycerol in the buffer, pH 5.4), eluted with a 50-mL linear NaCl gradient from 0.1 to 0.5 M at a flow rate of 1 mL/min. The active fractions were pooled and used as a purified enzyme preparation. The separated and purified active fractions were concentrated and dialyzed against water, then freeze-dried and stored at –20°C for further studies.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular mass under denaturing conditions was determined by SDS-PAGE performed using a Mini-gel system (Bio-Rad). The gels were cast with 0.75 mm spacers (Bio-Rad). SDS-PAGE was performed according to Laemmli's discontinuous Tris-glycine buffer system at 10 mA for 2 - 3 h with a 5% acrylamide stacking gel (pH 6.8) and 12% separating gel (pH 8.8) (Laemmli, 1970). Proteins in the gel were stained with Coomassie brilliant blue R-250 and destained with 0.5 M NaCl aqueous solution. Protein quantitative analysis was determined by the Bradford method (Bradford, 1976) with bovine serum albumin as a standard.

Enzyme activity assay

Enzyme activity was quantified by *p*-nitrophenol release from *p*-nitrophenyl-D-glucoside (*p*NPG). One unit was defined as the amount of enzyme that hydrolyzes 1 µmol of *p*NPG per minute at 1 mM *p*NPG and 30°C in 0.1 M sodium acetate, pH 5.5 (Chuankhayan et al., 2005).

Crude soy flour extraction

Ten grams of defatted soybean flour was extracted with 40 mL of 80% methanol by stirring overnight at room temperature. The solid was removed from the extract supernatant by centrifugation at 12,000 rpm for 15 min (Murphy et al., 2002; Chuankhayan et al., 2007).

Hydrolysis of crude soy flour extract

To test the hydrolysis efficiency of *Aspergillus* sp. MT-0204 *β*-glucosidase toward the isoflavonoid glycosides in crude soy flour extract, 0.5 mL of crude soybean extract were hydrolyzed with 0.05 unit of *β*-glucosidase in 1 mL of 0.1 M sodium acetate, pH 5.5. The reaction mixtures were incubated at 40 for 1 h, and then the reaction was stopped by boiled for 10 min. The stopped reactions were dried by speed vacuum and resuspended in 1 mL of 10% acetronitrile in 0.1% phosphoric acid/water. A control reaction of crude extract without enzyme was set up in the same manner. Then the reaction mixtures were analyzed by the HPLC method (Chuankhayan et al., 2007).

HPLC analysis was used to measure the amounts of daidzein, genistein, daidzin, genistin, and malonylgenistin to quantify changes in isoflavone content of crude extracts treated with the *Aspergillus* sp. MT-0204 *β*-glucosidases. Separation and quantification of isoflavonoids were achieved with an Eclipse XDB-C18 (4.6 × 250 mm, 5 µm) reverse phase column on an HP series 1100 HPLC (Agilent Corp., Palo Alto, CA) with the UV detector set at a wavelength of 260 nm (Gerhauser et al., 2003). The flow rate was 0.8 mL/min. Peaks of soy isoflavone glucosides and aglycones were identified by matching retention times with isoflavonoid standards (Gerhauser et al., 2003).

Determinations of kinetics parameters

The values of Km and Vmax for the *β*-glucosidase were determined by assaying purified enzyme at increasing substrate concentrations ranging from 1 to 20%. The temperature, pH and quantity of the enzyme were kept the same as the standard enzyme activity assay described above. Apparent Km and apparent Vmax were calculated according to Lineweaver-Burk plots.

Sequence submission

The nucleotide sequence has been deposited in the GenBank database under accession number DQ206869.

RESULTS

Isolation, characterization and identification of strain A*sp*ergillus *sp*. MT-0204

This study aimed at the isolation of strains capable of producing *β*-glucosidase, which hydrolyze the isoflavones. Various microbial strains were isolated from soil samples by the enriched culture. Under the conditions used in the experiments, the strain, *Asp*ergillus sp. MT-0204, with the maximum *β*-glucosidase activity, was isolated and selected as the best strain for further studies.

The detailed morphological and physiological properties of the *Asp*ergillus sp. MT-0204 are shown in Table 1. From the analysis of the ITS rDNA gene sequence (GenBank accession No. DQ206869), this strain was found to be similar to *A. niger* (EF105366) and *A. niger* strain ATCC 16888 (AY373852) (homology, 99.8%/623 bps, based on ITS rDNA). Phylogenetic relationships could be inferred through the alignment and cladistic analysis of homologous nucleotide sequences of known bacteria, and the approximate phylogenetic position of the strain is shown in Figure 1. By examining physiological, biochemical characteristics and comparing it's ITS rDNA gene sequence, the strain *Asp*ergillus sp. MT-0204 was identified as a strain of *A. niger*, and named *A. niger* MT-0204.

Purification of *β*-glucosidase and molecular properties

The *β*-glucosidase was purified in four steps. The results of the purification were summarized in Table 2. The recovery and the purification fold were 24.5 and 23.44%, respectively. The enzyme was stable in storage at 4°C in 20% glycerol for several weeks. The purified enzymes gave only one band on the SDS-PAGE, with a molecular weight of about 42 kD (Figure 2).

Determination of optimal pH and pH stability

To investigate the optimal pH and the effect of pH on the *β*-glucosidase activity, buffers including citric acid-Na_2HPO_4 (pH 3.4 - 8.0), Tris-HCl (pH 7.2 - 9.1) and glycine-sodium hydroxide (pH 8.6 - 10.6) with concentra-

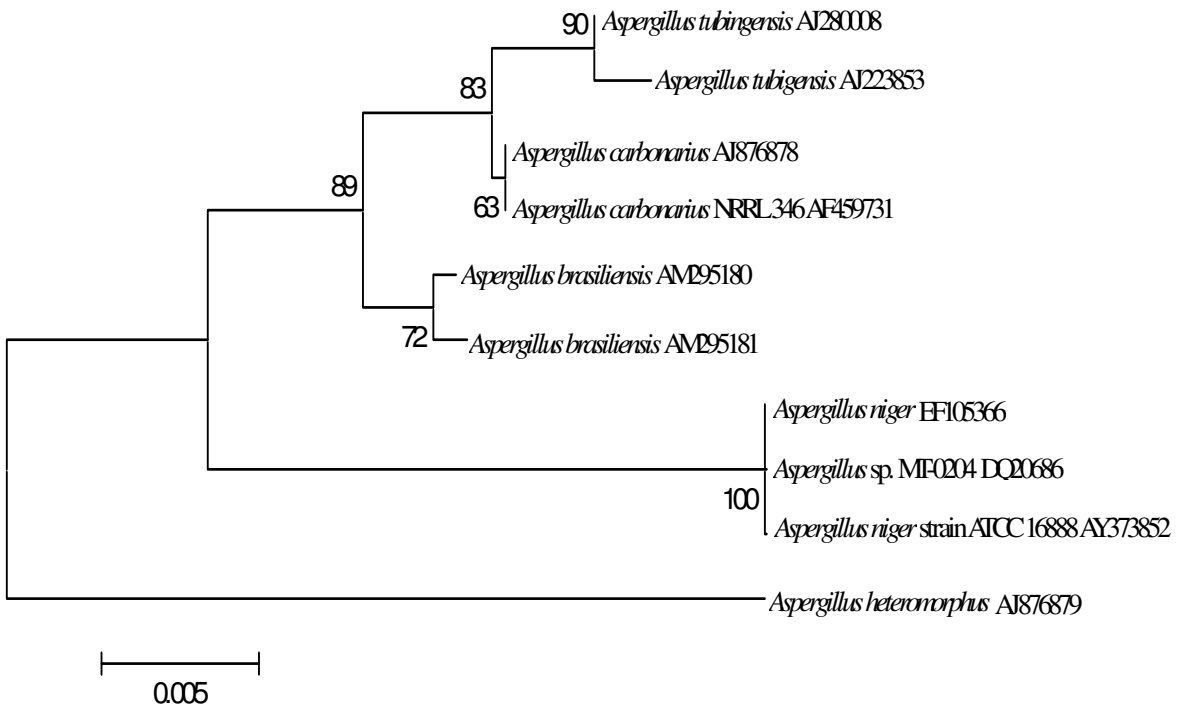

Figure 1. The phylogenetic dendrogram for *Aspergillus* sp. MT-0204 and related strains based on the ITS rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 replicates. *Mycobacterium petroleophilum* was used as the out-group.

Table 1. Morphological and physiological properties of the *Aspergillus niger* MT-0204

Characteristic		Properties
Milk	Solidification	-
	peptonization	-
Liquefaction of gelatin		-
Degradation of starch		-
Formation of H_2S		-
Formation of melanin		-
Growth on cellulose		+
Nitrate reduction		-
Carbon source utilizing	L-rhanose	+
	Mannose	+
	Mannitol	+
	Lactose	+
	D-galactose	+
	Soluble starch	+
	Sucrose	+
	Maltose	+
	Xylose	+
	D-sorbitol	+
	Glycerol	+
	Glucose	+
	Fructose	+
	Control	-

tions of 100 mM for each were used. The purified enzyme was dissolved in different buffers for 1 h at 50°C to achieve an initial activity, after that the relative residual activity was measured to assess the pH stability according to the standard method described above. Fig. 3 showed that the β-glucosidase activity was detectable in the pH range from 3.5 to 10.0. The results indicated that the β-glucosidase had a broad plateau of stability between pH 4.5 and 6.5. Analysis of β-glucosidase activity under different pH indicated a minimum activity at pH approximately 8.0 and a maximum activity between pH 5.0 and 6.0. A more rapid decrease in activity was observed under basic conditions, while neutral and acidic conditions favored the enzyme activity. There was 70% residue activity at pH 3.5. These data indicated that β-glucosidase might function in acidic as well as neutral conditions. The details were shown in Figure 3.

Effect of temperatures on β-glucosidase activity

To ascertain the correct temperature, reaction temperature of the enzyme was investigated in water bath calibrated by two thermometers. After incubated in a preheated water bath at various temperatures ranging from 20 to 100°C for 30 min, the relative residual activity were assayed. The β-glucosidase activity that assayed under the standard reaction was taken as 100%. The β-glucosidase activity increased slowly between 25 and

Table 2. Purification and properties of *β*-glucosidase.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude enzyme	55	21.2	0.39	100	1
$(NH_4)_2SO_4$	31	18.7	0.60	88.2	1.54
High Q IEX	11.5	12.5	1.09	59	2.59
HIC	1.62	6.22	3.83	29.33	9.82
High S IEX	0.56	5.12	9.14	24.5	23.44

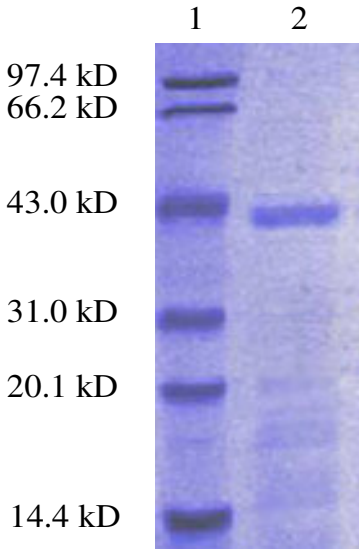

Figure 2. SDS-PAGE analysis of *β*-glucosidase. Lane 1. Molecular mass marker proteins. (Rabbit phosphorglase B, 97.4 kD. Bovine serum albumin, 66.2 kD. Rabbit actin, 43.0 kD. Bovine carbonic anhydrase, 31.0 kD. Trypsin inhibitor, 20.1kD. Lysozyme, 14.4 kD). Lane 2. The purified *β*-glucosidase.

40℃ and decreased between 55 and 70℃, while dropped significantly at 75℃, and the details were shown in Figure 4.

Effect of metal ions and other reagents on β-glucosidase activity

The effects of different metal ions and other reagents on the *β*-glucosidase activity were examined by incubating the enzyme in the presence of the reagents at 30℃ for 1 h. As shown in Table 3, *β*-glucosidase activity was inhibited by Hg^{+}, Cu^{2+}, Ag^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+} and Zn^{2+}. The *β*-glucosidase activity was also slightly inhibited by EDTA and Fumarate. The results of metal analysis showed that the K^{+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, and Mn^{2+} could activate the *β*-glucosidase activity, with Ca^{2+} being the best. The maximum hydrolysis activity was attained at 0.4 mM concentration of Ca^{2+} for *β*-glucosidase, but on a further increase in the concentration, the activity did not change significantly.

Table 3. Effect of metal ions and chemicals on *β*-glucosidase activity*.

Chemical	Concentration (mM)	Relative activity (%)
None		100
EDTA-Na_2	0.2	45.12
Citrate	0.5	61.0
$CoCl_2$	0.2	48.7
KCl	0.2	109.3
$FeCl_2$	0.2	114.3
$BaCl_2$	0.2	62.4
$CaCl_2$	0.2	119.2
$MgCl_2$	0.2	81.2
$MnSO_4$	0.2	111.5
$ZnSO_4$	0.2	83.38
$AgNO_3$	0.2	5.32
Hg_2Cl_2	0.2	13.21
$CuSO_4$	0.2	65.51

*Enzymes activities were determined in the presence of an additional test chemical substance under the standard assay conditions.

Determination of the kinetic parameters

The Michaelis constant Km and Vmax values for purified *β*-glucosidase was determined using the Lineweaver-Burk plots (not shown) and isoflavones as the substrate. The values of apparent Km and Vmax were calculated to be 22.47 and 5.2 mM min^{-1} mg^{-1} for *β*-glucosidase, respectively.

DISCUSSION

β-glucosidases could hydrolyze both conjugated and nonconjugated glycosides in crude soybean extract more rapidly, suggesting its application to increase free isoflavones in soy products. As the promising biocatalyst for food and pharmaceuticals industries, in recent years, *β*-glucosidases also have been put into industrial applications for the preparation of free isoflavones. This work focused on investigating the characteristics of a *β*-glucosidase from a newly isolated *Aspergillus* sp. MT-

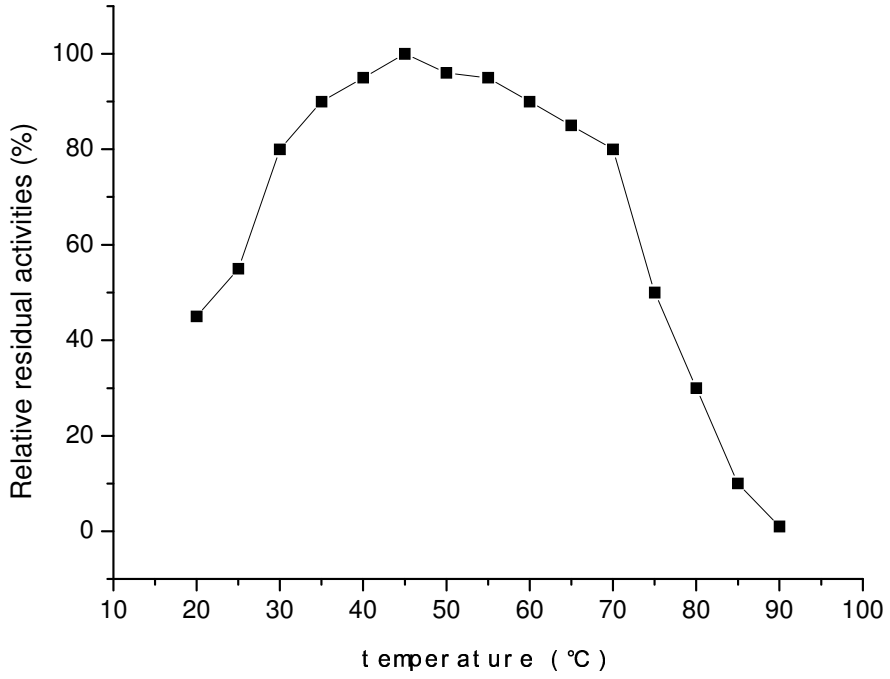

Figure 3. The relative residual activity of β-glucosidase at different pH. The enzymes were treated in different buffers for 30 min, and then the β-glucosidase activities were determined according to the standard enzyme assay method. The activities of enzymes that assayed under the standard reaction were taken as 100%.

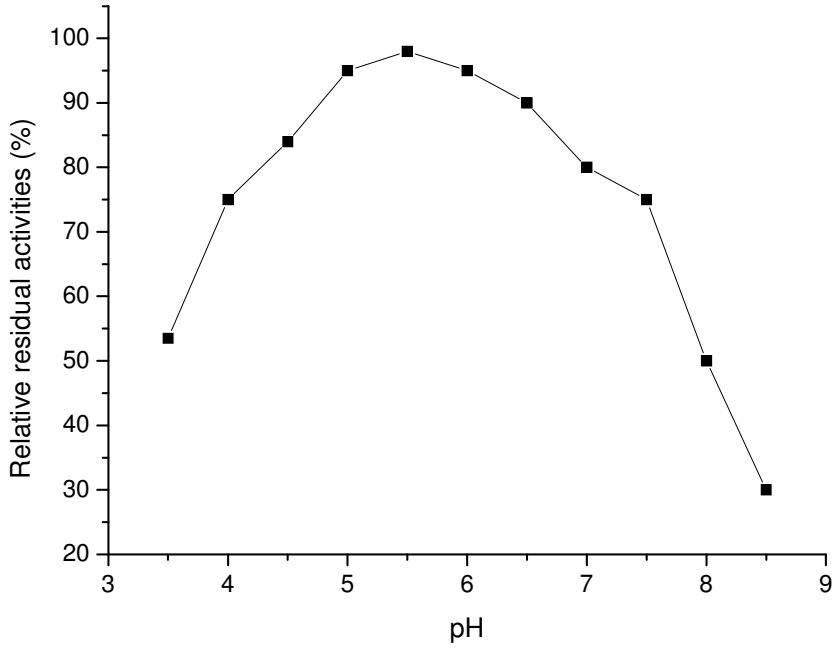

Figure 4. The relative residual activities β-glucosidase at different temperature.

0204. During the procedure of purification, the β-glucosidase was found to be extremely stable, which led to a high overall yield of β-glucosidase. This was very helpful to the separation of larger scale production and application for β-glucosidase, therefore it was time- and cost-efficient.

It is noteworthy that *Aspergillus* sp. MT-0204 *β*-glucosidase is able to hydrolyze almost all isoflavonoid conjugated and nonconjugated glycoside forms in the crude soy flour extract in a short time. The hydrolysis of isoflavonoid glycoside in methanolic extracts of soy flour by *Aspergillus* sp. MT-0204 *β*-glucosidase was promising.Addition of *Aspergillus* sp. MT-0204 *β*-glucosidase greatly increased the release of free isoflavones in the suspended soy flour. Furthermore, this hydrolysis resulted in increases of free isoflavones in the particles. The hydrolysis of isoflavonoid glycosides may increase their conversion to isoflavones, which have bioavailability and health benefits (Barnes and Messina, 1991; Anderson et al., 1995; Bahram et al., 1996; Song et al., 1999). *Aspergillus* sp. MT-0204 *β*-glucosidase is a very stable enzyme with a temperature optimum of 50℃, so it should be useful for industrial processes. This research suggests that *Aspergillus* sp. MT-0204 *β*-glucosidase may be useful for processing of soy foods to enhance their nutritional and economic value.

Several ions can activate the *β*-glucosidase activity which indicated its possible role as a metal ion cofactor in the enzyme-substrate reaction and having a stabilizing effect on the *β*-glucosidase. At the same time, the enzyme was stable to heat; it will be helpful for application of *β*-glucosidase into industries. Though the stability of *β*-glucosidase is good enough, its activity can not be of requirement for application. In order to improve the stability and activity of *β*-glucosidase, the gene encoding *β*-glucosidase should be cloned, and the *β*-glucosidase could be modified by rational design of proteins or by directed molecular evolution of proteins (Stemmer, 1994; Arnold and Volkov, 1999; Wong and Robertson, 2004; Liu et al., 2006), overexpression and modification of this kind of *β*-glucosidase would facilitate to better understand the structure of enzyme and could result in the production of large amounts of efficient *β*-glucosidase biocatalyst, which are undergoing in our further studies.

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