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# PURIFICATION AND CHARACTERIZATION OF SOY COTYLEDON β-GLUCOSIDASE

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## ABSTRACT

β-Glucosidase $F_{42}$ of soy cotyledons was purified by ammonium sulfate fractionation, ion-exchange chromatography (CM-Sephadex-C-50, Sigma, St. Louis, MO) and gel filtration (Sephadex G-100, Sigma). The enzyme was purified 111.8-fold relative to its concentration in the crude extract. It had an apparent molecular mass of 53 kDa in gel filtration experiments and produced a 33-kDa band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, suggesting that it is dimeric. The purified β-glucosidase $F_{42}$ was characterized as a glycoprotein after the identification of fucose, galactosamine and glucosamine by high-pressure anion-exchange chromatography–pulsed amperometric detector. Its highest activity was observed at pH 5.0 and 45C, and it was stable for up to 4 days at 25C. The $K_m$ of the enzyme was 0.12 mM *p*-nitrophenyl-β-D-glucopyranoside. β-Glucosidase $F_{42}$ showed specificity for different substrates, and its activity was inhibited by 1 mM $HgCl_2$, 10 mM glucono-δ-lactone or 150 mM glucose and increased by 10 mM $MnCl_2$.

## PRACTICAL APPLICATIONS

β-Glucosidase is an enzyme that hydrolyzes β-glucosidic bonds to liberate glucose and hydrolyzes isoflavones to release aglycones. Soy aglycones have been broadly investigated because of their biological activity in the prevention and treatment of some chronic diseases. Soy β-glucosidase can be used in the food industry to alter soy isoflavones for the production of functional foods that are rich in aglycone isoflavones. Therefore, it was an established method of purification of the enzyme that has great biotechnological potential.

## INTRODUCTION

β-Glucosidase (β-D-glucoside glucohydrolase, EC. 3.2.1.21) catalyzes the hydrolysis of the nonreducing terminals of compounds containing β-D-glucose residues, with glucose released as a result. This enzyme is produced by fungi, bacteria and many vegetables. In vegetables, it takes part in numerous functions, such as the hydrolysis of conjugated gibberellins, the conversion of stored cytokinins to active forms, stress metabolism and resistance to some phytopathogens (Esen 1992; Faure 2002; Morant *et al.* 2008).

The consumption of soy has increased in the last few decades because of the importance of its nutritional and biological properties. The nutritional properties of soy are its high protein content and high-quality oil. The biological properties of soy include the presence of isoflavones that have phytoestrogenic (Liu *et al.* 2010), antioxidant (Ma *et al.* 2010) and antitumor activities (Lai and Yen 2002). However, in humans, the bioavailability of soy isoflavones depends on their metabolism, which can differ depending on ethnic groups, dietary habits and the presence of appropriate intestinal microflora. These factors promote changes in the amount

and activity of the intestinal β-glucosidases that hydrolyze the β-glucosides in aglycones (Ismail and Hayes 2005). The β-glucosides show less estrogenic activity than aglycones with greater absorption properties (Izumi *et al*. 2000; Brouns 2002).

Isolation and purification of β-glucosidase has great potential in biotechnological applications in the food industry (Santosh *et al*. 1999; Bhatia *et al*. 2002; Chiou *et al*. 2010). β-Glucosides can be converted to soy aglycones by crude β-glucosidases from fungal or bacterial origins (Matsuda *et al*. 1992; Aguiar and Park 2004; Otieno and Shah 2007; Chun *et al*. 2008) or by β-glucosidases originating from the grain itself (Araújo *et al*. 1997). However, the purification and characterization of soy β-glucosidases is relatively unexplored, with investigations previously conducted only in whole flour and in the root of germinate soy, both of which yielded partially purified enzyme (Matsuura and Obata 1993; Hsieh and Graham 2001; Suzuki *et al*. 2006).

In this study, β-glucosidase $F_{42}$ of soy cotyledons was purified by fractionation with ammonium sulfate, ion-exchange chromatography and gel filtration. Its biochemical properties were then characterized.

## MATERIALS AND METHODS

### Raw Material

The soy cultivar BRS 213 (*Glycine Max* [L.] Merrill), 2002/2003 harvest, developed at the Embrapa Soy Experimental Farm in Londrina, PR, Brazil, was used.

### Extraction, Fractionation and Purification of β-Glucosidase

**Extraction.** The conditions for β-glucosidase extraction were as described by Matsuura and Obata (1993), with 60 g of soy cotyledon flour and 100 mM sodium phosphate buffer, pH 6.6, in a 1:10 proportion (w/v) slowly agitated for 1 h at 4C and then centrifuged at 4,000 × g at 4C for 15 min. The supernatant was acidified with 0.1 M HCl to pH 5.0, and the samples were centrifuged again under the same conditions. The supernatant obtained following this step was the crude extract used for fractionation and purification.

**Fractionation.** The crude extract was first precipitated by ammonium sulfate at 40% saturation. The sample was then placed at 4C until precipitation was complete. The precipitated protein ($P_{0–40}$) was collected after centrifugation at 4,000 × g for 15 min at 4C, and ammonium sulfate was added to the supernatant until 85% saturation was achieved. The second precipitate ($P_{40–85}$) was then obtained using the above conditions. The precipitates were resuspended in a 50 mM citrate phosphate buffer, pH 5.0. The supernatants ($S_{0–40}$ and $S_{40–85}$) and precipitates ($P_{0–40}$ and $P_{40–85}$) were dialyzed with the same buffer for 14 h at 4C. The activity of β-glucosidase and the soluble protein content were then determined. The $P_{40–85}$ fraction with the highest β-glucosidase activity was concentrated using an ultrafiltration cell (Amicon, model 8400; Millipore, Billerica, MA) with a 1-kDa molecular exclusion membrane (Regenerated cellulose, Millipore).

**Purification.** The concentrated $P_{40–85}$ fraction was applied to a CM-Sephadex C-50 ion-exchange column (2.5 cm × 55 cm). Ion-exchange chromatography starts with conditioning 50 mM citrate phosphate buffer, pH 5.0, without NaCl and the protein eluted is nonretained protein ($F_1$). Elution of retained protein ($F_2$, $F_3$ and $F_4$) is carried out by means of increasing the buffer ionic strength with NaCl (0–1 M). The elution flow was 27 mL/h, and 3.5 mL of fractions was collected in a test tube. Absorbance readings at 280 nm were measured for the eluted fractions and β-glucosidase activity was determined. Fraction $F_4$, which had the highest β-glucosidase–specific activity, was concentrated by membrane ultrafiltration at 1 kDa, dialyzed against a 100 mM citrate phosphate buffer at pH 5.0 and applied to a Sephadex G-100 gel filtration column that was equilibrated with a 100 mM citrate phosphate buffer at pH 5.0. The elution flow was 17 mL/h, and 2.3 mL of fractions was collected in a test tube. Absorbance readings at 280 nm were performed on the eluted fractions and β-glucosidase activity was determined. In this assay, three protein fractions were separated ($F_{41}$, $F_{42}$ and $F_{43}$). The β-glucosidase $F_{42}$ fraction showed the highest specific activity and was thus used for biochemical characterization.

**β-Glucosidase Activity.** β-Glucosidase activity in the different extraction and purification fractions was determined using the *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) substrate according to the procedures described by Matsuura and Obata (1993). The standard curve of *p*-nitrophenol (0.04–0.32 μmol) was prepared. A β-glucosidase activity unit (U) was defined as the amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol/min under assay conditions.

The soluble protein content was quantified using the method described by Lowry *et al*. (1951) with a bovine serum albumin standard solution, and the specific β-glucosidase activity of each fraction was determined as the relationship between the enzymatic activity and the protein content, and this value was expressed as U/mg.

## Biochemical Characterization of Purified β-Glucosidase $F_{42}$

**Homogeneity Assay by Native-Polyacrylamide Gel Electrophoresis (PAGE).** Native-PAGE (10% w/v) analysis of fractions (EB, $P_{0–40}$, $P_{40–85}$, $F_4$ and $F_{42}$) was performed according to the method of Davis (1964) and gels were stained with silver nitrate (Nielsen and Brown 1984).

**Molecular Mass Determination by Gel Filtration and Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE).** The molecular mass of native purified β-glucosidase $F_{42}$ was estimated using gel filtration in a Sephadex G-100 column (2.3 cm × 100 cm) that was equilibrated with 100 mM citrate phosphate buffer at pH 5.0. A 1 mL/h flow was used, 2.3 mL of fractions was collected in tubes, and absorbance readings were performed at 280 nm. The protein standards were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). Blue dextran (2,000 kDa) was used to determine the void volume. The molecular mass was graphically estimated as the *log* of the standard molecular mass in the ordinate versus $K_{av}$ in the abscissa. The $V_e$ value of each sample was the sum of the collected fraction volumes from the application of the sample until the fraction where the highest absorbance reading was recorded.

The molecular mass of the purified β-glycosidase $F_{42}$ was also estimated using SDS-PAGE with β-mercaptoethanol according to the method described by Laemmli (1970). A 4% stacking gel and a 10% separation gel buffered with 1.5 M Tris–HCl with SDS at pH 8.9 was used. SDS-PAGE was run in a vertical system at 100 mV and 4C. Samples were prepared in a 1:1 (v/v) sample buffer and boiled for 10 min before loading onto the gel. The molecular mass was estimated using the relationship between the *log* of the molecular weights and relative mobilities of 6- to 180-kDa standards (BenchMark Pre-stained Protein Ladder; Invitrogen, Carlsbad, CA). The protein bands were stained with silver nitrate (Nielsen and Brown 1984).

**Identification of Monosaccharides in the Purified β-Glucosidase $F_{42}$.** The purified β-glucosidase $F_{42}$ was evaluated for the presence of neutral and acidic monosaccharides using high-pressure anion-exchange chromatography (HPAEC) in a Dionex DX 500 system with a pulsed amperometric detector (PAD) and a CarboPac PA10 analytical column (4 × 250 mm) equipped with an Amino Trap (Dionex, Sunnyvale, CA) guard column. The elution conditions were 16 mM NaOH in deionized water (eluent 1) and 0.2 M NaOH (eluent 2) with a 1.0 mL/min flow rate (Elifio *et al*. 2000). The column was regenerated after 20 min with 100% eluent 2, followed by 15 min of 16 mM NaOH before addition of a new sample. Purified β-glucosidase $F_{42}$ samples containing 2% carbohydrates were previously subjected to acid hydrolysis in a heater (Digi-Block, Laboratory Devices Inc., Placerville, CA) at 120C for 2 h using trifluoroacetic acid (Merck KGaA, Darmstadt, Germany) and 4 M HCl to release the neutral and acidic monosaccharides (Corradi da Silva *et al*. 2005). The hydrolyzed sample was then completely evaporated using a vacuum centrifuge (HETO mod DNA Plus, Jouan Nordic, Allerød, Denmark), followed by three cycles of water addition and complete evaporation.

**Effects of Temperature, pH and *p*-NPG Concentration on β-Glucosidase $F_{42}$ Activity.** The optimal temperature for purified β-glucosidase $F_{42}$ was assayed at different temperatures from 4 to 70C for 30 min at pH 5.0. The optimal pH was determined under different pH conditions (3.0–8.0) at 45C. Activities are expressed as a percentage of β-glucosidase activity as a function of temperature or pH.

The effect of *p*-NPG concentrations (0.1–20 mM) on purified β-glucosidase $F_{42}$ activity was determined at pH 5.0, 30 min incubation at 45C. The results were expressed as U/mg protein. The Michaelis constant ($K_m$) was estimated using the Lineweaver–Burk plot method.

**Enzyme Thermal Stability.** The thermal stability of the purified β-glucosidase $F_{42}$ was evaluated by incubating the enzyme at different temperatures (25, 45, 50, 60 and 70C), after incubating for different intervals (5 min, 10 days) and after four successive freeze–thaw cycles. The β-glucosidase activity was measured by assaying the remaining activity at 45C for 30 min.

**Specificity of Purified β-Glucosidase $F_{42}$ Toward Different Substrates.** The specificity of β-glucosidase $F_{42}$ was investigated under optimal conditions, pH 5.0 and a 30-min incubation period at 45C using various substrates at a concentration of 1 mM. The specificity for daidzin and genistin was assessed using the methods described by Matsuura and Obata (1993) with 400 μL of 1 mM substrate and 100 μL of sample, followed by 2 mL of methanol to stop the reaction. The quantitative analysis of liberated daidzein and genistein aglycones was conducted using high-pressure liquid chromatography (HPLC) according to the methods described by Berhow (2002). One unit of enzyme (U) was defined as the amount of enzyme necessary to liberate 1 μmol aglycone/min under assay conditions.

The specificity of β-glucosidase $F_{42}$ activity was also investigated for substrates derived from β-D-glucosides: salicin, cellobiose, gentiobiose, amygdalin, esculin and methyl-β-D-glucopyranoside. β-Glucosidase $F_{42}$ activity was determined

using the glucose oxidase method with an automated system (Selectra 1, Vitalab/Merck). In this procedure, the liberated glucose was quantified by spectrophotometry at 420 nm. One unit of β-glucosidase (U) was defined as the amount of enzyme necessary to liberate 1 μmol glucose/min under assay conditions. The specificity of enzyme for substrates derived from *p*- and *o*-nitrophenol was compared with the *p*-NPG substrate (100% activity).

### Effects of Metallic Ions and Organic Compounds on the Activity of β-Glucosidase $F_{42}$

The effects of metallic ions and organic compounds on β-glucosidase $F_{42}$ activity were determined at pH 5.0 and 30C with 30-min incubation. The β-glucosidase $F_{42}$ was incubated in 100 mM citrate phosphate buffer with the addition of the metallic ions $HgCl_2$, $AgNO_3$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $ZnSO_4$ and $CuSO_4$ and the organic compounds glucone-δ-lactone, phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) salts at 1 and 10 mM concentrations. The results are expressed as the percentage of β-glucosidase activity compared with the control.

The effect of concentration of glucose (1, 10, 50, 100 and 150 mM) on the activity of β-glucosidase $F_{42}$ was tested in *p*-NPG (1 and 2 mM). The inhibition constant ($K_i$) was determined using the Dixon (1953) representation.

## RESULTS AND DISCUSSION

### Soy Cotyledon β-Glucosidase $F_{42}$ Purification

Purification of soy cotyledon β-glucosidase $F_{42}$ activity is summarized in Table 1. Sixty grams of soy cotyledons in 100 mM sodium phosphate buffer, pH 6.6, was acidified with 0.1 M HCl to pH 5.0. From this, it was possible to obtain a crude extract with 4.81 U and 2,782.0 mg of soluble protein. The crude extract was fractionated by ammonium sulfate precipitation, and most activity was detected in 40–85% saturation ($P_{40–85}$).

The $P_{40–85}$ fraction loaded onto an ion-exchange chromatography CM-Sephadex C-50 was eluted with a 50 mM citrate phosphate buffer to separate a protein fraction with β-glucosidase activity that was called fraction $F_1$. This fraction contained 1.8% of the β-glucosidase activity and 2% of the protein (Table 1 and Fig. 1), which may be a new alternative source of β-glucosidase that needs further study. After the application of a 0–0.6 M linear NaCl gradient in the same buffer, three protein fractions were separated, and these fractions were called $F_2$, $F_3$ and $F_4$, according to their separation and elution order. The recovery of these fractions in terms of protein level was 4.0, 0.8 and 1.1%, respectively. However, only the $F_4$ fraction showed β-glucosidase activity, with a recovery of 26.3% of the activity. In the ion-exchange chromatography separation process, the $F_1$ fraction showed a low specific activity, while the $F_4$ fraction showed a high specific activity of 20.0 and a purification factor of 23 compared with the crude extract (Table 1 and Fig. 1). Therefore, the $F_4$ fraction was used for further purification.

Using the same extraction, fractioning and ion-exchange chromatography conditions, Matsuura and Obata (1993) partially purified β-glucosidase from soy grains. In their chromatography study, three fractions with β-glucosidase activity were separated (A, B and C). Compared with the crude extract, they observed enzyme recoveries from the A, B and C fractions of 4.0, 6.0 and 22.0%, respectively. After the application of a NaCl gradient, fractions B and C showed purification factors of 8.5 and 44, respectively. In the present study, only two fractions had β-glucosidase activity; this difference from the separation performed by Matsuura and

**TABLE 1.** PURIFICATION STAGES OF β-GLUCOSIDASE FROM SOY COTYLEDONS

| Purification stage | Protein (mg) | Activity* (U) | Recovery (%) | Specific activity† | Purification factor |
|---|---|---|---|---|---|
| Raw extract | 2,782.6 | 4.81 | 100.0 | 0.0017 | 1.0 |
| Precipitation $(NH_4)_2SO_4$ | | | | | |
| $P_{(40–85)}$ | 684.8 | 2.74 | 57.0 | 0.0040 | 2.4 |
| CM-Sephadex C50 | | | | | |
| $F_1$ | 55.1 | 0.085 | 1.8 | 0.0015 | 0.9 |
| $F_2$ | 112.1 | 0.0 | 0.0 | 0.0000 | 0.0 |
| $F_3$ | 22.4 | 0.0 | 0.0 | 0.0000 | 0.0 |
| $F_4$ | 31.6 | 1.27 | 26.4 | 0.0402 | 23.6 |
| Sephadex G-100 – $F_4$ fraction | | | | | |
| $F_{41}$ | 0.9 | 0.0 | 0.0 | 0.0000 | 0.0 |
| $F_{42}$ | 2.7 | 0.51 | 10.6 | 0.1900 | 111.8 |
| $F_{43}$ | 10.8 | 0.043 | 0.9 | 0.0040 | 2.4 |

* U = μmol *p*-nitrophenol/min.

† U/mg protein.

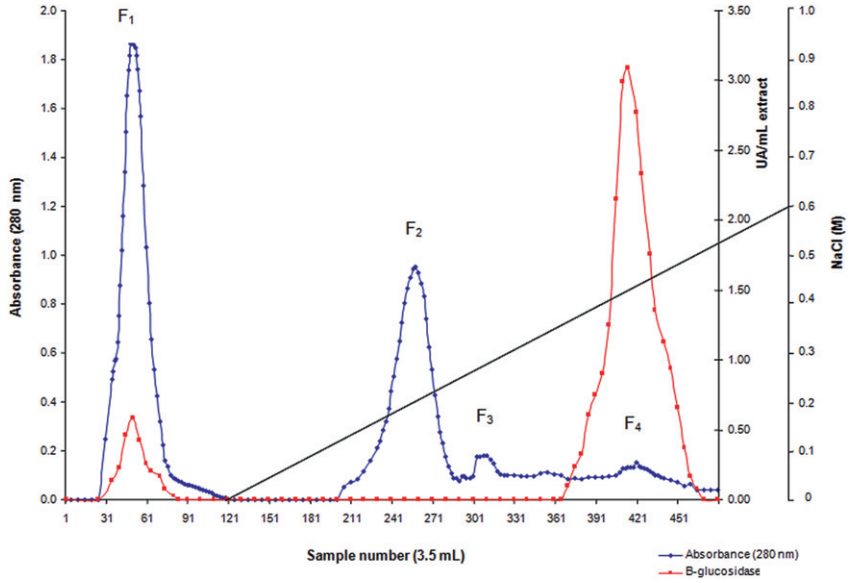


**FIG. 1.** CM-SEPHADEX C-50 ION-EXCHANGE CHROMATOGRAPHY OF ELUTED FRACTION $P_{40\text{-}85}$ WITH A LINEAR GRADIENT OF 0–600 mM NaCl IN 50 mM CITRATE PHOSPHATE BUFFER, pH 5.0, AND A FLOW RATE OF 27 mL/h

Obata (1993) may have been due to the characteristics of the crude extract, the initial treatment parameters or the different substrates used to determine β-glucosidase activity.

The $F_4$ fraction applied to the Sephadex G-100 gel filtration column separated into three protein fractions called $F_{41}$, $F_{42}$ and $F_{43}$ (Fig. 2), according to their separation and elution order. The $F_{42}$ and $F_{43}$ fractions showed β-glucosidase activity with a recovery of 10.6 and 0.9%, respectively. Furthermore, the purification factor of the $F_{42}$ fraction was 111.8 compared with the crude extract (Table 1), indicating possible enzyme purification. The separation profile and the number of fractions with β-glucosidase activity in the present study differed from that of Matsuura and Obata (1993) and from Hsieh and Graham (2001). Matsuura and Obata (1993) applied the B and C fractions independently to columns containing Sephadex G-150. After elution, they obtained partial purification of the B and C fractions. They obtained a recovery of β-glucosidase activity of 2.0% for the B fraction and 7.0% for the C fraction. Their purification factor was 87.5 for the B fraction and 95.9 for the C fraction compared with the crude extract. Hsieh and Graham (2001) partially purified β-glucosidase from germinated soy root extracts using anion-

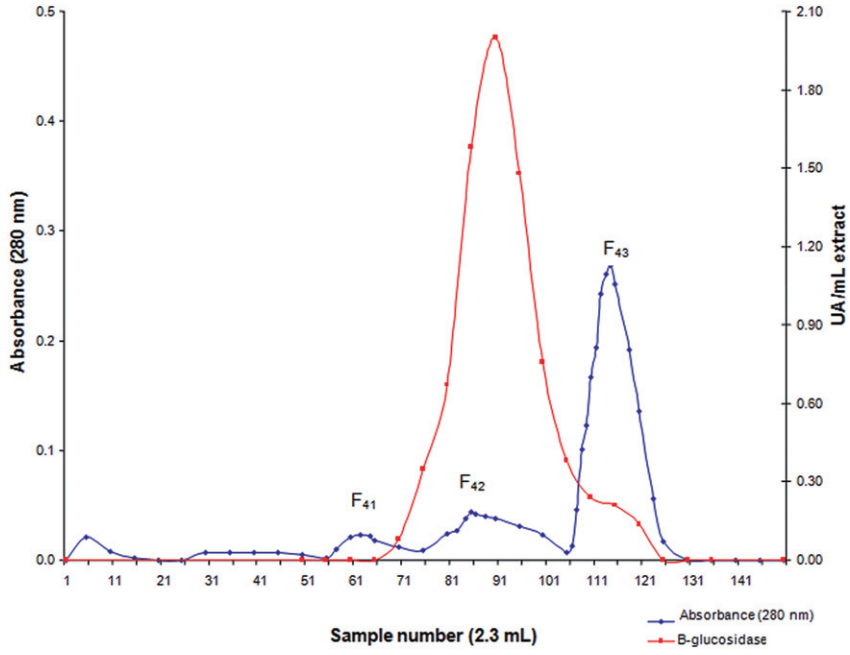


**FIG. 2.** SEPHADEX G100 GEL FILTRATION CHROMATOGRAPHY OF THE $F_4$ ELUTED FRACTION IN 100 mM CITRATE PHOSPHATE BUFFER, pH 5.0, WITH A 17 mL/h FLOW RATE

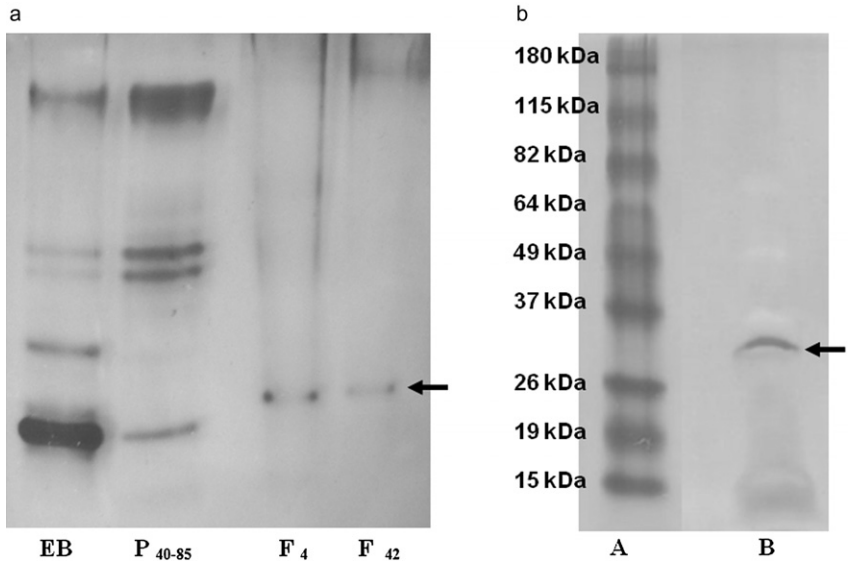


**FIG. 3.** (a) PROTEIN AT DIFFERENT STAGES OF PURIFICATION. LANE 1: CRUDE EXTRACT (20 μg). LANE 2: FRACTION P40–85 (20 μg). LANE 3: FRACTION F4 (30 μg). LANE 4: FRACTION F42 (30 μg). (b) SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS OF β-GLUCOSIDASE F42 PURIFIED FROM SOY COTYLEDONS (A) Molecular mass standards. (B) Purified β-glucosidase $F_{42}$ (40 μg).

exchange chromatography in DEAE-Sephadex and cation exchange in CM-Sephadex of extracts fractionated with ammonium sulfate at 40–60% saturation. They obtained only a partially purified fraction with high specific activity that had a recovery of 20% of the activity and a purification factor of 20 compared with the crude extract. The percentage of recovery and purification factor obtained for the β-glucosidase $F_{42}$ can be compared with the values observed for this enzyme extracted from microbial sources with recovery of 4.5–37.8% and purification factor of 10.2–176.9 (Riou *et al*. 1998; Traon-Masson and Pellerin 1998; Zanoelo *et al*. 2004; Nakkharat and Haltrich 2006; Kaur *et al*. 2007).

The different stages of purification of β-glucosidase were followed by electrophoresis in native-PAGE. It was applied to 20–30 μg of protein and separation of protein fractions was observed. The purified enzyme ($F_{42}$) migrated as a single band, demonstrating that the process of purification of this enzyme was effective (Fig. 3a).

## Biochemical Characterization of Purified β-Glucosidase $F_{42}$

**Molecular Mass Estimate.** The molecular mass of native purified β-glucosidase $F_{42}$ was estimated using Sephadex G-100 gel filtration with known molecular mass standards (Fig. 4). From the obtained linear equation ($y = -1.0951x + 3.4535 - R^2 = 0.9703$), the molecular mass of native β-glucosidase $F_{42}$ was estimated to be 53 kDa. The molecular mass estimated via SDS-PAGE revealed only one protein band with a relative mobility corresponding to a molecular mass of approximately 33 kDa (Fig. 3b), indicating that the enzyme was pure and was a dimer. These results are similar to those published by Matsuura and Obata (1993), who estimated the molecular mass of native β-glucosidase using gel filtration to be 52 kDa. However, Matsuura *et al*. (1995) used gel filtration/HPLC and SDS-PAGE to obtain molecular masses for β-glucosidase of 81 and 36 kDa, respectively.

The molecular mass of native β-glucosidase $F_{42}$ from soy cotyledons is similar to that of β-glucosidase obtained from corn (Esen 1992; Han and Chen 2008), rice (Akiyama *et al*. 1998) and orange bagasse (Cameron *et al*. 2001). However, other vegetable β-glucosidases show a higher molecular mass and are composed of different numbers of subunits. For example, soy root β-glucosidase has two subunits of 80 and 75 kDa (Hsieh and Graham 2001) or 58 kDa (Suzuki *et al*. 2006), rye β-glucosidase is an oligomer containing five 60 kDa subunits (Sue *et al*. 2000), and β-glucosidase from vanilla is composed of four 50 kDa subunits (Odoux *et al*. 2003). According to Hsieh and Graham (2001), many β-glucosidases contain identical or nonidentical subunits, and the number of subunits may vary from 2 to 10. Family 1 β-glucosidases have a very similar tertiary structure that tends to form homo- and hetero-oligomers. The quaternary structures of family 1 proteins show a number of oligomeric forms, including dimers, tetramers, octamers and large aggregate compounds formed by different numbers of subunits (Kim and Kim 2004).

**Identification of Monosaccharides.** HPAEC-PAD chromatography was used to evaluate the neutral monosaccharides in hydrolyzed β-glucosidase $F_{42}$. This detected components with retention times corresponding to fucose, galactose, glucose and mannose standards. The same technique was used for the amino monosaccharides. The detection of components with retention times corresponding to

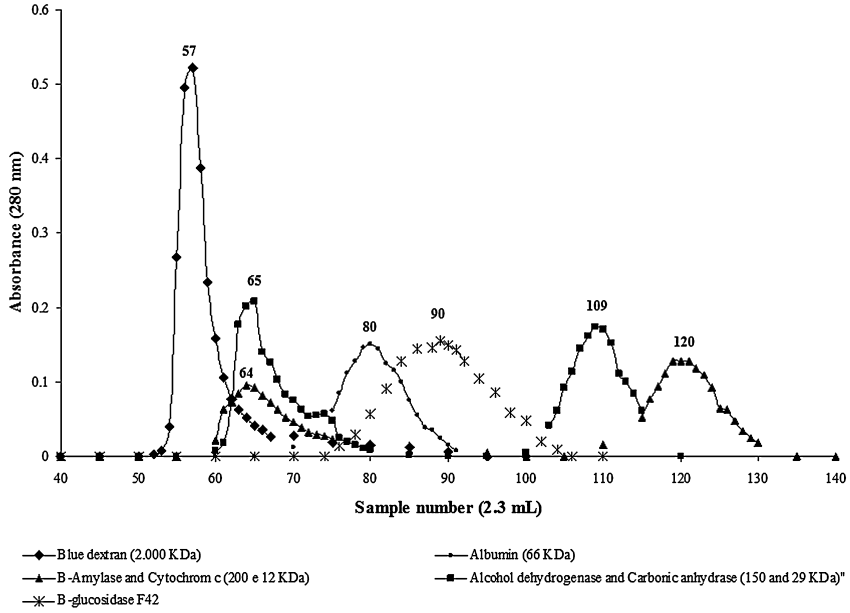


**FIG. 4.** MOLECULAR MASS OF NATIVE PURIFIED β-GLUCOSIDASE $F_{42}$ ESTIMATED USING SEPHADEX G-100 GEL FILTRATION ELUTED IN 100 mM CITRATE PHOSPHATE BUFFER, pH 5.0, WITH A 17 mL/h FLOW RATE

galactosamine and glucosamine indicated that the purified β-glucosidase $F_{42}$ is a glycoprotein, as fucose and glucosamine monosaccharides are commonly found in N- or O-linked glycoproteins (Montreuil *et al*. 1994).

## Effects of Temperature, pH and *p*-NPG Concentration on β-Glucosidase $F_{42}$ Activity

The optimal temperature for enzyme activity was 45C, and under such conditions, β-glucosidase $F_{42}$ had an optimal pH of 4.5–5.5 (Fig. 5). The enzyme activity was reduced by 71% at pH 3.5 and pH 7.0. The optimal temperature of 45C for soy cotyledon β-glucosidase is similar to that obtained in soy by Matsuura and Obata (1993) and by Cameron *et al.* (2001) in *Citrus sinensis* var. Valencia fruit (40–45C). Other β-glucosidase sources have distinct optimal temperatures, such as 25–30C in rye (Sue *et al*. 2000), 40C in the vanilla bean (Odoux *et al*. 2003), 50C in corn (Esen 1992) and 60C in barley (Leah *et al*. 1995). The optimal pH range of 4.5–5.5 for soy cotyledon β-glucosidase $F_{42}$ activity is similar to that described by Matsuura and Obata (1993) in soy, Akiyama *et al*. (1998) in rice and Leah *et al*. (1995) in barley. However, in corn, the optimal pH of this enzyme is higher, at 5.8 (Esen 1992).

The effect of *p*-NPG concentration on β-glucosidase activity was investigated. An increase in activity was observed up to 1 mM of *p*-NPG, and that activity was then maintained up to 20 mM of *p*-NPG, with no indication of inhibition or reduction in activity (Fig. 6). The $K_m$ at pH 5.0 and 45C was 0.12 mM, indicating high reaction specificity for *p*-NPG. Hsieh and Graham (2001) observed a $K_m$ of 1.3 mM for soy root β-glucosidase at 40C. In the vanilla bean (Odoux *et al*. 2003) and *Citrus sinensis* var. Valencia (Cameron *et al*. 2001), the $K_m$ values for *p*-NPG were reportedly 1.1 mM (40C, pH 7.0) and 0.12 mM (40C, pH 5.0), respectively.

The β-glucosidase enzyme was stable at 25C for up to 4 days, with a 35% reduction in activity observed after 10 days. After 1 h of incubation period at 45C, the enzyme activity decreased by 60%. Thus, it appears that the enzyme is not

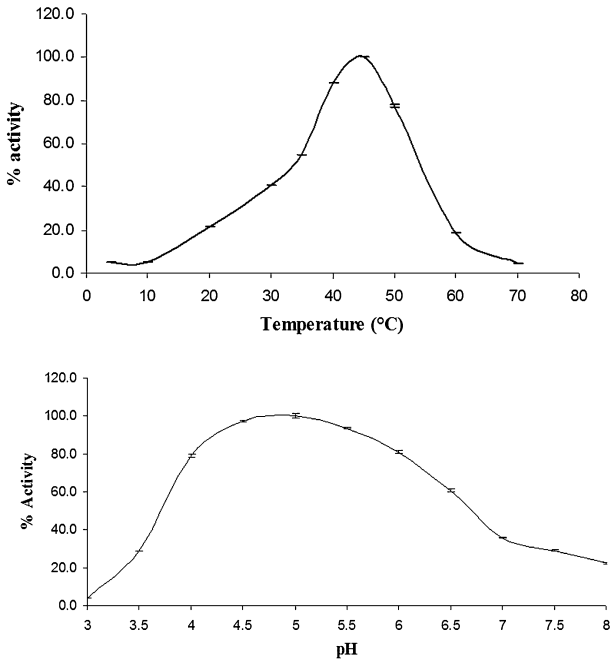


**FIG. 5.** EFFECTS OF TEMPERATURE AND pH ON THE ACTIVITY OF β-GLUCOSIDASE F42 PURIFIED FROM SOY COTYLEDONS

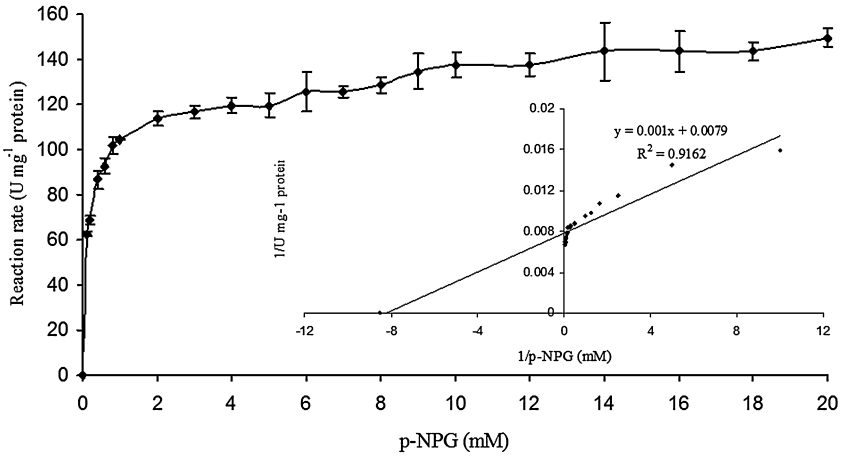


**FIG. 6.** EFFECT OF *p*-NITROPHENYL-β-D-GLUCOPYRANOSIDE (*p*-NPG) CONCENTRATION ON THE ACTIVITY OF β-GLUCOSIDASE $F_{42}$ PURIFIED FROM SOY COTYLEDONS

stable for long periods at its optimal temperature. After 20 min of incubation at 50C, the enzyme activity was reduced by 91%. After 5 min of incubation at a temperature higher than 60C, the enzyme activity was reduced by 95% (Fig. 7). After four successive freeze–thaw cycles, the β-glucosidase activity remained stable.

## β-Glucosidase $F_{42}$ Substrate Specificity

Regarding the specificity of β-glucosidase $F_{42}$ for different substrates, *p*-NPG showed the highest specificity compared with other tested substrates, followed by cellobiose and gentiobiose (Table 2). The high specificity of vegetable β-glucosidase for *p*-NPG has also been observed in ripe cherries and in corn (Esen 1992; Gerardi *et al.* 2001). β-Glucosidase $F_{42}$ showed a low specificity for daidzin and genistin. However, Matsuura and Obata (1993) observed that the daidzin and genistin extracted from soy were better substrates than other natural glucosides, such as amygdalin and esculin. It is possible that differences in the sample origins may explain the differences in the observed results.

The action of the enzyme on *p*-NPG indicates a high specificity for β-D-glucose. However, the enzyme also showed specificity for D-mannose (*p*-nitrophenyl-β-D-mannopyranoside) and D-galactose (*p*-nitrophenyl-β-D-galactopyranoside) (Table 3). Therefore, β-glucosidase may also be considered a β-D-glycosidase (Odoux *et al.* 2003). However, when the same substrate is switched from a *para* to an *ortho* bond position, the enzyme relative activity is reduced, indicating greater specificity of activity for the *para* bond position.

The bond position between two glucoses may also affect the enzyme activity. However, β-glucosidase $F_{42}$ hydrolyzed

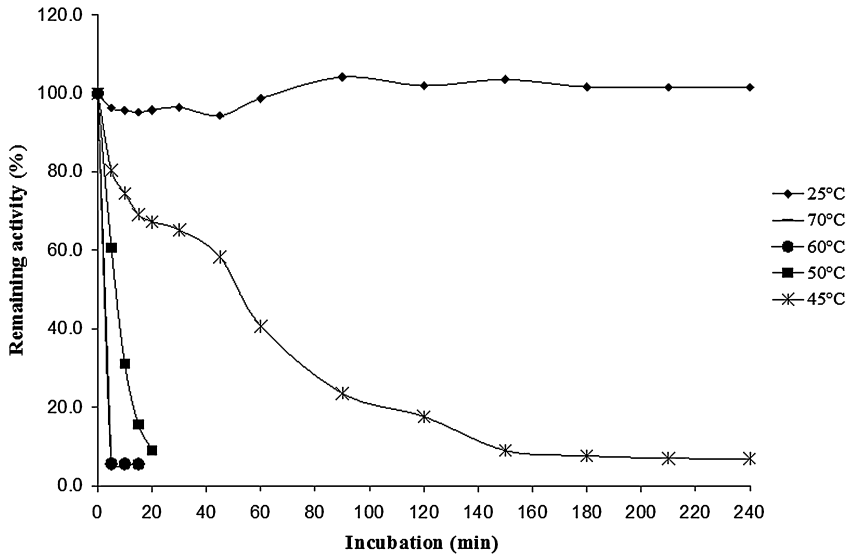


**FIG. 7.** EFFECT OF INCUBATION TIME AND TEMPERATURE ON THE SOY COTYLEDONS PURIFIED β-GLUCOSIDASE $F_{42}$

**TABLE 2.** SPECIFICITY OF β-GLUCOSIDASE $F_{42}$ FOR β-D-GLUCOSIDE SUBSTRATES

| Substrate (1 mM) | Relative activity* (%) |
|---|---|
| *p*-Nitrophenyl-β-D-glucopyranoside | 100 |
| Cellobiose | 46 |
| Gentiobiose | 44 |
| Daidzin | 25 |
| Genistin | 24 |
| Amygdalin | 20 |
| Esculin | 17 |
| Salicin | 11 |
| Methyl-β-D-glucopyranoside | 8 |

* Expressed as a percentage of activity compared with the activity with *p*-nitrophenyl-β-D-glucopyranoside as a substrate.

both cellobiose (1→4) and gentiobiose (1→6) (Table 2). A similar result was observed in *Citrus sinensis* var. Valencia (Cameron *et al*. 2001). However, Matsuura and Obata (1993) used purified soy enzyme and did not observe activity for these substrates, indicating that β-glucosidase $F_{42}$ is unique in its specificity for some substrates.

**TABLE 3.** SPECIFICITY OF β-GLUCOSIDASE $F_{42}$ FOR DIFFERENT *PARA*- OR *ORTHO*-NITROPHENYL GLUCOSIDE SUBSTRATES

| Substrate (1 mM) | Relative activity* (%) |
|---|---|
| *p*-Nitrophenyl-β-D-glucopyranoside | 100.0 |
| *p*-Nitrophenyl-β-D-mannopyranoside | 99.3 |
| *p*-Nitrophenyl-α-D-galactopyranoside | 88.7 |
| *o*-Nitrophenyl-β-D-glucopyranoside | 25.9 |
| *p*-Nitrophenyl-α-D-mannopyranoside | ND |

* Expressed as a percentage of activity compared with the activity with *p*-nitrophenyl-β-D-glucopyranoside as a substrate.
ND, not detected.

## Effects of Metallic Ions and Organic Compounds on β-Glucosidase $F_{42}$ Activity

β-Glucosidase $F_{42}$ activity was strongly inhibited by 1 mM $HgCl_2$ and 10 mM $CoCl_2$ (84 and 75%, respectively). In the presence of 10 mM $CuSO_4$, 1 mM $AgNO_3$ or 1 mM $ZnSO_4$, an inhibitory effect of greater than 50% was observed (Table 4). The inhibition by $Ag^+$ and $Hg^{2+}$ suggests that the sulfhydryllic group may have an essential role in β-glucosidase activity (Hsieh and Graham 2001). The inhibitory effect of $Ag^+$ and $Hg^{2+}$ ions at low concentrations may be explained by the fact that they interact with cysteine residues, forming an insoluble complex with sulfur, whereas the other ions form soluble complexes (Esen 1992). According to Yang *et al*. (2004), cysteine residues are involved in enzyme stability and activity. Thus, inhibition by these metals may be prevented in the presence of β-mercaptoethanol (Hsieh and Graham 2001).

$MnCl_2$ at concentrations of 1 mM and 10 mM increased β-glucosidase activity by 10 and 63%, respectively (Table 4). $Mn^{2+}$ may be a positive modulator connected to the active site of β-glucosidase. According to Esen (2003), the presence of $Mn^{2+}$ in the crystal structure of myrosinase (β-s-glucosidase) suggests that this divalent cation may be necessary for enzyme activity. The lack of inhibition of β-glucosidase by $Ca^{2+}$ is notable, as this enzyme may be used in lactose hydrolysis, decreasing the effects of this sugar in lactose-intolerant individuals (Pessela *et al*. 2003).

Among the organic compounds, glucono-δ-lactone was a strong inhibitor of β-glucosidase, inhibiting 94% of the enzyme activity at a concentration of 10 mM. Glucono-δ-

**TABLE 4.** EFFECTS OF METALLIC IONS AND ORGANIC COMPOUNDS ON β-GLUCOSIDASE $F_{42}$ ACTIVITY

| Metallic ion (mM) | | Relative activity (%)* | Organic compound (mM) | | Relative activity (%)* |
|---|---|---|---|---|---|
| $HgCl_2$ | 1 | 16 | Glucono-δ-lactone | 1 | 38 |
| | 10 | n.d. | | 10 | 6 |
| $AgNO_3$ | 1 | 47 | PMSF | 1 | 78 |
| | 10 | n.d. | | 10 | 64 |
| $ZnSO_4$ | 1 | 44 | EDTA | 1 | 79 |
| | 10 | 86 | | 10 | 77 |
| $CuSO_4$ | 1 | 86 | | | |
| | 10 | 36 | | | |
| $CoCl_2$ | 1 | 90 | | | |
| | 10 | 25 | | | |
| $CaCl_2$ | 1 | 96 | | | |
| | 10 | 96 | | | |
| $MnCl_2$ | 1 | 110 | | | |
| | 10 | 163 | | | |

* Expressed as a percentage of activity compared with the activity with *p*-nitrophenyl-β-D-glucopyranoside as a substrate.
EDTA, ethylenediaminetetraacetic acid; n.d., not determined; PMSF, phenylmethylsulfonyl fluoride (in 95% ethanol).

lactone has been described as a strong inhibitor of β-glucosidase from different vegetable sources (Matsuura and Obata 1993; Cameron *et al*. 2001; Hsieh and Graham 2001). PMSF and EDTA had moderate effects on the enzyme, inhibiting only about 20% of its activity. The lack of inhibition by EDTA indicates that the enzyme does not require a metal cofactor in its active site. However, the low level of inhibition may be related to a possible change in amino acid load at the active site caused by this organic compound (Harnpicharnchai *et al*. 2009). The lack of inhibition by PMSF, which interacts with histidine and serine residues in the active site, indicates that these amino acids do not have an essential role in β-glucosidase activity (Wolosowska and Synowiecki 2004).

Glucose has an inhibitory effect on β-glucosidase $F_{42}$ activity, acting as a competitive inhibitor. Dixon's (1953) report established a $K_i$ of 2 mM. According to Yang *et al*. (2004), competitive inhibition from glucose is a common characteristic of β-glucosidases vegetable and microbial. Cameron *et al*. (2001) and Han and Chen (2008) reported inhibition of β-glucosidase by glucose in orange bagasse and corn forage, respectively. However, vanilla β-glucosidase is not inhibited by glucose at concentrations lower than 2 M (Odoux *et al*. 2003).

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