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The fungal metabolite eugenitin as additive for *Aspergillus niveus* glucoamylase activation

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ABSTRACT

Endophytic microorganisms live inside tissues of host plants apparently do not causing warning to them, and are a promising source of bioactive molecules as antimicrobial and antitumoral drugs. In this work, we report the isolation of eugenitin from cultures of the endophyte *Mycoleptodiscus indicus* and its potential as additive for *Aspergillus niveus* glucoamylase activation. The glucoamylase hydrolytic activity increased twofold using 5 mM of eugenitin and this activation could be explained by the binding mode of eugenitin with the three-dimensional structure of glucoamylase. The *in silico* prediction of ligand binding sites revealed at least 9 possible interaction sites able to accommodate eugenitin on glucoamylase from *Hypocrea jecorina*. Besides, we evaluated the effect of pH and temperature on activity and stability, as well as in the hydrolysis of different substrates and kinetic parameters either in presence or absence of eugenitin. The results displayed by eugenitin as additive to glucoamylase activation are promising and provide novel perspectives for applications of fungal metabolites.

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1. Introduction

Endophytes could be defined as microorganisms (fungi or bacteria) that can be detected at a given moment within the tissues of an apparently healthy host plant [1]. The metabolic interactions of an endophyte with its host may also favor the biosynthesis of different bioactive natural products. The study of natural products from plants and their endophytes has shown that this hypothesis is feasible, and endophytes have been found to produce a significant number of interesting novel and bioactive natural products [2–5]. The chemical diversity produced by endophytes is noteworthy and includes polyketides, shikimate derivatives, terpenes and steroids, alkaloids, and peptides. Novel and bioactive natural products have continued to be discovered from endophytic fungi after the previously published comprehensive reviews covering small molecules discovered up to 2005 [3,6,7]. In a study to uncover the chemical diversity of plant-associated microorganisms, we have undertaken the investigation of the secondary metabolites from cultures of *Mycoleptodiscus indicus*, an endophytic fungus isolated from leaves of the South American medicinal plant *Borreria verticillata* (L.) G.F.W. Meyer.

Amylases are enzymes present in many organisms. In plants they participate in the synthesis of starch in some types of roots, and in animals for the digestion of starch in food, and are found in many prokaryotic and eukaryotic microbes that use starch as a carbon source [8]. Glucoamylases hydrolyze α-1,4 and α-1,6 linkages of starch and related polymers to produce glucose as the only end product. Glucoamylases also hydrolyze other starch related oligo and polysaccharides, and show a preference for malto oligosaccharides of at least six residues [9]. One of most important applications of glucoamylases is the production of high glucose syrups from starch, and these enzymes are also used in the production of ethanol and in the baking and brewing industries [10].

In the present study, we report the isolation of eugenitin, a compound belonging to chromone class, and its potential as additive for *Aspergillus niveus* glucoamylase activation, an unusual biological application for natural products from fungi. Generally, fungal metabolites are used as antitumoral, antimicrobial and antihypercholesterolemic agents, which are successfully employed as

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therapeutical drugs, including β-lactams, lovastatin and its derivatives, the immunosuppressant cyclosporin, and ergotamine [11]. Therefore, our purpose was to investigate a promising and unusual biological application for eugenitin, the major secondary metabolite obtained from rice–oat cultures of *M. indicus*.

2. Experimental

2.1. General experimental procedures

Optical rotation was measured in $CHCl_3$ (chloroform) using a Jasco Digital Polarimeter – DIP 370 at room temperature; IR (infra-red) spectra was obtained using a Bruker Tensor 27 FTIR instrument; UV spectra was obtained in $CHCl_3$ on an UV VARIAN – 50 BIO UV-Visible Spectrophotometer. The HPLC (high performance liquid chromatography) system consisted of a Model Shimadzu (SCL-10Avp, Japan) multisolvent delivery system, SPD-M10Avp Photodiode Array Detector, and an Intel Celeron computer for analytical system control, data collection and processing. Using a reverse phase column CLC-ODS (M) – (Shimpack, 4.6 mm × 250 mm I.D.; 5 μm particle size, Shimadzu) protected by a precolumn PELLIGUARDth LC-18. The NMR (nuclear magnetic resonance) spectra were acquired on a VARIAN Avance DRX-400 instrument at 400 MHz for ^1H NMR and, 100 MHz for ^{13}C NMR in $CDCl_3$ (deuterated chloroform) using the residual solvent as int. standard; multiplicity determinations (DEPT – Distortionless Enhancement by Polarization Transfer) and 2D NMR spectra (HMQC – Heteronuclear Multiple Quantum Coherence and HMBC – Heteronuclear Multiple Bond Correlation) were obtained using standard Varian pulse programs. The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. HREIMS (high resolution mass spectra) were obtained by direct injection using a Bruker Bioapex-FTMS with electro-spray ionization (ESI).

2.2. Fungal material

The endophytic fungus used in this study was isolated from the medicinal plant *B. verticillata* (L.) G.F.W. Meyer, Rubiaceae, which is native to South America. The isolate was identified as *M. indicus* by one of authors based on sequence analysis of the ITS region of the rDNA (to be published).

2.3. Cultivation, extraction and isolation of metabolites of *Mycoleptodiscus indicus*

The endophytic fungus *M. indicus* was grown on PDA plates for 7 days at 30 °C. Then, 10 plugs were transferred to five Erlenmeyer flasks (500 mL), each containing 100 mL of PDB prepared with distilled water. Flasks were shaken on rotary shaker at 30 °C and 120 RPM for 48 h. After that, 10 mL were transferred to 50 flasks, containing 90 g of solid medium (rice–oat) each, and were grown for 30 days. On day 30th, the mycelial mass was macerated with ethanol overnight and filtrated. The filtrated was concentrated under vacuum to obtain a crude ethanolic extract (22 g). Then, it was dissolved in water–methanol 9:1 and partitioned, three times, with equal volumes of hexanes and CH_2Cl_2 (dichloromethane), in sequence, furnishing hexanic (6 g) and dichloromethanic (6.9 g) fractions. The hexanic fraction was dissolved in methanol and centrifuged (3000 rpm/3 min) giving a precipitate fraction (2.52 g) and a soluble fraction (3.48 g). The precipitate fraction was submitted to crystallization process in Erlenmeyer flask using a mixture of methanol and hexanes, and this step afforded the compound eugenitin (1.70 g) with high purity (98%), according to HPLC and NMR spectra profiles.

2.4. Aspergillus niveus glucoamylase

Approximately 10^7 conidia/mL from 3-day-old cultures were inoculated into 125 mL Erlenmeyer flasks containing 25 mL modified Khanna medium [12] as follows: 0.1% yeast extract, 5% Khanna salt solution (2% NH_4NO_3, 1.3% KH_2PO_4, 0.36% $MgSO_4{\cdot}7H_2O$, 0.1% KCl, 0.07% $ZnSO_4{\cdot}H_2O$, 0.014% $MnSO_4{\cdot}H_2O$, 0.007% $Fe_2(SO_4)_3{\cdot}6H_2O$, 0.006% $CuSO_4{\cdot}5H_2O$) and 1% starch. After inoculation, the media were maintained in BOD under static conditions at an initial pH 6.5 and 40 °C for 72 h. Culture filtrates were obtained by filtration through filter paper in a Buchner funnel. The filtrate was used as a source of crude extracellular amylolytic activity

2.5. Glucoamylase purification

The purification steps as followed according to Silva et al. [13]. The culture filtrate was dialyzed overnight against 10 mM Tris–HCl buffer, pH 7.5, and adsorbed on DEAE-Fractogel TSK 650 M column (2.0 cm × 97.0 cm) equilibrated with the same buffer, and eluted with 200 mL of a linear gradient (0–1 M) of sodium chloride prepared with the same buffer. The fractions showing amylolytic activity were pooled, dialyzed against distilled water, lyophilized and suspended in 2 mL of 100 mM sodium acetate buffer, pH 5.0. This sample was adsorbed on Sephacryl S-200 gel filtration column (2.0 cm × 85.0 cm) equilibrated and eluted in 100 mM sodium acetate buffer, pH 5.0 at a flow rate of 18 mL h^{-1}. The absorbance of the eluate was monitored at 280 nm, and a volume of 1 mL was collected in each fraction. The fractions containing enzymatic activity were pooled and used for further characterization.

2.6. Glucoamylase activity assay

Glucoamylase activity was determined by measuring the production of reducing sugar using 3,5-dinitrosalicylic acid (DNS) as described by Miller [14]. The assay was carried out at 65 °C using 1.0% starch solution in 0.1 M sodium acetate buffer, pH 5.0. In addition, the enzyme activity was measured according to Cereia et al. [15], using soluble starch, glycogen and malto oligosaccharide as substrate, in which the amount of glucose released was estimated by peroxidase/glucose oxidase.

2.7. Enzymatic characterization and kinetic parameter

The pH optimum was determined at 60 °C using citrate phosphate buffer (pH range 3.0–7.0). The pH stability was determined at 25 °C, for 24 h, after pre-incubation of the diluted enzyme in citrate phosphate buffer at different pH values (pH range 3.0–9.0) in the presence and absence of eugenitin. The optimum temperature was determined with 0.1 M sodium acetate buffer, pH 5.5 at different temperature values (40–80) in the presence and absence of eugenitin. The thermal stability was determined by measuring the residual activity after incubation of the diluted enzyme in the absence of substrate at 60 °C in 0.1 M sodium acetate buffer, pH 5.0, for 4 h. For determination of the pH and temperature stabilities, the enzymatic assays were carried out using 1% soluble starch as substrate. The K_m and V_{max} values for the purified enzyme were determined by incubating the enzyme with 1–60 mg soluble starch in 100 mM sodium acetate buffer, pH 5.5, at 65 °C (optimum pH and temperature). The data obtained were fitted to a standard Hanes model using linear regression [16].

2.8. Molecular modelling

Prediction of ligand binding sites was performed with Q-SiteFinder web server [17,18] using the three-dimensional structure of glycoside hydrolyse family 15 glucoamylase from

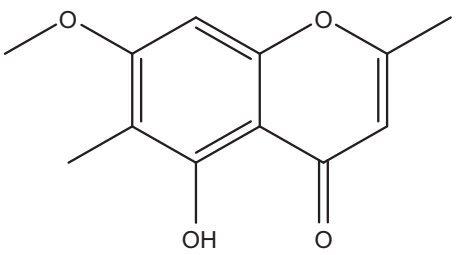

Fig. 1. Eugenitin, a secondary metabolite from *Mycoleptodiscus indicus*, was obtained from cultures in rice–oat fermented for 30 days at 30 °C. The isolation was performed using chromatographic techniques and the identification was done by NMR spectra analysis.

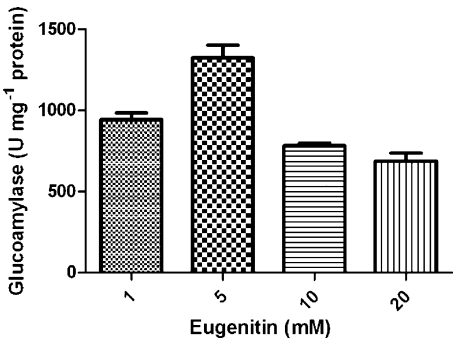

Fig. 2. Glucoamylase activation by eugenitin. The assays for glucoamylase measures were carried out as described in Section 2 by using starch as substrate. Eugenitin (100 mM in DMSO) was added to the assay reaching 1, 5, 10 and 20 mM at final concentration. The results are relative to controls, to which it was added the same amount of eugenitin, free of DMSO.

Hypocrea jecorina available at the PDB (Protein Data Bank) [PDB structure code: 2VN4]. Docking simulations were performed with GOLD software [19] using the Goldscore scoring function.Among the genetic algorithm parameters, 100.000 operations, 95 mutations, 95 crossovers and a population of 100 were set. These calculations were performed inside the protein regions covering the ligand binding sites identified previously with Q-SiteFinder [17,18].

3. Results and discussion

3.1. Eugenitin

Eugenitin (5-hydroxy-7-methoxy-2,6-dimethylchromone) (Fig. 1) was identified by NMR means in comparison with data previous reported [20], and it is a chromone derivative, which had been obtained earlier from mild cloves, *Eugenia caryophyllata* [21]. This class of compounds have been isolated from a wide variety of plants and fungi [22]. Previous studies suggested that they possess a spectrum of activities including anticancer [23,24] and the inhibition of gray hair by promotion of melanin formation [25]. Fungi that produce chromone derivatives include *Tolypocladium extinguens*, *Paraphaeosphaeria quadriseptata*, *Chaetomium chiversii*, *Chaetomium brasiliense* among others.

3.2. Effect of eugenitin on glucoamylase hydrolytic activity

Particular secondary metabolites produced by endophytic fungi are believed to benefit the host plants as they may be plant growth regulators, antimicrobials, antivirals, and insecticides, or even mediate resistance to some types of abiotic stress [26]. To explore the high number of functional groups in eugenitin, we studied the possible positive or negative effect of this compound

Fig. 3. Tertiary structure of glycoside hydrolyse (PDB code: 2VN4) and suggested ligand binding sites.

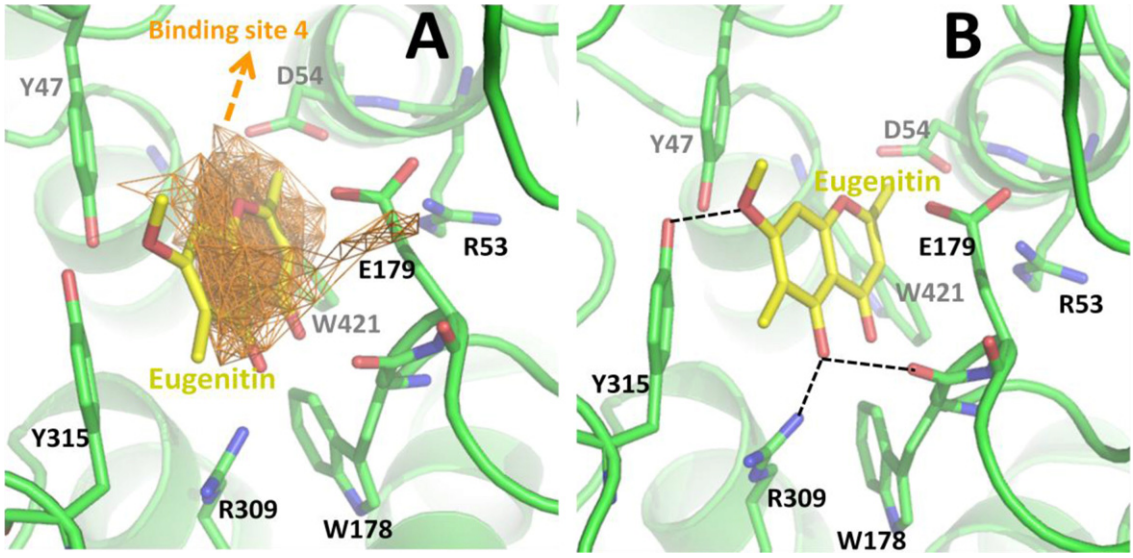

Fig. 4. Suggested binding mode for eugenitin in glycoside hydrolyses. (A) Preferred orientation of eugenitin inside binding site 4 of glycoside hydrolyse. (B) Suggested hydrogen bonds (dashed lines) performed for eugenitin with R309, W421 and Y315 residues of glycoside hydrolyse.

on glucoamylase hydrolytic activity (Fig. 2). The glucoamylase assays were carried out using 1% starch as substrate adding eugenitin at crescent concentrations. The glucoamylase was activated about twofold at 5 mM eugenitin, but this activation was not dose-dependent because at higher concentrations of eugenitin the effect of activation was reverted.

In order to propose a binding mode of eugenitin with its molecular target, it was used the three-dimensional structure of glucoamylase from *H. jecorina* [27] available at the PDB (Protein Data Bank) [PDB structure code: 2VN4]. Firstly, the protein structure was mapped *in silico* with Q-SiteFinder server [17,18] in order to search for potential binding sites that could accommodate possible ligands. The prediction of ligand binding sites revealed at least 9 possible interaction sites able to accommodate eugenitin (Fig. 3).

Molecular docking simulations were performed for eugenitin inside the protein structure, comprising the 3D space covered for all the 9 possible binding sites previously identified (Fig. 4A). Energetically, binding site 4 was the preferred interaction site for eugenitin structure during simulations, which corresponds to the binding site reported to acarbose in glucoamylase from *Aspergillus awamori* [28]. The binding site 4 is a virtual space inside the protein influenced by Y47, D54, R53, E179, Y315, W421, R309 and W178 protein residues. These residues present chemical characteristics able to recognize eugenitin. According to the energetically most favorable binding mode obtained for eugenitin in the binding site 4, three ligand-protein hydrogen bonds could be performed simultaneously with R309, W421 and Y315 residues. This suggested binding mode for eugenitin (Fig. 4B) give insights to future investigations of glucoamylase activation by natural products, since the model presented here could be a starting point to understand the molecular basis of such biological activity.

Based on the discussion about the dose response correlation and molecular docking simulations, it was hypothesized that the glucoamylase dose dependent activation could be related to the number of sites accessed by eugenitin.

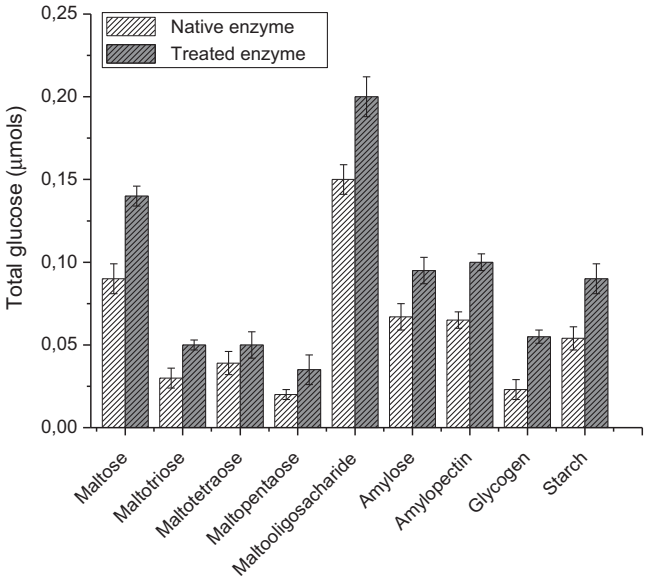

Fig. 5. Glucoamylase specific activity on different substrates in the presence and absence of eugenitin. The glucoamylase was obtained in Khanna medium after 72 h of cultivation. Maltooligosaccharide is an oligosaccharide with 10 glucose molecules. The amount of glucose released during 10 min of reaction was estimated by the peroxidase/glucose oxidase method.

3.3. Hydrolysis of different substrates and kinetic parameters

The enzymatic activity of the glucoamylase from *A. niveus* against various substrates in the presence and absence of eugenitin is presented in Fig. 5. In general, it was evident that the presence

Table 1
Influence of eugenitin on kinetic parameters of the glucoamylase from *A. niveus*.

Substrate	Eugenitin	K_m (mg mL^{-1})	V_{max} (U mg^{-1} protein)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mg^{-1} mL^{-1})
Starch	–	17.5	660	60.5	3.46
	+	19.2	1391	127.5	6.64

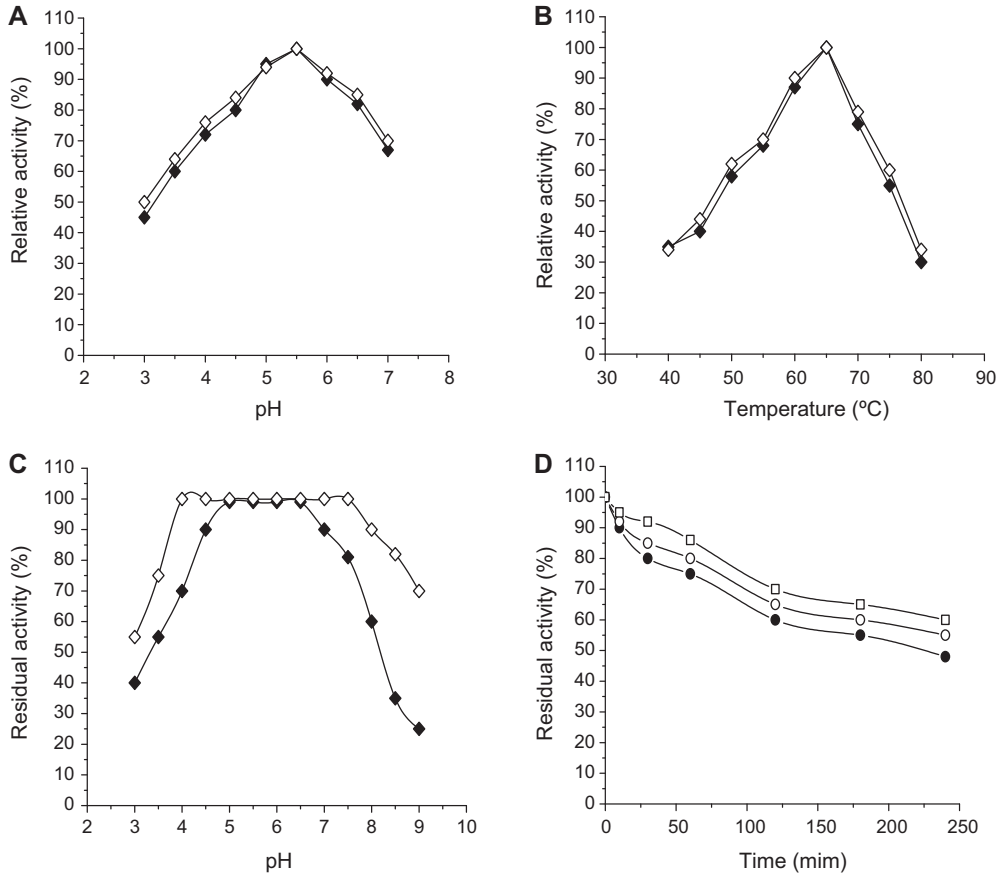

Fig. 6. Effect of pH and temperature in glucoamylase activity and stability after eugenitin addition. A pH optimal, B temperature optimal, C stability at different pHs and D stability at 60 °C. The activity assay was carried out as in "Section 2", modifying pH and temperature in according to each experiment. Legends: (●) and (♦) – no eugenitin; (◊) – 5 mM eugenitin; (○) – 2 mM eugenitin; (□) – 5 mM eugenitin.

of eugenitin improved the performance of the enzyme. The glucoamylase hydrolyzed, preferentially, malto oligosaccharide G10 and maltose independently of the presence or absence of eugenitin.

In Table 1, it is summarized the kinetic analysis of the enzyme using soluble starch as substrate. When eugenitin was added, there were no significant changes in the K_m values. However, the V_{max}, turnover number and catalytic efficiency increased about twofold, i.e., these results showed that although there were no changes in affinity for the substrate, the enzyme released larger amounts of product. The literature reports that the fungal glucoamylases have two domains, an amino-terminal catalytic domain and a carboxy-terminal no catalytic domain with starch-binding capability [29]. Thus, the eugenitin certainly binds to the enzyme catalytic domain, promoting changes in the active site and increasing the rate of hydrolysis of the substrate. The model for glucoamylase action describe the formation of an oxocarbenium ion, where the Glu179 acts as the general acid that transfer a proton to oxygen atom of the glycosidic bond, resulting in bond cleavage and formation of a glucopyranosyl cation. In parallel, the Glu 400 acts as general base, assisting the hydrolysis by removing a proton from a neighboring molecule of water, followed by nucleophilic attack by water to glycosidic carbon C1 [30,31]. It is reported that nonbonding electrons at OH of Tyr48 are involved in stabilizing the intermediate oxocarbonium [32,33]. According to Fig. 3, the binding site 4 of eugenitin involves a Tyr47, so the proximity of the molecule with Tyr48 could alter the electronic charge of this residue, influencing the stabilization of the intermediate complex, increasing the substrate hydrolyses.

3.4. Effect of pH and temperature on activity and stability

Studies of the temperature and pH effect on the enzyme activity in the presence and absence of eugenitin were carried out over the 40–80 °C temperature range (in 100 mM sodium acetate buffer, pH 5.5) and citrate phosphate buffer in the range of pH 3.0–9.0, respectively. Optimum pH and temperature were estimated to be 5.5 and 65 °C, respectively, independent of the eugentin addition (Fig. 6A and B). The glucoamylase activity was acid tolerant remained completely stable at pH 5.0–6.5. However, the presence of eugenitin expended this range to 4.0–7.5 (Fig. 6C). Thermal inactivation by incubating the glucoamylase in the absence of starch revealed that the purified enzyme was more stable in the presence of eugenitin (Fig. 6D). This result is important because it increases the possibility for using this enzyme as an additive in biotechnological process that involves extreme conditions.

4. Conclusions

The chromone compound eugenitin, obtained from rice–oat cultures from endophyte *M. indicus* displayed a promising biological activity as additive for *A. niveus* glucoamylase activation, increasing the hydrolytic activity in twofold at 5 mM. Molecular docking simulations revealed, at least, 9 possible interaction sites able to accommodate eugenitin inside glucoamylase from *H. jecorina*, where binding site 4 was the preferred interaction site, which corresponds to the binding site reported to acarbose in glucoamylase from *A. awamori*. Furthermore, the presence of eugenitin

improved the glucoamylase activity in different substrates and slightly affected the thermal stability and kinetics parameters. Our findings provide new perspectives for the use of natural products from fungi in biotechnological process involving enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.08.003.

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