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Research Article

Production and Characterization of Naringinase from New Fungal Isolate of *Lasiodiplodia theobromae*

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Abstract

Lasiodiplodia theobromae was selected from ten isolates of fungi for the production and characterization of naringinase enzyme for the first time. Naringinase is essential for bitterness removal of citrus fruit juices. Optimum temperature and pH for the enzyme activity was found to be 50oC and 4 respectively. The kinetic parameters were found to be k_m 2.5 mM and V_{max} 47.2 $Umin^{-1}$. Molecular characterization of the purified naringinase revealed that it is a single enzyme showing one subunit on the SDS-PAGE with a molecular weight at 67 KDa.

Keywords: Naringinase; *Lasiodiplodia theobromae*; Debittering; Kinetic Parameters

Introduction

Naringinase is an enzyme which hydrolyzes the glycoside naringin (4,5,7-trihydroxyflavonone 7-rhamnoglucoside) the principal bitter component of grapefruit, and it was reported to possess anti-inflammatory, anti-ulcer, and antioxidant activities [1-4]. Naringinase is a complex enzyme consisting of α-rhamnosidase (EC 3.2.1.40) and β-glucosidase (EC 3.2.1.21). In typical processing, naringinase converts naringin to naringenin in a two-step process. The substrate naringin, 4′, 5, 7-trihydroxyflavanone-7- rhamnoglucoside, is hydrolyzed by the rhamnosidase component to produce prunin (4′, 5, 7 -trihydroxyflavanone-7-glucoside), which is then converted by the flavonoid β-glucosidase to naringenin (4′, 5, 7-trihydronyflavonone) [5-7]. Naringenin is only one-third as bitter as naringin; however, prunin is less bitter than naringenin and only the first hydrolyzing activity of naringinase is in fact essential for bitterness removal.

Naringinase enzyme has industrial and pharmaceutical applications such as; preparation of rhamnose [8], preparation of pruning [9], preparation of the antibiotic chloropolysporin [10], steroid transformation [11,12], debittering and clarifying of the citrus juices (sweeten of fruit juice) [13-15], Production of ginsenoides [16], production of glycolipids [17] and Improvements of the beneficial effects of the *Cleome arabica* leaf extracts.

Many microorganisms produce naringinase enzyme such as *Penicilium decumbens* [18], *Aspergillus niger* [19] and *Aspergillus sojae* [20].

According to literature this is the first record of *Lasiodiplodia theobromae* (AUMC 4953), for the production of naringinase enzyme.

Materials and Methods

Microorganisms

Soil samples were collected from different localities in Egypt. Theses samples were serially diluted and inoculated on Czapek's yeast extract medium. Other fungal isolates were obtained from rotten orange, lemon and grapefruit fruits by scraping off spores on same medium. Isolates were identified in Assiut University Mycological Center (AUMC). Five

discs (size 1 cm) of each isolate were inoculated into separate 500 ml flask (150 rpm) that contained 100 ml of half strength Czapek's liquid media supplemented with 0.01g/l naringin, which was incubated for 72h at 28° C, in dark for screening and enzyme production.

Assay Method

Naringinase activity was estimated using spectrophotometer at 420 nm by determining remaining naringin using the Davis method [21]. One unit of naringinase activity was defined as 1μmol of naringin hydrolyzed under the assay conditions.

Optimization Studies

Different carbon sources (glucose, maltose, sucrose and starch) at concentration 10 g/l and nitrogen sources (sod. nitrate, amm. sulphate, amm. phosphates and peptone) at concentration 3 g/l, were added to different flasks containing Cz-yeast media supplemented with 0.01% naringin, and yeast extract with different concentrations (0.5, 1.0 and 2.0 g/l) then inoculated with L. *theobromae* and incubated at 28° C (in dark). Aliquots were withdrawn after 5, 7, and 10 days from incubation.

L. theobromae was inoculated into flasks containing Cz-yeast supplemented with 0.01% naringin and incubated at different temperatures 28° C, 37° C and 50° C. Another set of flasks containing the same medium was adjusted the pHs of 3, 4,5,6,7 and 8 and incubated at 28° C (in dark). Aliquots were withdrawn after 5, 7, and 10 days from incubation.

Naringinase Purification

The mycelia was filtrated through Whatman No.1, 2x acetone was added to the filtrate which was chilled for 4 hours, then centrifuged at 14,000 rpm for 10 mins to precipitate the protein. The crude protein was dissolved in 0.1M sodium acetate buffer pH 4 then applied to the Sephadex G-100 column. Protein concentration and naringinase bioassay was carried out for all fractions. The positive fractions were combined and concentrated down to 1ml, then applied to 5 ml Q-Sepharose column (GE Healthcare, UK) using peristaltic pump (HBI, multistaltic pump, USA), with a flow rate of 1ml/min. 20 mM Tris HCl buffer (pH 7.0) were used as a mobile phase with increasing gradient of KCl from 100 mM to 1M. Positive fractions were combined, precipitated and were then dissolved in 0.1M sodium acetate buffer pH 4 and analyzed using SDS-PAGE.

Characterization of the Pure Enzyme

100 μl of pure naringinase incubated with 0.1% naringin at different temperatures and pHs and the activity was estimated using spectrophotometer as mentioned above.

Enzyme Kinetics

Pure naringinase enzyme was incubated with different substrate concentrations (0.025%, 0.05%, 0.1%, 0.2% and 0.3%) in 0.1 M sod. acetate buffer pH 4 at 50° C. Km and Vmax were calculated.

7.5 % SDS-PAGE was performed using the method of [22], using prestained protein molecular weight marker (Gene Direx). The current was adjusted to 22 mA. Protein bands were visualized by the ProteoSilver Plus Silver Stain kit (Sigma, USA).

Native-PAGE was carried out with constant current of 19 mA using 7.5% polyacrylamide gel, after electrophoresis, the gel was soaked in 0.1% Naringin in 0.1 M acetate buffer pH 4 overnight, then stained with 0.1% Congo Red in NaOH at pH 9.

Results

Screening of Ten Fungal Isolates for Naringinase Production

Silica gel / TLC-sheets were used for screening for naringinase production, while mobile phase was Ethyl acetate 8: Isopropanol 2: H_2O 0.5, seven isolates showed their ability to utilize naringin producing naringinase (Table 1).

Lasiodiplodia theobromae was selected for further study of naringinase production in this study, as there is no previous record of being producer of this enzyme.

Table1. Identification and Screening of the Fungal Isolates for Naringinase Production

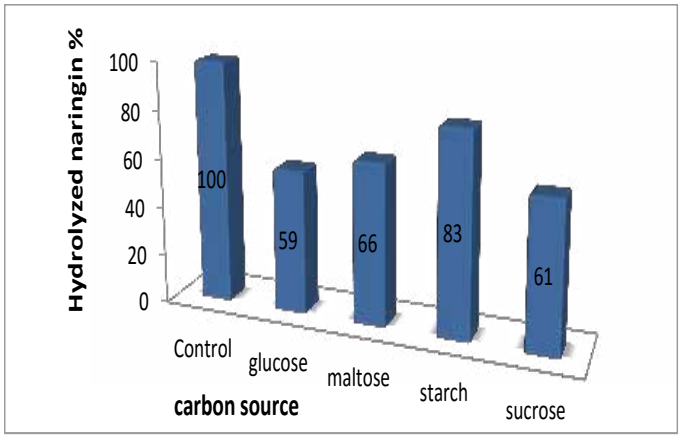
Isolate No.	Origin	Species	(AUMC) No.	Result of screening
1	Rotten lemon	*Penicillium oxalicum*	4951	(–)
2	Rotten orange	*Emircella phoenicis*	4952	(+)
3	Rotten orange	*Lasiodiplodia theobromae*	4953	(+)
4	Cairo University garden soil	*Aspergillus aculeatus*	4954	(+)
5	Giza air	*Aspergillus carbonarius*	4955	(+)
6	Giza air	*Aspergillus niger*	4956	(+)
7	Fayoum soil	*Emircella rugulosa*	2501	(–)
8	Fayoum soil	*Emircella nidulans*	190	(–)
9	Fayoum soil	*Aspergillus aegyptiacus*	150	(+)
10	Fayoum soil	*Emircella variecolor*	3302	(+)

Optimum Conditions for Fungal Naringinase Production

Carbon Source

All carbon sources in the medium showed naringinase production but the yield was lower comparable with the control(no carbon source except naringin), the production of naringinase was repressed by glucose and sucrose, although both gave the highest mycelial growth, while starch yielded the highest naringinase production (Figure1).

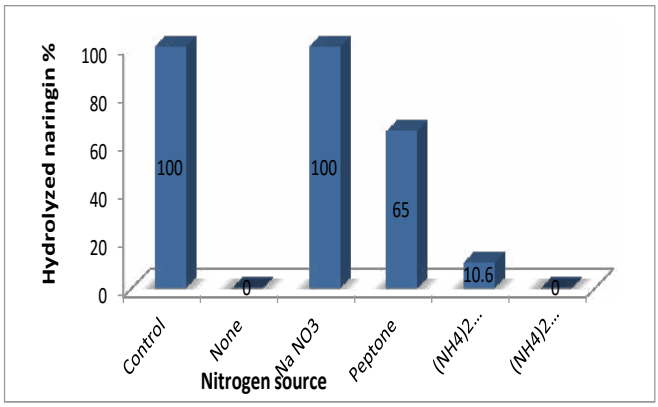
Figure 1. Effect of Carbon Source Affecting Naringinase Production from L. theobromae; Control: with no Carbon Source Except Naringin.



Nitrogen Source

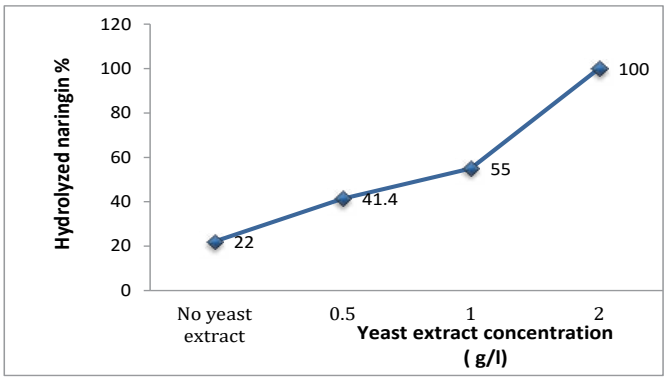
The highest naringinase production was revealed after 10 days of incubation in presence of sod. nitrate, while $NH_4H_2PO_4$ exhibited the lowest enzyme production (Figure 2a).

Figure 2a. Effect of Nitrogen Source on Naringinase Production by B. theobromae; Control: with no Nitrogen Source Except Yeast Extract



The addition of of yeast extract in the medium stimulated the mycelial growth and the naringinase production 2g/l yeast extract exhibited the highest naringinase production (Figure 2b).

Figure 2b. Effect of Yeast Extract Concentration on Naringinase Production by L. theobromae



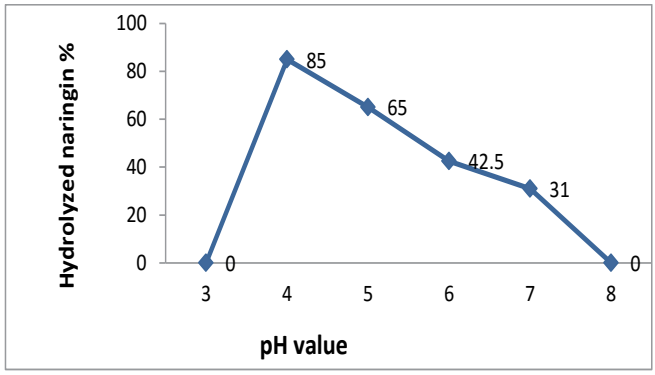
Effect of Temperature

Naringinase production was lowered with the increased of incubation temperature, and the optimum incubation temperature for naringinase production for was found to be 28° C

Effect of pH

Naringinase production showed a wide pH range (4–7), where the maximum naringinase production was found to be at pH 4 and decreased with the increase of pH (Figure 3).

Figure 3. Effect of Initial pH on Naringinase Production by L. theobromae

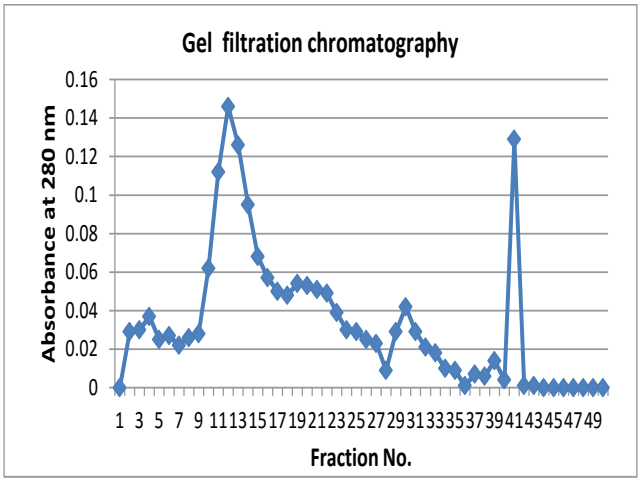


Purification of Naringinase Enzyme

Naringinase was purified using three-step process, 2 x acetone precipitation of protein was used as an initial step, then dissolved protein was applied to Sephadex G-100 gel filtration column where elution was done using 50 mM sodium acetate buffer at pH 4. Bioassay was performed for eluted fractions and also visualized on TLC silica sheet, and enzyme positive fractions were combined. Further purification was performed using ion exchange chromatography; Q-Sepharose column (strong anion-exchanger) in 20 mM Tris-HCl buffer pH 7

which is higher than its isoelectric point. Naringinase positive fractions were eluted off the column between 200-300 mM KCl. Protein fractions were separated by (SDS-PAGE) and visualized using silver staining kit. The naringinase enzyme from this study was found to be of two subunits, at 62 KDa and 67 KDa, (Figure 4)

A



B

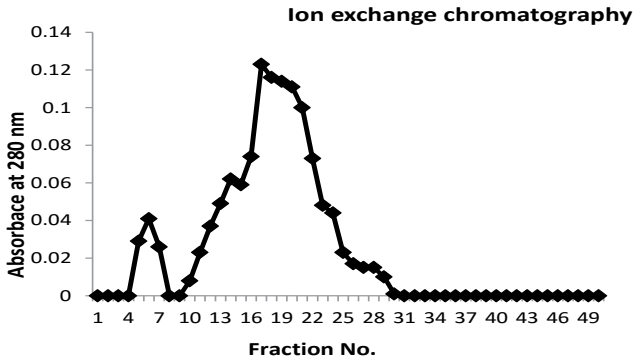


Figure 4. A,B. Elution Profiles of Naringinase Enzyme from Chromatographic Columns; (A) Gel Filtration Column and (B) Q Sepharose Column.

These purification steps resulted in a 1.14 fold purification of naringinase; also Native-PAGE was visualized using Congo red which showed one band only Figure (4d).

C

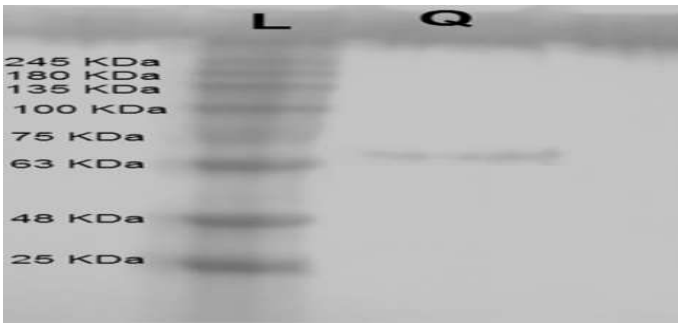


Figure 4C. 7.5% SDS-PAGE of the Purified Naringinase Enzyme, Stained by Silver Staining. L; Standard Protein Leader; Q, Peak Fraction of Q-Sepharose.

D

Figure 4d. 7.5% Native-PAGE Gel for Naringinase.

Characterization of Pure Naringinase

The activity of pure enzyme was recorded at a wide range of temperature degrees (20˚ C - 60˚ C), and was completely inhibited at 70˚ C. The optimum temperature of naringinase activity was found to be 50˚ C, Figure (5).

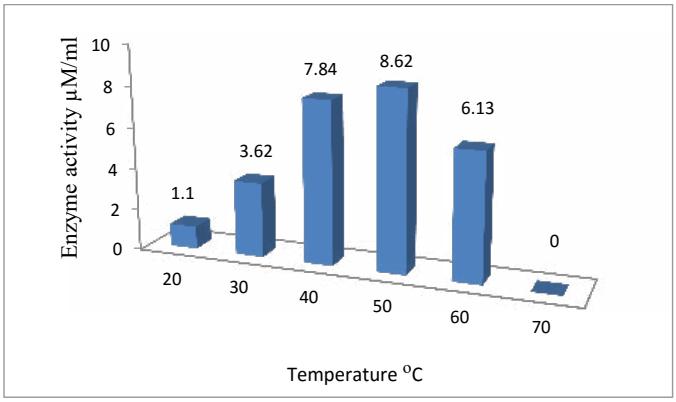


Figure 5. Effect of Temperature on the Purified Naringinase Enzyme Activity by *L. theobromae.*

The optimum pH for the purified naringinase was pH 4, although at pH 3 there was no activity but at pH 5 the activity of naringinase was decreased by 25%, Figure (6).

Determination of Kinetic Properties

Hanes–Woolf plot was constructed and K_m and V_{max} were calculated and found to be:

K_m = 2.5 mM. V_{max} = 47.2 U min^{-1}

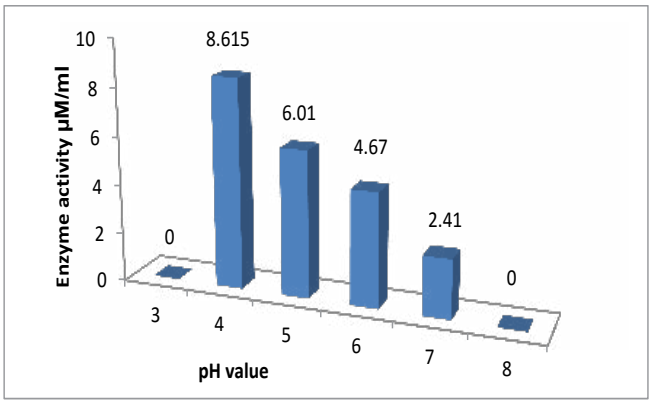


Figure 6. Effect of pH Value on Purified Naringinase Enzyme Activity, Using by *L. theobromae.*

Discussion

Naringinase has industrial and pharmaceutical applications such as; preparation of rhamnose [8] Daniels *et al*.1990, preparation of pruning [9], preparation of the antibiotic chloropolysporin [10], steroid transformation [11,12] debittering and clarifying of the citrus juices (sweeten of fruit juice).The production of naringinase was repressed by glucose and sucrose; although both contributed the highest mycelial growth [23]. Production of the naringinase complex was carried out in submerged cultures by using naringin as the inducer and carbon source [24,25].

Peptone as an organic nitrogen source in the medium induced the highest enzyme production, while $NH_4H_2PO_4$ exhibited the lowest, comparable with $NaNO_3$ and peptone, presumably because of the release of ammonium ions which increases the pH of the medium. Using $(NH_4)_2SO_4$ as a nitrogen source represses the naringinase production, also in case of absence of nitrogen source, although yeast extract was available in the medium, it gave moderate mycelial growth with no naringinase production due to deficiency in nitrogen which is essential for protein synthesis [19,23,26].

The optimum incubation temperature for naringinase production by *L. theobromae* was found to be 28° C while no growth was observed at 50° C similar to that of [23].

The maximal naringinase production was recorded at pH 4 and regularly decreased with the increase of pH (17). pH 4 was also the optimum pH for purified naringinase activity from *Asoergillus niger* BCC 25166 [19], while that from *Penicillium* DSM 6825 had an optimum pH 5 - 5.5 [14]. In the case naringinase isolated from [20], the α-L-rhamnosidase activity of this enzyme was optimal at pH 6. Optimum temperature of naringinase activity was found to be 50° C, which is in an agreement with that of [23]. The purified naringinase from *A. niger* 1344 had an optimum temperature of 50° C, and from *Penicillium sp.* between 50 - 55° C (16), while that from *Coniothrium diplodiella* was between 60 - 65°C[27].

The molecular masses of naringinases (from different sources) ranged from 70 to 240 kDa [28]. Some naringinase comprised two identical subunits that were produced by fermentation of *Penicillium* DSM 6825, with a molecular weight of 60-100 kDa [14].The naringinase isolated from *A. sojae*, presented a molecular weight of 70 kDa [20]. The naringinase enzyme from this study was found to be one subunit at 67 KDa, which also showed one band on the native-PAGE.

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