World Journal of Microbiology & Biotechnology (2005) 21:753–758
DOI 10.1007/s11274-004-5488-7

Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids

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Keywords: *Aspergillus niger*, debittering, naringin, naringinase, purification, transformation

Summary

An extracellular naringinase (an enzyme complex consisting of α-L-rhamnosidase and β-D-glucosidase activity, EC 3.2.1.40) that hydrolyses naringin (a trihydroxy flavonoid) for the production of rhamnose and glucose was purified from the culture filtrate of *Aspergillus niger* 1344. The enzyme was purified 38-fold by ammonium sulphate precipitation, ion exchange and gel filtration chromatography with an overall recovery of 19% with a specific activity of 867 units per mg of protein. The molecular mass of the purified enzyme was estimated to be about 168 kDa by gel filtration chromatography on a Sephadex G-200 column and the molecular mass of the subunits was estimated to be 85 kDa by sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme had an optimum pH of 4.0 and temperature of 50 °C, respectively. The naringinase was stable at 37 °C for 72 h, whereas at 40 °C the enzyme showed 50% inactivation after 96 h of incubation. Hg^{2+}, SDS, *p*-chloromercuribenzoate, Cu^{2+} and Mn^{2+} completely inhibited the enzyme activity at a concentration of 2.5–10 mM, whereas, Ca^{2+}, Co^{2+} and Mg^{2+} showed very little inactivation even at high concentrations (10–100 mM). The enzyme activity was strongly inhibited by rhamnose, the end product of naringin hydrolysis. The enzyme activity was accelerated by Mg^{2+} and remained stable for one year after storage at −20 °C. The purified enzyme preparation successfully hydrolysed naringin and rutin, but not hesperidin.

Introduction

The presence of bitterness has been a major limitation in the commercial acceptance of citrus juices. Citrus tissues possess naringin, a flavonoid responsible for the bitter taste (Hasegawa & Maier 1993). Different approaches have been used to avoid naringin bitterness or to eliminate this flavonoid from citrus juices but few have achieved significant success (Johnson & Chandler 1988; Kimball 1991).

Naringinase (EC 3.2.1.40), an enzyme detected in different species of microorganisms, is widely used to remove rhamnose and glucose from naringin and other glycosides to obtain the aglycone (Puri *et al.* 1996). Naringin is 4,5,7-trihydroxyflavonone 7-rhamnoglucoside and on hydrolysis with naringinase yields rhamnose and naringenin (4,5,7-trihydroxyflavonone), a non-bitter derivative, which cannot be reconverted to naringin (Chandler & Nicol 1975; Habelt & Pittner 1983).

Although naringinases are not common enzymes, several industrial applications have been reported (for details see review, Puri & Banerjee 2000). The use of naringinase for removing naringin, the main bitter component of several citrus juices, is a common industrial practice (Thomas *et al.* 1958; Ono *et al.* 1978; Habelt & Pittner 1983). The deglycosylation of the novel glycopeptide antibiotic, chloropolysporin from *Faenia interjecta*, was achieved successfully by the rhamnosidase activity of naringinase (Sankyo 1988). Also, the hydrolysis of hesperidin by α-L-rhamnosidase to release L-rhamnose and hesperetin glucoside, an important precursor in sweetener production has been described (Manzanares *et al.* 1997). The enzyme is used to produce L-rhamnose which is a chiral intermediate in organic synthesis and it is used as a pharmaceutical and plant protective agent (Daniels *et al.* 1990). The production and characterization of an *Aspergillus terus* α-L-rhamnosidase activity of naringinase in combination with β-D-glucosidase is considered suitable for aroma enhancement in wine making (Caldini *et al.* 1994).

Among the naringinase-producing fungi, the enzyme preparation from *Penicillium decumbens* (Young *et al.* 1989) is commercially available. Naringinase obtained from *P. decumbens* had been used for immobilization studies and for the transformation of flavonoids (Manjon *et al.* 1985; Romero *et al.* 1985; Puri *et al.* 2001).

On using commercial naringinase, incomplete hydrolysis of naringin was observed (Puri *et al.* 1996, 2001). In

order to improve upon its hydrolysis, a new strain of *Aspergillus* niger MTCC 1344 was tested. Recently, we reported optimization of process parameters for the production of naringinase (Puri *et al*. 2004). Hence, in the present study an attempt has been made to purify an extracellular naringinase from *A. niger* 1344 to improve upon the transformation of flavonoids. The aim of our research is to use the purified enzyme in debittering reactors for the transformation of inactive chloropolysporin to the active form and for treatment of fruit juices. In addition, the enzyme could eventually be used to develop biosensors for the determination of naringin level in juices. The purified enzyme has also been characterized, particularly with respect to substrate specificity.

Materials and methods

Chemicals

Naringin, rhamnose, rutin and hesperidin were obtained from Sigma (St. Louis, USA). Acrylamide, high molecular weight markers for gel filtration and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio Rad (Bio Rad Laboratories, Hercules, California, USA). Q Sepharose and Sephadex G-200 were purchased from Pharmacia fine chemicals (Uppsala, Sweden). All other chemicals were of analytical grade.

Fungal strain and production of enzyme

Aspergillus niger MTCC 1344 was isolated, identified, maintained and cultivated for naringinase production as described earlier (Puri *et al*. 2004). The medium used for the production of naringinase had the composition (g l^{-1}): sucrose, 10; sodium nitrate, 2; KH_2PO_4, 1; KCl, 0.5; $MgSO_4$, 0.5 and naringin 0.025. The pH of the medium was adjusted to 4.5. The seed culture was prepared by inoculating a few spores from the maintenance plate into the culture medium and incubated at 27 °C for 72 h. The inoculum (10%, v/v) was used for the production of enzyme in a 7 l bioreactor (Chemap, Switzerland) containing 5 l of medium. The bioreactor was run under controlled temperature (30 °C), at 350 rev min^{-1} for 120 h. The broth was centrifuged at 19,000 × *g* at 4 °C for 15 min and the supernatant was assayed for enzyme activity and used for purification and characterization.

Assay of naringinase activity

Naringinase activity was assayed with respect to naringin at 50 °C with minor modifications (Davis 1947). A typical assay mixture comprised 0.8 ml of naringin (0.1%) dissolved in 0.1 M sodium acetate buffer (pH 4.0) and 0.2 ml enzyme solution. The assay mixture was incubated at 50 °C for 60 min after which a 0.1 ml aliquot was added to 5 ml diethylene glycol (90%) and 0.1 ml NaOH (4 M) was then added to the mixture. The samples were maintained at ambient temperature for 10 min. The intensity of the resultant yellow colour was determined at 420 nm. One unit of activity (IU) was defined as the amount of enzyme required to hydrolyse 1 μmol of naringin under the above assay conditions.

Enzyme purification

The broth from the bioreactor, containing extracellular naringinase, was concentrated by ultrafiltration in a Pellicon system (Millipore Corporation, US) using a membrane cartridge with an M_r cut-off of 30 kDa. The retentate from this step was then precipitated using ammonium sulphate at 65% saturation. The precipitated protein obtained after centrifugation (19,000 × *g* for 5 min) was collected and dialyzed against phosphate buffer (0.05 M, pH 7.0). The concentrated sample was applied to an anion exchange column (Q Sepharose, 2.5 × 10 cm). The column had been previously equilibrated with 0.05 M buffer, pH 7.0. Elution of the column was performed with a linear gradient of NaCl (0–1 M) in phosphate buffer (0.05 M, pH 7.0) with a flow rate of 1 ml min^{-1} and 6 ml fractions were collected. The active enzyme fractions were pooled, dialyzed against phosphate buffer and concentrated by Ultrafiltration (Centricon YM-30, Amicon, USA) before loading onto a Sephadex G-200 column (1.5 × 45 cm) that had been previously equilibrated with 0.05 M phosphate buffer (pH 7) containing 0.15 M sodium chloride. Elution was performed at a flow rate of 0.5 ml min^{-1} (0.05 M phosphate buffer). Fractions (1 ml) corresponding to naringinase activity were collected and purity checked on native PAGE. Extracellular protein was measured using Lowry's method with bovine serum albumin as a standard. The purified enzyme was subsequently used for characterization work.

Polyacrylamide gel electrophoresis

PAGE was done on polyacrylamide slab gels with 0.1% SDS according to Laemmli's method (Laemmli 1970). Urease (272, 542 kDa), BSA (66, 132 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa) were used as molecular mass markers. The gels were run at 15 mA for stacking and at 35 mA for resolving and were then stained by CBB G-250 (Commassie Brilliant Blue).

For calculation of the protein molecular mass of subunits, a 10% SDS-polyacrylamide gel was used. Reference proteins (rabbit muscle phosphorylase *b*, 97.4 kDa; bovine serum albumin 66 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa; and lysozyme, 14 kDa) were used as molecular markers according to the instructions provided by the manufacturer (Sigma Chemical Co., St. Louis, US, High Molecular Weight Markers Kit, Cat no. SDS-6H).

Purified enzyme with sample buffer (1 ml 0.5 Tris buffer pH 6.8, 1 ml glycerol and 1 ml water containing 0.25 mg bromophenol blue) and reference proteins were boiled for 3–5 min and applied to 10% SDS-PAGE with 4% stacking gel. After running the gel, the proteins were stained by Coomassie brilliant blue (acetic acid 100 ml, methanol 400 ml, water 500 ml and CBB 1 g).

Characterization of purified enzyme

To find out the optimum pH, temperature and thermostability of naringinase, assays were carried out at different temperatures (20–70 °C) and at different pH values using sodium acetate (0.05 M, pH 3–6) and phosphate buffer (0.05 M, pH 7–8). A mixture containing the purified enzyme solution, metal ions and EDTA (2.5–100 mM) was incubated for 60 min at 50 °C and enzyme activity was assayed. Apparent K_m and V_{max} values for naringin were calculated using a Lineweaver–Burk plot.

Stability studies

Thermal stability studies were conducted without any additives. The enzyme solution was kept at various temperatures (25–60 °C) in a temperature-controlled water bath for 8–10 days and residual enzyme activity was measured from time to time. The relative activity was calculated as the percentage ratio of activity at a given pH/temperature to the activity at optimum pH/temperature.

Substrate specificity towards rhamnoglucosides

To study the hydrolytic behaviour of purified naringinase on different substrates, 0.5 U of purified enzyme was incubated with naringin, rutin or hesperidin (0.25%, w/v) in 50 mM sodium acetate buffer, pH 4.5, for up to 6 h. Hydrolysis products were identified by reverse phase HPLC using a C-18 Hibar column (E. Merck, Germany), operating at a flow rate of 1.0 ml min^{-1}. Glucose and rhamnose were used as standards.

Results and discussion

Purification of enzyme

A typical purification of naringinase from *Aspergillus niger* MTCC 1344 is summarized in Table 1. The crude extract was concentrated 2.5-fold by ultrafiltration. The concentrated extract after ultrafiltration was subjected to fractional precipitation with ammonium sulphate (65%). This step yielded 7-fold purification with a specific activity of 158 units per mg protein. The ammonium sulphate precipitates were dialyzed against phosphate buffer (pH 7.0, 0.05 M) overnight at 6 °C. The dialyzed precipitates were then lyophilized and dissolved in minimum quantity of phosphate buffer (pH 7.0, 0.05 M). The protein was further purified on a Q Sepharose column and naringinase activity eluted at ~ 0.45 M NaCl. Fractions from the Q Sepharose column with high naringinase activity were pooled and concentrated. The Q Sepharose step yielded a purification factor of 10.5 with a specific activity of 237 units per mg protein while the percentage recovery was 33 (Table 1). Further purification was performed using a Sephadex G-200 column. A major peak eluted using phosphate buffer (0.1 M, pH 7.0) containing 0.15 M NaCl. By this procedure, naringinase was purified approximately 38-fold from the culture filtrate with a specific activity of 867 units per mg protein while the recovery was 19% (Table 1).

Table 1. Summary of the purification of naringinase from *A. niger* 1344.

Purification step	Total protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Activity recovery (%)	Fold purification
Crude extract	360	8180	22.7	100	1
Ultrafiltration	128	7168	56	87	2.5
$(NH_4)_2SO_4$	36	5480	152	67	6.7
Q Sepharose	11.5	2730	237.3	33	10.5
Sephadex G-200	1.8	1560	867	19	38.2

Characterization of enzyme

The relative molecular weight of the purified enzyme was estimated by gel filtration chromatography on a Sephadex G-200 column. The molecular mass of the purified enzyme was 168 kDa, however on SDS-PAGE, the purified naringinase gave two bands with an apparent molecular mass of 85 and 80 kDa, respectively (data not shown). This protein therefore appears to be a heterodimer, probably consisting of α-L-rhamnosidase and β-D-glucosidase subunits. By comparison, molecular masses of the naringinases have been reported to range from 70–240 kDa, and some of these comprise two identical subunits. The molecular mass of the enzyme subunit reported here is similar to that of the extracellular α-L-rhamnosidase from *Aspergillus terrus* (90 kDa) (Gallego *et al.* 2001). Also, values of 87 and 90 kDa have been described for subunits of naringinase from *A. aculeatus* (Mutter *et al.* 1994).

Effect of pH and temperature

Figure 1 shows the optimum pH for purified naringinase activity to be 4.0. The enzyme showed maximum activity at pH 4.0, though at pH 3 and 5 it was still relatively active, having lost only 14% of its maximum activity. A broad pH optimum is preferable for applications of naringinase in the food and pharmaceutical industries. This pH optimum, under the conditions used, was similar to those reported for naringinase from other microorganisms. The optimum pH values of the

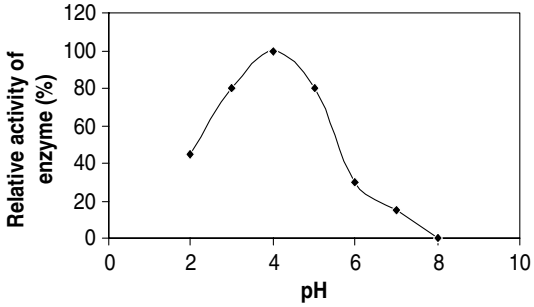

Figure 1. Profile of purified naringinase as a function of pH.

enzymes from *Turbo cornutus*, *A. terreus* (Kurosawa *et al.* 1973), *F. esculentum* (Bourbouze *et al.* 1976) and *Bacteroides* JY-6 (Jang & Kim 1996) have been reported to be 2.8, 4.5, 5.0 and 7.0, respectively. The enzymes from *Penicillium paucimobilis* FP 2001 and *Sphingomonas* sp. RI (Hashimoto & Murata 1998) had pH optima at a slightly alkaline pH.

The optimum temperature for the purified enzyme at pH 4.0 was found to be 50 °C (Figure 2). The enzyme lost 5% of its maximum activity at 40 °C. The enzyme activity declined very sharply at temperatures above 60 °C and was completely abolished at 70 °C. However, earlier studies of Manzanares *et al.* (1997) reported the optimum temperature for purified α-L-rhamnosidase to be 65 °C.

The stability of the purified naringinase was measured during incubation at different temperatures (25–60 °C) (Figure 3). The enzyme was stable at 25 °C (ambient temperature) for a longer duration with no inactivation at 37 °C, as activity was stable up to 5 days. At 40 °C the enzyme showed 50% inactivation after 4–5 days incubation. At its optimum temperature 50 °C, the rate of inactivation was higher, with a calculated half-life of 90 min. At this temperature the enzyme retained 60% of its initial activity after 1 h of incubation. At 60 °C the half-life was 15 min (Figure 2). For α-L-rhamnosidase from various species of *Aspergillus* similar temperature optima have been reported ranging from 50 to 60 °C (Kurosawa *et al.* 1973; Ono *et al.* 1978; Mutter *et al.* 1994; Gallego *et al.* 2001).

Kinetic parameters

Kinetic parameters [maximum reaction velocity (V_{max}) and apparent Michaelis constant (K_m)] were determined

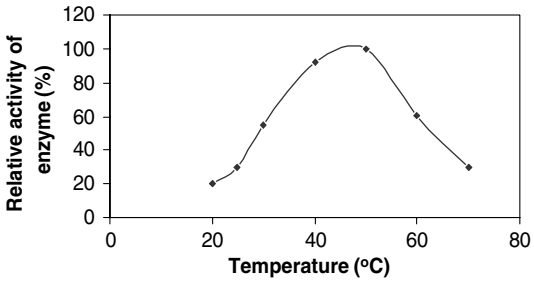

Figure 2. Profile of naringinase as a function of temperature.

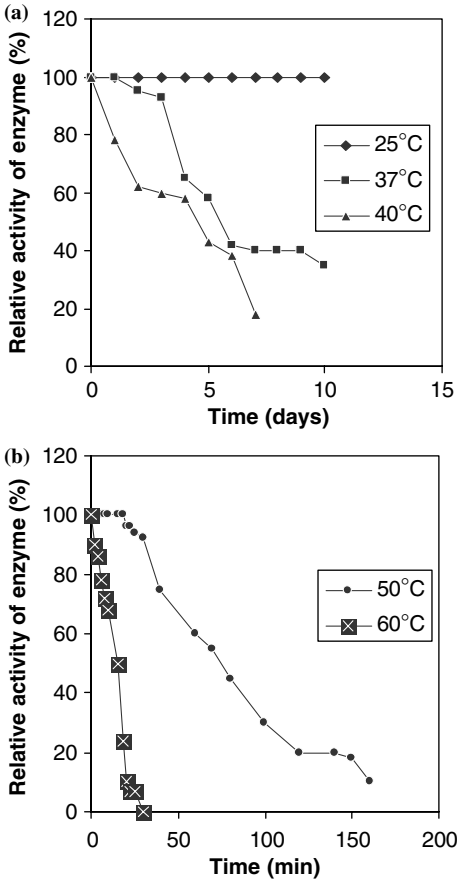

Figure 3. Thermal denaturation of the naringinase in (a) days (25, 37 and 40 °C), (b) minutes (50 and 60 °C).

for purified naringinase with respect to the natural substrate naringin at 50 °C by Lineweaver–Burk plot. Under optimal conditions (50 °C, pH 4.0), naringinase activity exhibited Michaelis–Menten type kinetics. The Michaelis constant (K_m) measured for naringin was 1.9 mM and V_{max} was found to be 21 U mg^{-1}, respectively. The K_m value was less than that for *A. niger* (2.32 and 2.65 mM) α-L-rhamnosidase (Kurosawa *et al.* 1973; Caldini *et al.* 1994), whereas a lower K_m value, 1.52 mM was found for the *Penicillum* sp α-L-rhamnosidase activity (Manjon *et al.* 1985).

Substrate specificity

A natural substrate for the *A. niger* naringinase could be the flavonoids of plant origin, such as naringin, rutin and hesperidin. Naringinase was active towards 0.05% (w/v) naringin (relative rate 100%), and rutin (relative rate 63%), but was unable to release L-rhamnose from hesperidin. In naringin, the L-rhamnose residue is α-1,2 linked to the β-D-glucoside whereas in rutin and hesperidin it is α-1,6 linked. Thus, the enzyme seems to hydrolyse both α-1,2 and α-1,6 linkages to β-D-glucosides. The reason naringinase did not release rhamnose from hesperidin may be due to steric hinderance due to attachment of rutinose (mannopyranosyl-D-glucose) to the chromene nucleus of the flavone molecule via C_7 in

Table 2. Effect of metal ions and organic compounds on naringinase activity of *A. niger* 1344.

Metal	Percentage activity retained at concentration (mM)							
	2.5	5	10	20	30	50	70	100
Ca^{2+}	100	100	100	100	100	100	100	100
Cu^{2+}	100	86	82	75	63	32	nd	nd
Co^{2+}	100	100	100	100	89	83	79	72
Fe^{2+}	72	61	35	nd	nd	nd	nd	nd
Mg^{2+}	100	100	100	100	100	100	100	100
Mn^{2+}	100	86	66	9	nd	nd	nd	nd
Ni^{2+}	100	100	100	100	100	86	72	36
Zn^{2+}	100	100	100	100	100	80	65	50
EDTA	100	100	100	100	86	57	nd	nd
SDS	23	9	0	nd	nd	nd	nd	nd
p-CMB	2	nd	nd	nd	nd	nd	nd	nd
Hg^{2+}	6	nd	nd	nd	nd	nd	nd	nd

p-CMB, *p*-chloromercuribenzoate;
nd, not detected.

hesperidin, whereas the attachment is via C_3 in rutin. The α-L-rhamnosidases from different *A. niger* preparations were active towards naringin and rutin but not against hesperidin (Kurosawa *et al.* 1973; Gallego *et al.* 2001), or only active against naringin (Ono *et al.* 1978) and hesperidin (Sanchez *et al.* 1987). The α-L-rhamnosidase from *A. aculeatus* has been described as active towards naringin and hesperidin (Young *et al.* 1989).

To understand the molecular mechanism underlying the degradation of substrate it is necessary to elucidate the three dimensional structure of the naringinase.

Effect of metal ions and EDTA

Table 2 shows the effect of metal ions and organic compounds on naringinase activity. Cu^{2+} and Mn^{2+} completely inhibited the enzyme activity at a concentration range of 10–50 mM, whereas Fe^{2+} and Ni^{2+} at this concentration showed 40% inhibition. Ca^{2+}, Co^{2+} and Mg^{2+} showed very little inhibition even at 10–100 mM. Zn^{2+} showed 20% inhibition at a concentration of 50 mM. EDTA at a concentration up to 20 mM did not show any inhibition of enzyme activity. At 50 mM concentration, 50% of the enzyme activity was inhibited. Also, Fe^{2+} ions formed a precipitate in the assay mixture. Hg^{2+}, *p*-chloromercuribenzoate (*p*-CMB) and SDS completely inhibited enzyme activity at a concentration of 2.5–5 mM. The enzyme activity was significantly inhibited by a sulphydryl reagent (*p*-CMB) and sulfhydryl oxidant metal (Hg^{2+}), suggesting that a sulphydryl group may be important for enzyme activity. Many of these metal ions are present in the media, either as trace elements or mineral ions, so it is important that these levels should not reach concentrations inhibitory to naringinase activity. Jang & Kim (1996) observed that the metal ions Pb^{2+}, Cu^{2+} and Zn^{2+} at 2 mM concentration did not activate α-L-rhamnosidase from *Bacteroides* (Jang & Kim 1996). Yanai & Sato (2000) observed that the enzyme did not show any requirement for divalent cations for the activity.

Conclusion

The thermostability, kinetic properties and broad substrate specificity displayed by *Aspergillus niger* naringinase described here suggests its possible application in biotechnological processes such as debittering of citrus fruit juice, aroma enhancement during wine-making and transformation of the antibiotic, chloropolysporin. In order to overproduce this enzyme, cloning of the corresponding gene is now in progress.

Acknowledgements

Author M.P. thanks Dr U.C. Banerjee, Biochemical Engineering Research and Process Development Centre (BERPDC), Institute of Microbial Technology, Sector 39 A, Chandigarh, India for carrying out part of this work during a Biotechnology National associateship programme funded by the Department of Biotechnology, Government of India, New Delhi, India. S.K. thanks the Department of Biotechnology, Punjabi University, Patiala for providing research facilities.

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