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Hydrolysis of soybean isoflavones by *Debaryomyces hansenii* UFV-1 immobilised cells and free β-glucosidase

Gabriela P. Maitan-Alfenas, Lorena G. de A. Lage, Maíra N. de Almeida, Evan M. Visser, Sebastião T. de Rezende, Valéria M. Guimarães *

Dep. Bioquímica e Biologia Molecular, BIOAGRO, Universidade Federal de Viçosa, Viçosa, MG 36.570-000, Brazil

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ABSTRACT

An intracellular β-glucosidase from *Debaryomyces hansenii* UFV-1 was produced in an YP medium with cellobiose as the carbon source. This enzyme was purified, characterised and presented a *M*r of 65.15 kDa. Yeast cells containing the intracellular β-glucosidase were immobilised in calcium alginate. The free β-glucosidase and immobilised cells containing the enzyme presented optima values of pH and temperature of 6.0 and 45 °C and 5.5 and 50 °C, respectively. The free enzyme maintained 62% and 47% of its original activity after 90 days at 4 °C and after 15 days at room temperature, respectively. The immobilisation process resulted in higher enzyme thermostability at 45 and 50 °C. Soy molasses treatment with the free enzyme and the immobilised cells containing β-glucosidase, for 2 h at 40 °C, promoted efficient hydrolysis of isoflavone glicosides to their aglycon forms. The results suggest that this enzyme could be used in the food industry, in the free or immobilised forms, for a safe and efficient process to hydrolyse isoflavone glycosides in soy molasses.

1. Introduction

β-Glucosidases (β-D-glucoside glucohydrolases; EC 3.2.1.21) are enzymes that catalyse the hydrolysis of the β-glycosidic linkage from the non-reducing end of isoflavone glucosides, disaccharides, oligosaccharides, aryl-glucosides and alkyl-glucosides (Cairns & Esen, 2010; Kaya et al., 2008; Xue, Yu, & Song, 2009). These enzymes have been used in several biotechnological applications, including food detoxification, biomass conversion, flavor enhancement in wines and other beverages (Cairns & Esen, 2010; Pal, Banik, Ghorai, Chowdhury, & Khowala, 2010) and, also, the conversion of soybean isoflavone glycosides into their aglycon forms (Song, Xue, Wang, & Wu, 2011; Yeom, Kim, Kim, & Oh, 2012).

Isoflavones are diphenolic secondary metabolites of plants, which have a structural and functional similarity to human estrogen, and can act in the prevention of osteoporosis, cancer, cardiovascular diseases and postmenopausal syndromes (Barbosa et al., 2010; Luthria, Biswas, & Natarajan, 2007; Nielsen & Williamson, 2007). Soybeans are considered a rich source of isoflavones (Chen et al., 2012a) and they contain 12 isoflavone chemical forms, including the three aglycones, daidzein, genistein and glycitein, and their glycosides, acetyl-, malonyl-, and β-glycosides (Kaya et al., 2008; Xue et al., 2009). Bioavailability of soy isoflavones differs between isoflavone aglycones and glycosides, and studies have revealed that isoflavone aglycones are superior to isoflavone glycosides with respect to various bioactivities, due to their effective absorption into the human body (Chen, Lo, Su, Chou, and Cheng, 2012c). Therefore, there is great interest in increasing the amounts of isoflavone aglycones in soy products mainly because, naturally, most of the isoflavones exist in the glycosylated forms (Cheng, Wu, Lin, & Liu, 2013).

The enzymatic processing of isoflavone glycosides in soybean products using isolated β-glucosidases has proved to be effective in increasing the concentration of isoflavone aglycones (Horri et al., 2009; Xue et al., 2009; Yang, Wang, Yan, Jiang, & Li, 2009). It has previously been demonstrated that the use of the immobilised microorganism cells containing enzymes of interest in bioconversion processes is advantageous when compared to the use of the purified enzymes, since the purification step is not necessary and enzymatic stability is higher (Junior et al., 2009). *Debaryomyces hansenii* is the most common yeast species in protein-rich fermented food, where this species metabolises organic acids and amino acids to regulate the acidity, and also provides lipolytic and proteolytic activities that contribute to flavor development; the potential of *D. hansenii* UFV-1 to produce hydrolytic enzymes, specially α-galactosidases, has previously been explored (Viana et al., 2007). The present study reports the purification and characterisation of an intracellular β-glucosidase from *D. hansenii* UFV-1, the immobilisation of *D. hansenii* cells in calcium alginate, and the application of the free and immobilised enzymes for the hydrolysis of isoflavone glucosides in soy molasses.

* Corresponding author. Tel.: +55 (31) 3899 2374; fax: +55 (31) 3899 2373.
E-mail address: vmonteze@ufv.br (V.M. Guimarães).

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2. Materials and methods

2.1. Microorganism

The yeast strain used in this study was isolated from a dairy environment in Minas Gerais, Brazil, and maintained in the culture collection of the Laboratory of Microorganism Physiology, BIOAGRO, Federal University of Viçosa (UFV), Brazil. The yeast was identified by the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as *D. hansenii* (Zopf) Lodder & Kreger-van Rij var *fabryi* Nakase & Suzuki. In this study it is designated as *D. hansenii* UFV-1.

2.2. β-Glucosidase production

A stock culture of *D. hansenii* UFV-1 was maintained at −80 °C in glycerol and YPD medium (1% yeast extract, 2% peptone and 2% glucose). *D. hansenii* UFV-1 was streaked on an YPD agar surface (1.5% agar) and maintained in an incubation chamber at 30 °C for 36 h. The yeast was then activated in YPD liquid medium and incubated for 12–15 h, 180 rpm at 28 °C. The cells obtained after centrifugation (5000*g* for 5 min at 4 °C) were inoculated in an YP medium (1% yeast extract, 2% peptone) containing cellobiose, glucose, maltose or cellulose (1%) as the carbon source. After incubation at 28 °C, 180 rpm, for 12, 24, 36 and 48 h, the supernatant was separated by centrifugation (15,000*g* for 20 min at 4 °C) and the biomass was utilised as a source of the intracellular enzyme. The activity of the intracellular β-glucosidase was estimated by measurement of the fresh cell mass for the different cultivation times.

2.3. Extraction of the intracellular enzyme

D. hansenii UFV-1 cells (15 g) were ground with liquid nitrogen and resuspended in 40 mL of 0.1 M sodium acetate buffer, pH 5.0, containing 0.25% (by weight) Triton X-100. This mixture was submitted to a series of nitrogen freezing and thawing at 40 °C in a water bath (Branson, USA). It was then submitted to an ultrasonic bath for 10 min and centrifuged (25,000*g* for 20 min at 4 °C). The supernatant was used as the source of intracellular enzyme.

2.4. Purification of intracellular β-glucosidase

The enzymatic extract was submitted to dialysis against 4 L of 10 mM sodium phosphate buffer for 15 h at 4 °C. A dialysis membrane with 3 kDa of pore exclusion was used. After this procedure, the sample was loaded onto a DEAE-Sepharose anion exchange column (6.8 × 2.0 cm), equilibrated with 50 mM sodium phosphate buffer, pH 7 at 4 °C. Elution was performed at the flow rate of 60 mL/h, with a linear gradient formed with 150 mL of 50 mM sodium phosphate buffer and 150 mL of the same buffer containing 0.8 M NaCl. Fractions containing β-glucosidase activity were pooled and concentrated by Amicon ultrafiltration with a 3 kDa molecular membrane cut off at 4 °C, 3500*g* for 1 h. The concentrated sample was subjected to FPLC (Fast Protein Liquid Chromatography) with a Sephacryl S-300 gel filtration column (26 × 60 cm), equilibrated with 25 mM sodium phosphate buffer, pH 7. The proteins were eluted at a flow rate of 60 mL/h. Fractions containing β-glucosidase activity were pooled and loaded onto a phenyl-sepharose column (2.5 × 1.6 cm) coupled to the FPLC, equilibrated with 0.5 M ammonium sulfate in 25 mM sodium phosphate buffer, pH 7. The proteins were eluted at a flow rate of 240 mL/h with a linear gradient of ammonium sulfate (0.5–0 M) in 25 mM sodium phosphate buffer, pH 7. The active fractions were pooled and analysed for purity by SDS–PAGE.

2.5. Enzyme assay

β-Glucosidase activity was assayed by measuring the amount of *p*-nitrophenol (*p*NP) released from the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPβGlc) as substrate. Both, *p*NP and *p*NPβGlc, were purchased from Sigma Aldrich (USA). The standard reaction mixture contained 2 mM *p*NPβGlc, 50 mM sodium phosphate buffer (pH 6.0) and the enzyme preparation in a final volume of 1.0 mL. After incubation at 40 °C for 15 min, 1.0 mL of 0.5 M sodium carbonate was added to the mixture to stop the reaction. The absorbance of the mixture was then measured at 410 nm. The amount of *p*NP released was calculated according to a standard curve, and one unit of enzyme activity (*U*) was defined as the amount of enzyme that releases 1.0 μmol of ρNP per min under the assay conditions. For β-glucosidase activity determination in immobilised cells, the assays were conducted with the same reagents but replacing the enzyme preparation with 4 alginate beads and modifying the pH of the phosphate buffer from 6.0 to 5.5.

The activities against cellobiose, maltose, gentiobiose, lactose and melibiose were determined by the glucose oxidase method. Glucose liberated was quantified using a commercial analytical kit based on glucose oxidase and peroxidase (Quibasa Basic Chemical, Belo Horizonte, MG, Brazil). The production of reducing sugar was determined using the 3.5-dinitrosalicylate reagent where sucrose and cellulose were used as substrates (Miller, 1959). One unit of enzyme activity (*U*) was defined as the amount of enzyme that releases 1.0 μmol of product per min under the assay conditions.

Data presented for β-glucosidase activity is the mean of assays performed in triplicate.

2.6. Protein determination

Protein concentration in the enzymatic extracts was determined by the BCA (bicinchoninic acid) method (Smith et al., 1985) with bovine serum albumin (BSA) as the standard.

2.7. Molecular mass determination

The molecular weight (MW) of the purified enzyme was estimated by SDS–PAGE using a 12.5% (w/v) polyacrylamide gel (Laemmli, 1970). The molecular mass standards were obtained from Sigma Aldrich (Sigma Markers Wide Range MW 6500–200,000 Da, St. Louis, MO, USA). After electrophoresis, the proteins were visualised by silver staining (Blum, Beier, & Gross, 1987).

2.8. D. hansenii UFV-1 cells permeabilisation

The protocol used for permeabilisation of *D. hansenii* UFV-1 cells was the same as that reported by Junior et al., 2009, with some alterations. Yeast culture samples were centrifuged (25,900*g* for 5 min at 4 °C) and the pellet was resuspended in a 50% (v/v) ethanol solution at the proportion of 450 μL of this solvent to 0.2 g of cells. After agitation for 5 min at room temperature, the suspension was centrifuged (4000*g* for 5 min at 4 °C) and the permeabilised cells were dried for 1 h at 37 °C.

2.9. Immobilisation of permeabilised cells in calcium alginate beads

The protocol used for immobilisation of permeabilised *D. hansenii* UFV-1 cells was the same as that reported by Junior et al., 2009, with some alterations. The dry permeabilised cells were mixed with a 2% (w/v) sodium alginate solution, in a proportion of 4 g of cells to 1 g of alginate. This suspension was extruded through a hypodermic needle using a peristaltic pump to obtain a uniform particle size. The droplets eluted from the hypodermic needle were collected in a flask, containing 0.1 M $CaCl_2$ solution to form

alginate beads. The beads were maintained in a 0.1 M $CaCl_2$ solution for 12 h at 4 °C. They were subsequently washed three times with 0.1 M sodium phosphate buffer pH 5.5 and kept at 4 °C in the same buffer until utilisation.

2.10. Re-use of beads

The assay of re-use of the alginate beads was performed using *p*NPβGlc or isoflavones as substrates. Ten millilitres of 2 mM *p*NPβGlc in 50 mM sodium phosphate buffer pH 5.5 and 40 alginate beads were added to 25 mL Erlenmeyer flasks and incubated under agitation (100 rpm) at 50 °C. After 15 min incubation time, an aliquot (100 μL) of solution was taken and the amount of *p*NP was determined. The isoflavones hydrolysis assay was performed according item 2.12, except that the temperature was 50 °C. After this first cycle, the beads were separated by filtration, washed with 50 mM sodium phosphate buffer pH 5.5, and incubated with a fresh 2 mM *p*NPβGlc solution (10 mL) for an additional 15 min incubation period or with soy molasses suspension during 2 h. This cycle was repeated until the enzymatic activity become null.

2.11. Enzymatic characterisation

2.11.1. Effects of pH and temperature

The influences of pH and temperature on β-glucosidase activities were determined using the standard assay for the free and immobilised enzymes, except that the pH values were modified to a range of 2.0–8.0 (Mcllvaine, 1921) and the temperature values ranged from 10 to 60 °C.

The pH stability of β-glucosidase was determined by incubating the free enzyme solution or the alginate beads in the pH range of 2.0–8.0 for 30 min, on ice. After incubation, the mixture was used for determining residual activity, according to standard assay, using *p*NPβGlc as the substrate. Thermal stability was investigated by incubating the enzymatic solution or the alginate beads in 50 mM sodium phosphate buffer, pH 6.0 or 5.5, respectively, at temperatures of 45 and 50 °C for different times. After pre-incubation, aliquots of the enzymes or 4 alginate beads were collected and submitted to the standard assay, measuring the remaining activity. The relative activities were calculated in relation to β-glucosidase activity without pre-incubation, which was considered to be 100%. Results of the analyses are presented as mean ± SD for three measurements.

2.11.2. Determination of kinetic parameters

The Michaelis–Menten constant (K_M) and V_{max} for substrate hydrolysis by the free enzyme and the K_{Mapp} value for the immobilised enzyme were calculated by the Michaelis–Menten plot. Concentrations of *p*NPβGlc varied from 0.2 to 5.0 mM. The inhibition constant (K_i) for the free enzyme using glucose as inhibitor was determined by varying the *p*NPβGlc concentrations from 0.05 to 1.2 mM in the presence of 50, 100 or 120 mM of glucose.

2.11.3. Substrate specificity

Enzymatic assays were performed with various synthetic, natural and polymeric substrates. The reaction mixtures contained 650 μL of 50 mM sodium phosphate buffer pH 6.0, 0–100 μL of enzyme solution and 250 μL of synthetic substrates (0.5 mM) or cellobiose, lactose, maltose, gentiobiose, melibiose and sucrose (2.5 mM) or cellulose (0.025%). Activities were measured under standard assay conditions at 40 °C. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

2.11.4. Effect of ions, simple sugars and reducing agents

The effects of ions, simple sugars and reducing agents on enzyme activity were assayed by the standard methods. Reaction mixtures contained 450 μL of 50 mM sodium phosphate buffer pH 6.0, 0–100 μL of the enzyme solution and 200 μL of the compounds (0.2 and 2 mM). The data presented for all enzyme activity assays are mean values ± SD of measurements performed in triplicate.

2.12. Treatment of soy molasses with free and immobilised β-glucosidase

The soy molasses samples were kindly donated by Melaços Brasileiros Ltda., Saltinho, São Paulo, Brazil. One gram samples of soy molasses were incubated with either 10 U of free β-glucosidase in 50 mM sodium phosphate buffer pH 6.0 (10 mL) or with a calculated number of beads corresponding to 10 U of β-glucosidase in 50 mM sodium phosphate buffer pH 5.5 (10 mL) in an orbital shaker (100 rpm) at 40 °C, for 0, 15, 30, 60 and 120 min. The reactions were stopped by freezing the flasks at −80 °C and the hydrolyzed samples were lyophilised.

Isoflavones were extracted from the lyophilised samples (1 g) with 5 mL of 80% methanol by stirring for 2 h at room temperature. The mixtures were centrifuged at 16,100*g* for 10 min and the supernatants were filtered through a 0.45 μm filter for analysis of the isoflavones via HPLC.

2.13. HPLC analysis of isoflavones

The contents and compositions of isoflavones were determined quantitatively by HPLC. The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AD pump, a UV detector (SPD-10AV) and a Shim-pack CLC-ODS (M) column (4.6 × 250 mm) (Shimadzu Co., Kyoto, Japan). The mobile phase consisted of solvent (A) composed of 0.1% (v/v) acetic acid in filtered MilliQ water, and (B) solvent consisting of 0.1% (v/v) acetic acid in acetonitrile. The following gradient for solvent B was applied: 15–25% from 0 to 35 min, 25–26.5% over the next 12 min and 26.5–50% over 30 s followed by isocratic elution for 14.5 min. The flow rate was 1.0 mL/min, column temperature was 40 °C and the absorbance was measured at 254 nm. Isoflavone content of the samples was calculated by interpolation of the calibration curves prepared using varying concentrations of the 12 isoflavone standards.

3. Results and discussion

D. Hansenii UFV-1 grown in YP medium containing cellobiose as carbon source presented expressive biomass production and intracellular β-glucosidase activity (data not shown). The yeast exhibited intracellular β-glucosidase activity and biomass production of 0.016 U/mL and 4.36 mg/mL, respectively, when cultivated during 12 h in the YP medium with cellobiose. Cellobiose was the most effective sugar tested for induction of growth and intracellular β-glucosidase activity in *D. hansenii* UFV-1. Extracellular β-glucosidase production induced by cellobiose was reported for *Debaryomyces vanrijiae* and *Debaryomyces pseudopolymorphus* (Belancic, Gunata, Vallier, & Agosin, 2003; Villena, Iranzo, Gundllapalli, Otero, & Perez, 2006). Different from the others, *D. hansenii* UFV-1 did not secrete β-glucosidase when grown on cellobiose. The presence of this intracellular enzyme could suggest that *D. hansenii* presents a cellobiose transporter. Several yeast species including *Clavispora lusitaniae*, *Candida wickerhamii*, *Debaryomyces polymorphus* and *Pichia guillermondii* have the ability to transport cellobiose across the plasma membrane (Freer, 1991; Freer & Greene, 1990). *Kluyveromyces lactis* produces an intracellular β-glucosidase, implying

that this yeast also has the ability to transport cellobiose into the cell (Tingle & Halvorson, 1972).

Results of *D. hansenii* UFV-1 β-glucosidase purification are summarised in Table 1. After dialysis, the enzymatic extract was subjected to ion exchange chromatography, resulting in the separation of one protein fraction with β-glucosidase activity, which was eluted with 0.1 M NaCl. This step promoted considerable specific activity enrichment (Table 1). The concentrated enzymatic fraction was submitted to gel filtration chromatography, which also yielded a single active peak. In the last stage of the purification process, the active fractions were eluted from a phenyl-sepharose column when the salt gradient had been exhausted. This procedure resulted in a purification factor of 99.3 with 8% recovery of the original β-glucosidase activity. The electrophoretic profile of the enzyme in SDS–PAGE confirmed the presence of a single protein band with an estimated molecular mass of 65.15 kDa (Fig. 1).

Substantial activity against *p*NPβGlc was observed for the purified enzyme within a pH range of 5.5–7.0 and temperature range of 30–50 °C. The optimum pH for the enzyme was 6.0 (Fig. 2A) and the β-glucosidase achieved maximal substrate hydrolysis at 45 °C (Fig. 2B). This optimum pH value is the same as those reported for hydrolysis of *p*NPβGlc by the β-glucosidase from apple seed (Yu, Xu, Lu, & Lin, 2007), from *Pyrococcus furiosus* (Yeom et al., 2012) and from the endophytic bacterium *Pseudomonas* ZD-8 (Yang, Ning, Shi, Chang, & Huan, 2004). The β-glucosidase from *Termitomyces clypeatus* also exhibited maximal activity against *p*NPβGlc at 45 °C (Pal et al., 2010).

The purified *D. hansenii* UFV-1 β-glucosidase maintained approximately 51% of its original activity after 6 h of pre-incubation at 45 °C and 30% after 60 min at 50 °C (Fig. 2C). The half-life of *D. hansenii* UFV-1 β-glicosidase at 45 and 50 °C was 312 and 73 min, respectively. Stability of this enzyme was also evaluated at 4 °C and at room temperature (25 °C). The enzyme maintained 97% and 62% of its original activity after 30 and 90 days of incubation at 4 °C, respectively. When kept at room temperature, the enzyme maintained 67% and 47% of its original activity after 5 and 15 days, respectively.

The *D. hansenii* UFV-1 β-glicosidase showed significant stability over a wide pH range. This enzyme retained more than 90% of its activity after incubation for 30 min in a pH range of 5.5–8.0. About 85% and 64% of its activity was maintained after incubation at pH 4.5 and 4.0, respectively, and enzymatic activity was null after incubation at pH values below 3.5 (Fig. 2A). The enzyme showed significant stability for a wide pH range and reasonable temperature levels which is desirable for industrial applications, especially for hydrolysis of isoflavones in soybean products.

Immobilised *D. hansenii* UFV-1 cells containing β-glucosidase showed substantial activity within the same pH range of the free enzyme (5.5–7.0) and over an even larger temperature range (20–55 °C). In this case, the optimum pH was 5.5 (Fig. 2D), lightly more acidic than the optimum pH of the free enzyme, which was 6.0. This decrease in optimum pH after immobilisation in calcium alginate can be partially explained by the effect of the micro-environment in the calcium alginate gel matrix, particularly due to the presence of positively charged Ca^{2+} ions (Adami, Cavazzomi, Trezzi, & Craveri, 1998). Furthermore, the charged hydrophilic groups present in the polymer structure of the alginate gel lead to swelling by water absorption. The swelling equilibrium is affected by the pH and at pH 5.5 the ability of substrate and product diffusion within the three-dimensional network of the gel is likely increased. The effect of a decrease in the optimum pH value of enzymes after cell immobilisation in alginate was also observed by other authors (Junior et al., 2009; Wang, Su, Qi, Zhang, & He, 2010).

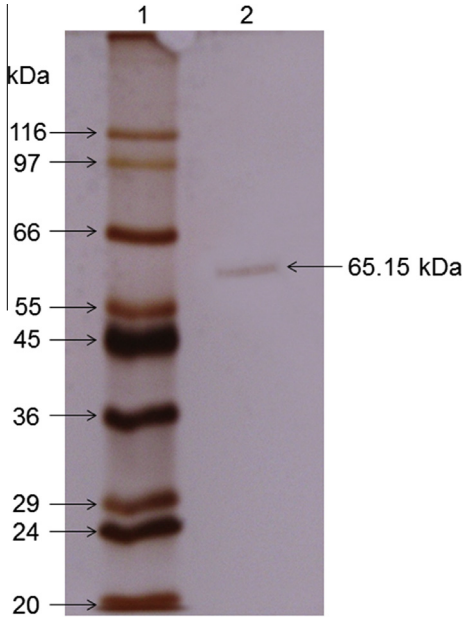

Fig. 1. Denaturing electrophoresis (12.5% SDS–PAGE) stained with silver. 1 – Molecular weight marker. 2 – Purified enzyme fraction.

High enzyme activities were maintained after pre-incubation of *D. hansenii* UFV-1 immobilised cells in pH values between 2.0 and 8.0, where more than 90% of its activity was preserved after pre-incubation in pH 6.0–8.0 (Fig. 2D). Immobilisation in calcium alginate probably protected the enzyme contained in the cells, since the enzyme pre-incubated in extreme pH values recovered its activity when the pH was returned to the optimum level.

The optimum temperature of the immobilised enzyme was 50 °C (Fig. 2E), higher than the value obtained for the free enzyme, 45 °C. At 50 °C the free enzyme presented only 73% of its maximum activity. Furthermore, the immobilised β-glucosidase presented 58% of its activity at 55 °C (Fig. 2E) and at this temperature the free enzyme showed only 21% of its maximum activity (Fig. 2B). This increase in the optimum temperature of the immobilised enzymes was also observed by Junior et al. (2009) in their studies with α-galactosidase from *D. hansenii* UFV-1. The temperature of 50 °C probably favours the swelling equilibrium and led to a greater rate of diffusion of substrate and product through the three dimensional gel network. Therefore, the immobilisation in calcium alginate protects the enzyme against the deleterious effects of high temperatures, giving greater stability to this molecule.

Table 1
Summary of *D. hansenii* UFV-1 intracellular β-glucosidase purification.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold (X)	Yield (%)
Crude extract	1171.81	174.02	0.15	1.0	100
Dialysis	621.06	125.43	0.2	1.4	72
DEAE-sepharose	22.05	111.61	5.06	33.9	64
Ultrafiltration	8.88	55.05	6.19	41.6	32
Sephacryl S-300	4.75	45.6	9.6	64.4	26
Phenyl-sepharose	0.97	14.53	14.8	99.3	8

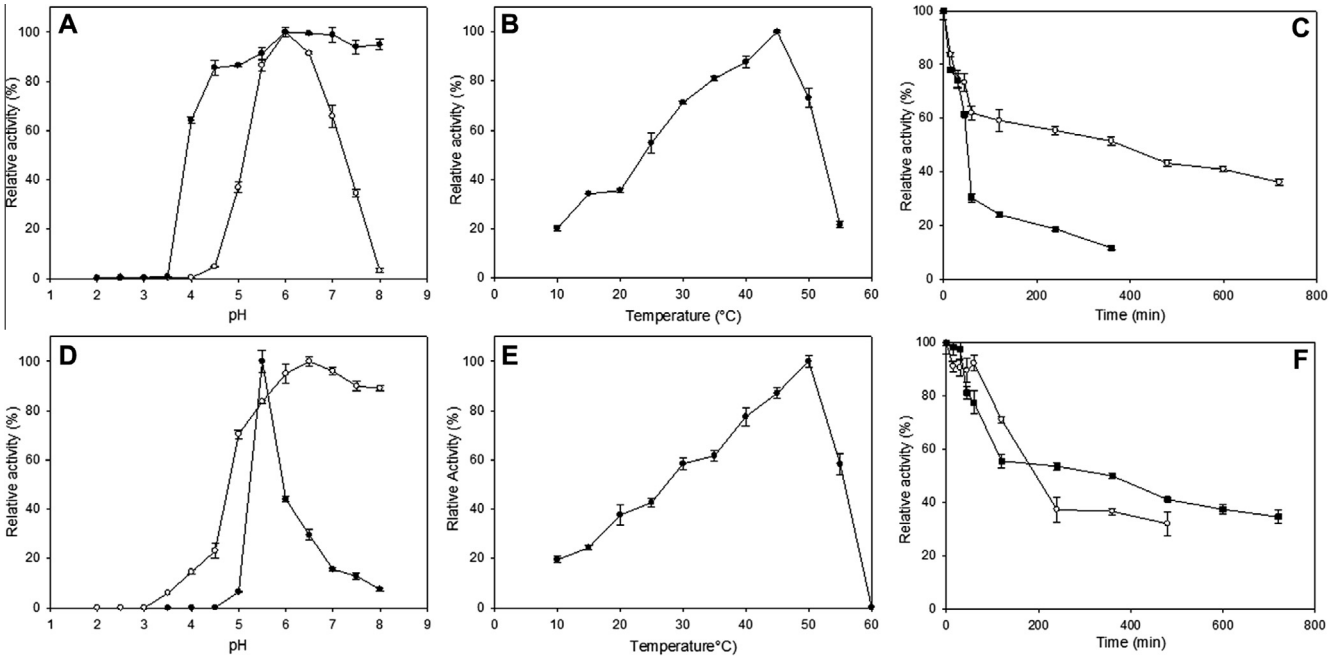

Fig. 2. Effect of pH and temperature on β-glucosidase from *D. hansenii* UFV-1. Free enzyme: (A) effect of pH on activity (○) and stability (●). (B) Effect of temperature. Relative activities were calculated in relation to activities determined at optima pH and temperature. (C) Thermostability at 45 (○) and 50 °C (■). Immobilised enzyme: (D) effect of pH on the activity (●) and stability (○). (E) Effect of temperature. Relative activities were calculated in relation to activities determined at optima pH and temperature. (F) Thermostability at 45 (○) and 50 °C.

In general, we could assume that the immobilisation process can contribute to conformational changes in the protein structure. And, when there is a change in the pH value, there are some alteration in the concentrations of charged species (substrate, product, ions) in the environment of the immobilised enzyme which could result in a change of the optimum pH value. The immobilisation also leads to a higher value of optimum temperature due to the binding of the enzyme to the support, which could prevent unfolding of the tertiary structure.

Thermostability assays at 45 and 50 °C indicate that alginate beads containing immobilised β-glucosidase exhibited higher thermostability (Fig. 2F) than the free enzyme (Fig. 2C). According to Wang et al. (2010), the immobilisation process leads to increased enzymatic rigidity, commonly reflected by an increase in thermal stability. These authors reported that the immobilisation preserves the tertiary structure of the protein and prevents conformational changes to this structure, in different environments. The immobilisation of *D. hansenii* cells in calcium alginate probably protected the β-glucosidase against the deleterious effects of high temperatures, resulting in greater stability of this molecule. Thus, the immobilisation process makes the enzyme more useful for biotechnological applications.

The free enzyme displayed classical Michaelis–Menten kinetics towards ρNPβGlc. The K_M value determined for free β-glucosidase from *D. hansenii* UFV-1 for hydrolysis of this substrate was 0.43 mM, lower than the K_M of 0.77 mM reported for *D. vanrijiae* β-glucosidase (Belancic et al., 2003). These results suggest that β-glucosidase from *D. hansenii* UFV-1 has a higher apparent affinity for ρNPβGlc compared to the other, and complex ES formation is probably not the limiting step for the reaction. The K_{Mapp} value for the immobilised enzyme against ρNPβGlc was 4.35 mM, ten times higher than the K_M value of the free enzyme (0.43 mM). This result suggests that the immobilisation process resulted in lower enzyme accessibility to the substrate ρNPβGlc.

Activity of the free β-glucosidase against several substrates is shown in Table 2. Under the experimental conditions, the *D. hansenii* UFV-1 β-glucosidase proved to be highly selective for the synthetic substrates with glucose in the β position, since only ρNPβGlc and *o*NPβGlc were hydrolyzed, with the latter to a lesser extent. The enzyme did not hydrolyze the synthetic substrates with non-glucose sugar residues or containing the α glycosidic bond. In contrast, one β-glucosidase from *D. hansenii* reported by Riccio et al. (1999) was capable of hydrolyzing different synthetic substrates with β and α configurations, indicating different features between β-glucosidases from these two strains. In relation to the natural substrates, the *D. hansenii* UFV-1 β-glucosidase was highly specific for the β-(1,4) linkage of glucose residues, since the enzyme was only able to hydrolyze cellobiose and cellulose. Generally β-glucosidases show greatest activity against the natural substrate cellobiose, such as the enzymes from *D. pseudopolymorphus* and *Termitomyces clypeatus* (Pal et al., 2010; Villena et al.,

Table 2
Hydrolysis of several substrates with β-glucosidase from *D. hansenii* UFV-1.

Substrate	Final concentration	Activity (μmol min^{-1}) ±SD
ρNPβGlc	0.5 Mm	0.102 ± 0200
ρNPαGlc	0.5 mM	0
ρNPβGal	0.5 mM	0
ρNPαGal	0.5 mM	0
ρNPαMan	0.5 mM	0
ρNPβMan	0.5 mM	0
ρNPβXyl	0.5 mM	0
ρNPαAra	0.5 mM	0
*m*NPαGal	0.5 mM	0
*o*NPαGal	0.5 mM	0
*o*NPβGlc	0.5 mM	0.030 ± 0080
Cellobiose	2.5 mM	0.002 ± 0002
Lactose	2.5 mM	0
Maltose	2.5 mM	0
Melibiose	2.5 mM	0
Gentiobiose	2.5 mM	0
Sucrose	2.5 mM	0
Cellulose (Avicel®)	0.025%	0.018 ± 0050

2006). The ability of the *D. hansenii* UFV-1 β-glucosidase to more efficiently hydrolyze the cellulose polymer compared to cellobiose is interesting. The activity against cellobiose was 11% of the activity against cellulose (Table 2). This result indicates that this enzyme presents greater affinity to cellulose compared to cellobiose, suggesting that in addition to β-glucosidase activity, this enzyme could display a 4-β-D-glucanglucohydrolase activity and acts on 1,4-β-D-glucans and related oligosaccharides, but slowly hydrolyses cellobiose. As shown on Table 2, the activity of *D. hansenii* UFV-1 β-glucosidase was higher against artificial substrates than the natural ones. Moreover, this activity against *o*NPβGlc is only 29% of that against *ρ*NPβGl. Different β-glucosidases reported in the literature present a wide variation in their activities when considering different substrates (Gueguen, Chemardin, & Arnaud, 2001; Korotkova et al., 2009; Krogh et al., 2010).

The free β-glucosidase from *D. hansenii* UFV-1 showed distinct sensitivities to sugars, ions and reducing agents (Table 3). The enzyme was completely inhibited by iron chloride, silver nitrate and SDS in both concentrations tested. In the assay conditions, the denaturing action of SDS probably affected the integrity of the enzyme tridimensional structure which is fundamental for its catalytic activity. Inhibition caused by SDS (1 and 10 mM) was also demonstrated by Li, Jiang, Fan, and Liu (2012) for cloned β-glucosidase using metagenomic DNA from mangrove soil. *D. hansenii* UFV-1 β-glucosidase activity was greatly increased by β-mercaptoethanol, glucose, urea and aluminium chlorid at both concentrations tested. Non-inhibition by EDTA implies that divalent cations are not essential to enzyme activity (Chen, Li, & Zong, 2012b) and it is not a metalloenzyme. Many works reported that EDTA does not inhibit β-glucosidases as in the case of *Pyrococcus furiosus* β-glucosidase that was considered metal-independent (Yeom et al., 2012). β-Mercaptoethanol was the agent which best promoted enzyme activation in both final concentrations tested. The activation by this reducing agent can be explained by the fact that some reduced chemical ligations in the enzyme structure are favourable for the catalytic activity. Calcium and magnesium have a stimulatory effect on *D. hansenii* UFV-1 β-glucosidase. It has been reported that these two ions are enhancers of β-glucosidase activity (Oyekola, Ngesi, & Whiteley, 2007).

Glucose was found to be a competitive inhibitor of *D. hansenii* UFV-1 β-glucosidase and the K_i value was 11.36 mM. In general, β-glucosidases are inhibited by glucose and this inhibition is competitive (Yang et al., 2004).

Soy molasses is a by-product generated in the production of soy protein concentrate, in which isoflavones and other phytochemicals are enriched (Hosny & Rosazza, 1999). This by-product in the soy industry is used as an inexpensive animal feed, but the processing and use of soy molasses as a functional food has been suggested (Najafpour & Shan, 2003).

The potential of *D. hansenii* UFV-1 intracellular β-glucosidase to hydrolyze isoflavones in soy molasses to their aglycon forms was demonstrated for the free β-glucosidase and the alginate immobilised cells containing this enzyme (Table 4). Prior to hydrolysis, glucoside isoflavones were predominant in the soy molasses, representing approximately 80%, where aglycones made up about 10%. After 2 h of treatment with the free or immobilised β-glucosidase the isoflavone glucosides were almost completely hydrolyzed after which there remained about 3% of these compounds. There was no change in the amounts of isoflavone glucosides and aglycones after 4 or 8 h of enzymatic treatment of soy molasses compared to the assay after 2 h of hydrolysis (data not shown). This indicates that a short incubation period is preferred over a prolonged incubation with the free or immobilised enzyme. A new immobilisation system of β-glucosidase, in glass microspheres, was capable to hydrolyze isoflavone glucosides in black soymilk in only 30 min (Chen et al., 2012c).

It is clearly shown in Table 4 that the contents of daidzein, genistein and glycitein increased after 2 h of hydrolysis for both experiments. Additionally, it has to be considered that there are other types of isoflavone glucosides in the soy molasses, which can also be converted to other forms at different proportions. The immobilised β-glucosidase presented minimal difference in conversion

Table 3
Relative activity of *D. hansenii* UFV-1 intracellular β-glucosidase submitted to different effectors. Relative activities were calculated in relation to the β-glucosidase activity without pre-incubation which was considered to be 100%.

Effector	Final concentration	Relative activity (%) ±SD	Final concentration	Relative activity (%) ±SD
Na_2SO_4	0.2 mM	117.92 ± 2.13	2 mM	127.96 ± 3.27
NaCl	0.2 mM	122.73 ± 0.40	2 mM	126.78 ± 1.33
NaF	0.2 mM	124.57 ± 0.20	2 mM	117.40 ± 0.20
KI	0.2 mM	130.68 ± 1.23	2 mM	112.25 ± 1.34
KCl	0.2 mM	124.24 ± 0.27	2 mM	0
$CaCl_2$	0.2 mM	116.31 ± 0.80	2 mM	124.8 ± 1.80
$CaCO_3$	0.2 mM	120.46 ± 1.87	2 mM	122.02 ± 0.73
$CoCl_2.6H_2O$	0.2 mM	111.60 ± 2.29	2 mM	120.412 ± 2.07
$MgSO_4.7H_2O$	0.2 mM	115.40 ± 1.63	2 mM	127.22 ± 3.78
$ZnSO_4.7H_2O$	0.2 mM	120.84 ± 0.67	2 mM	125.89 ± 2.20
$ZnCl_2$	0.2 mM	120.79 ± 1.80	2 mM	57.64 ± 1.07
$MnCl_2.4H_2O$	0.2 mM	117.44 ± 2.13	2 mM	126.69 ± 0.27
$MnSO_4.H_2O$	0.2 mM	127.25 ± 3.2	2 mM	119.66 ± 0.07
$AlCl_3.6H_2O$	0.2 mM	118.58 ± 0.40	2 mM	134.85 ± 0.07
$FeCl_3$	0.2 mM	0	2 mM	0
$AgNO_3$	0.2 mM	0	2 mM	0
SDS	0.2 mM	0	2 mM	0
Urea	0.2 mM	129.61 ± 0.53	2 mM	134.80 ± 0.53
Glucose	0.2 mM	121.69 ± 2.07	2 mM	136.64 ± 2.07
EDTA	0.2 mM	123.39 ± 0.94	2 mM	109.80 ± 1.39
Maltose	0.2 mM	93.28 ± 1.81	2 mM	131.52 ± 0.48
Cellobiose	0.2 mM	97.63 ± 1.65	2 mM	93.19 ± 2.41
Cellulose (Avicel®)	0.02%	105.50 ± 1.72	0.20%	94.68 ± 0.96
Glycerol	0.2 mM	102.60 ± 1.30	2 mM	90.21 ± 1.65
Ethanol	0.2 mM	92.05 ± 3.05	2 mM	100.80 ± 2.06
Acetic acid	0.2 mM	100.85 ± 0.67	2 mM	96.36 ± 0.24
Triton X-100	0.02%	95.46 ± 2.33	0.20%	89.87 ± 1.99
β-mercaptoethanol	0.2 mM	132.85 ± 2.73	2 mM	170.26 ± 0.34
Control	0.2 mM	100 ± 1.05	2 mM	100 ± 1.80

Table 4
Hydrolysis of isoflavone glucosides to aglycones in soy molasses by *D. hansenii* UFV-1 free β-glucosidase and immobilised cells containing the intracellular enzyme. Values represent the means of three measurements.

	Time (min)	Isoflavone (mg/g)[b]						Aglycone rate (%)[c]
		β-glucoside			Aglicone			
		Din[a]	Glin[a]	Gin[a]	Dein[a]	Glein[a]	Gein[a]	
Beads	0	3.93	2.16	6.56	0.05	0.3	1.28	11.41
	15	3.05	1.89	4.47	0.12	0.34	1.63	18.17
	30	1.87	1.26	2.98	0.16	0.4	2.72	34.93
	60	0.44	0.55	1.03	0.21	0.69	4.08	71.14
	120	0.02	0.08	0.24	0.23	0.71	4.6	94.22
Free enzyme	0	3.93	2.16	6.56	0.05	0.3	1.28	11.41
	15	2.62	1.79	4.39	0.05	0.52	3.72	32.77
	30	0.84	1.18	1.68	0.07	0.93	5.94	65.22
	60	0.23	0.14	0.17	0.06	1.48	6.48	93.69
	120	0.01	0.04	0.2	0.07	1.68	6.73	97.14

[a] Din, daidzin; Glin, glycitin; Gin, genistin; Dein, daidzein; Gein, genistein; Glein, glycitein.
[b] Amount of isoflavone (mg)/g of soy molasses.
[c] Aglycone rate = (the amount of aglycones/the total amount of isoflavones) * 100%.

efficiency of isoflavones compared to the free enzyme, but this result may be explained by the lower enzyme accessibility to the substrate caused by the immobilisation process. This lower isoflavone hydrolysis efficiency exhibited by the immobilised enzyme can be compensated by reuse of the beads. The operational stability of immobilised cells containing β-glucosidase was evaluated at 50 °C using *p*NPβGlc or soy molasses isoflavones as substrates. In the first cycle the activity with *p*NPβGlc was 0.26 U/g; followed by 0.12 U/g and 0.04 U/g in the second and third cycles, respectively. The reaction product was not detected in the subsequent cycles. In the first cycle of isoflavone glucosides conversion, the rate of aglycones present in the total of isoflavones was 11.3%, 23.7%, 39.1%, 72.7% and 95.3% with 0, 15, 30, 60 and 120 min of hydrolysis, respectively. There was no change in the concentration of aglycones during the second cycle of hydrolysis. These results indicate that the immobilisation system presents low stability at 50 °C. β-Glucosidase from *Paecilomyces thermophila* was capable of converting nearly all isoflavone glucosides (above 95%) and malonyl glucosides were little hydrolyzed by this enzyme in soybean flour extract (Yang et al., 2009). The results of isoflavone glucoside conversion in soy molasses by intracellular β-glucosidase from *D. hansenii* UFV-1 are interesting because *D. hansenii* is a nonpathogenic yeast and it is found in various types of food. The use of immobilised yeast cells in calcium alginate by the food industry makes the enzyme more stable; thus the process is more economical and the conversion of isoflavone glycosides to their aglycone forms is possible, which means that the bioavailability of these compounds in soy molasses will be higher.

Conclusions

Intracellular β-glucosidase from *D. hansenii* UFV-1 was produced, purified, characterised and also immobilised in calcium alginate. This enzyme presents promise for industrial applications since it showed great ability to hydrolyze isoflavone glucosides from soy molasses, in both its free and immobilised forms. The results reported indicate that the *D. hansenii* UFV-1 β-glicosidase may be used for establishment of a process to improve the nutritional value of soy products by hydrolyzing isoflavones in soy molasses to their aglycon forms.

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