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# α-L-RHAMNOSIDASE FROM *Aspergillus clavato-nanicus* MTCC-9611 ACTIVE AT ALKALINE pH

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An α-L-rhamnosidase secreting fungal strain has been isolated and identified as *Aspergillus clavato-nanicus* MTCC-9611. The enzyme was purified to homogeneity from the culture filtrate of the fungus using concentration by ultrafiltration membrane and ion-exchange chromatography on CM-cellulose. The native PAGE analysis confirmed the homogeneity of the purified enzyme. The SDS-PAGE analysis of the purified enzyme revealed a single protein band corresponding to the molecular weight 82 kDa. The α-L-rhamnosidase activity of *Aspergillus clavato-nanicus* MTCC-9611 had optimum at pH 10.0 and 50°C. The $K_m$ values of the enzyme were 0.65 mM and 0.95 mM using p-nitrophenyl α-L-rhamnopyranoside and naringin as a substrates respectively. The enzyme transforms naringin to prunin at pH 10.0 and further hydrolysis of prunin to naringenin does not occur under these reaction conditions that makes α-L-rhamnosidase activity of *Aspergillus clavato-nanicus* MTCC-9611 promising enzyme to get prunin for pharmaceutical purposes.

α-L-rhamnosidase (EC 3.2.1.40) [1] cleaves terminal α-L-rhamnose specifically (Fig. 1) from large number of natural products [2]. The enzyme occurs widely in nature and has been found in animal tissues [3–4], plants [5–6], fungi [7–12] and bacteria [13–15]. It is a biotechnologically important enzyme due to its various applications, like debittering of citrus fruit juices [16–19], enhancement of wine aromas [20], preparation of many drugs and drug precursors by derhamnosylating the terminal L-rhamnose containing substrates [21–23].

α-L-rhamnosidases with different properties are suitable for various biotechnological applications. For example, α-L-rhamnosidases which have pH optima in the range 3.0–4.0 are more suitable for debittering of citrus fruit juices because they also have pH in the range 3.0–4.0. α-L-Rhamnosidases having pH optima in the alkaline range will be more suitable for the enzymatic conversion of naringin to prunin because β-glucosidase activity which is responsible for further hydrolysis of prunin to naringenin will not be active in alkaline pH range even if it is present in the preparation of α-L-rhamnosidase. The most of the α-L-rhamnosidases reported so far have pH optima either in the acidic [3, 11, 24, 25] or in neutral [4, 13, 26] pH ranges. No α-L-rhamnosidase with pH optimum greater than pH 8.0 has been reported so far.

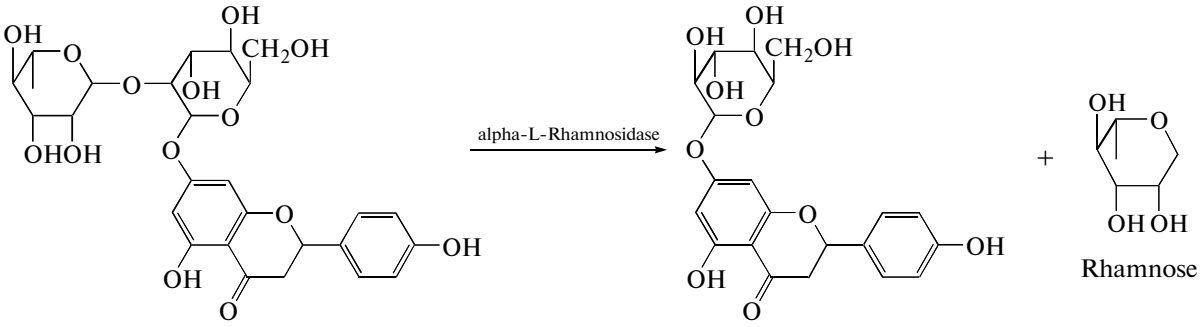
The aim of the study is to purify an α-L-rhamnosidase from *Aspergillus clavato-nanicus* MTCC 9611 having pH optimum 10.0. The enzymatic properties like $K_m$, pH and temperature optima of the purified enzyme have been determined and its use in the enzymatic conversion of naringin to prunin has been demonstrated.

## MATERIALS AND METHODS

**Chemicals.** p-nitrophenyl-α-L-rhamnopyranoside, naringin, L-rhamnose, rutin, DEAE cellulose and CM cellulose were purchased from Sigma (USA). All the chemicals used in the PAGE including the protein molecular weight markers were procured from Bangalore Genei Pvt. Limited (India). All other chemicals were either from Merck Ltd Mumbai (India) or from S. D. Fine Chem. Ltd Mumbai (India) and were used without further purification.

**Isolation of fungal strain and production of the enzyme.** The fungal strain was isolated in our laboratory from decaying rice on the soil by streaking method on Petri plate using potato dextrose agar medium [27]. The fungal strain was identified as *A. clavato-nanicus* at MTCC Centre, Institute of Microbial Technology in Chandigarh (India) and was deposited there with identification number MTCC-9611. The fungal strain was maintained on slants of potato dextrose agar medium.

The secretion of α-L-rhamnosidase by the *A. clavato-nanicus* MTCC 9611 cells was studied in the liquid culture medium having composition (g/l): $CaCl_2$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 3.0, $KH_2PO_4$ – 20.0, $N(CH_2COONa)_3$ – 1.5, $MnSO_4$ – 1.0, $ZnSO_4 \cdot 7H_2O$ – 0.1, $CuSO_4 \cdot 5H_2O$ – 0.1, $FeSO_4 \cdot 7H_2O$ – 0.1, sucrose – 40.0, ammonium tartarate – 8.0 and $H_3BO_3$ – 10.0 mg/l. One ml of the spore suspension (spore density $8 \times 10^6$ spores/ml) from the agar slant was inoculated aseptically into the liquid culture medium (20 ml) kept in 100 ml culture flask which also contained from 0.5 to 1.5% of naringin or rutin as inducer. The experiments were performed in triplicates. The culture flasks were incubated in a Biological Oxygen Demand incu-




Narinigin Prunin

**Fig. 1.** The α-L-rhamnosidase reaction.

bator at 25°C under stationary conditions. Aliquots of one ml were withdrawn at the regular intervals of 24 h, filtered through Millex syringe filters (0.22 μm) and analyzed for the presence of α-L-rhamnosidase activity as described below.

**Assay of α-L-rhamnosidase activity.** The activity of α-L-rhamnosidase was assayed using naringin as the substrate following the Davis method [28]. The reaction solution consisted of 2.5 ml of 0.86 mM naringin dissolved in 0.2 M Na-acetate-acetic acid buffer (pH 4.5) maintained at 60°C. 0.25 ml of the enzyme extract was added to the mixture, then 0.1 ml aliquot was withdrawn immediately and added to 2.5 ml of 90% diethylene glycol followed by the addition of 0.1 ml 4 N NaOH. 0.1 ml aliquots were withdrawn at the intervals of 5 min and treated in the same manner as mentioned above. The samples were maintained at the ambient temperature at least for 10 min and $OD_{420}$ were measured spectrophotometrically. The absorbance values were converted into the concentrations of naringin values by using a calibration curve for naringin. The rate of naringin hydrolysis was calculated from the plot of the concentration of naringin versus time. One unit of enzyme activity (IU) was defined as the amount of enzyme required to hydrolyze 1 μmol of naringin per min of under the above assay conditions.

During the purification of α-L-rhamnosidase and the determination of $K_m$, pH, and temperature optima of the purified enzyme, the activity of α-L-rhamnosidase was assayed using p-nitrophenyl-α-L-rhamnopyranoside as the substrate and monitoring the formation of p-nitrophenol spectrophotometrically at 400 nm by the method reported Romero et al. [29]. The molar extinction coefficient value 21.44 $mM^{-1}$ $cm^{-1}$ was used for the calculation of enzyme unit. UV-Visible spectrophotometer Hitachi (Japan) model U-2000 which was fitted with electronic temperature control unit was used for spectroscopic measurements. The steady state velocity measurements were reproducible within 5% errors.

**Purification of α-L-rhamnosidase.** For the purification of α-L-rhamnosidase, the fungus was grown in 15 sterilized 100 ml culture flasks containing 20 ml of liquid culture medium supplied with 1.0% rutin. The maximum activity of α-L-rhamnosidase was revealed on the third day of the growth. The fungal cultures were pooled and the mycelia were removed by filtering the culture medium through 4 layers of cheese cloth. The culture filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and supernatant (250 ml) was concentrated to 15 ml using Amicon concentration cell model 8200 and ultrafiltration membrane PM10 with molecular weight cut off value of 10 kDa (Millipore, USA). Then it was concentrated by putting in the dialysis tube and covering with sucrose and dialyzed against 0.01 M citric acid-phosphate buffer, pH 4.0, for 24 h. The appropriate condition for the binding of α-L-rhamnosidase on CM cellulose was determined experimentally by the reported method [30]. Three ml of the dialyzed enzyme solution was loaded on CM-cellulose column of size 2.5 × 15 cm equilibrated with 0.01 M citric acid-phosphate buffer, pH 4.0. The column was washed with the same buffer and α-L-rhamnosidase activity was eluted using the linear 0–1.0 M NaCl gradient in the same buffer (50 ml of the buffer + + 50 ml buffer containing 1.0 M NaCl). 5 ml fractions were collected and analyzed for the α-L-rhamnosidase activity and for protein concentration (Lowry's method). The α-L-rhamnosidase active fractions were combined and concentrated using sucrose. The purified concentrated enzyme sample 2.8 ml was stored at –20°C until use.

**SDS-PAGE and native PAGE analysis of the purified enzyme.** The homogeneity of the enzyme preparation was checked by SDS-PAGE analysis using the method reported by Weber and Osborn. The resolving gel was 10% acrylamide in 0.375 M Tris-HCl buffer (pH 8.8) and stacking gel was 5% acrylamide in 0.063 M Tris-HCl buffer (pH 6.8). The electrophoresis buffer was 0.025 M Tris-glycine buffer (pH 8.5). The gel was run at constant current of 20 mA. The mo-

Purification steps of α-L-rhamnosidase from *Aspergillus clavato-nanicus* MTCC-9611

| Steps | Total volume, ml | Activity, IU/ml | Protein, mg/ml | Specific activity, IU/mg | Total activity, IU | Total protein, mg | Purification, fold | Recovered, % |
|---|---|---|---|---|---|---|---|---|
| Culture filtrate | 250 | 0.03 | 0.08 | 0.37 | 7.50 | 20.0 | 1 | 100 |
| Concentration by ultrafiltration and dialysis | 15 | 0.35 | 0.31 | 1.13 | 5.25 | 4.65 | 3 | 70 |
| Eluted from CM-cellulose column | 24.5 | 0.06 | 0.01 | 6.00 | 1.47 | 0.25 | 16 | 20 |

lecular weight markers used were phosphorylase-97.4, bovine serum albumin-66.0, ovalbumin-43.0, carbonic anhydrase-29.0 and soyabean trypsin inhibitor 20.1 kDa.

The native polyacrylamide gel electrophoresis was done using the reagent kit supplied by Bangalore Genei Pvt. Limited (India). The resolving gel was 8% acrylamide in 0.39 M Tris-HCl buffer (pH 8.8) and the stacking gel was 5% acrylamide in 0.068 M Tris-HCl buffer (pH 6.8). The molecular weight markers used was catalase (240.0 kDa). In both PAGE the proteins were visualized by silver staining.

**Determination of the enzymatic characteristics.** The $K_m$ value of the purified α-L-rhamnosidase for the substrate p-nitrophenyl-α-L-rhamnopyranoside was determined by measuring the steady state velocity of the enzyme catalyzed reaction at different concentrations of p-nitrophenyl-α-L-rhamnopyranoside (from 0.1 to 5.0 mM) using the method reported Romero et al. [29]. The $K_m$ value was determined by linear regression analysis of the data points (average of triplicate measurements) of the double reciprocal plots. The value of $K_m$ of the purified enzyme for the natural substrate naringin was also determined in the similar way except that the steady state velocity of naringin hydrolysis was determined by Davis method [28].

The pH and temperature optimum of the purified enzyme was determined by using p-nitrophenyl-α-L-rhamnopyranoside as the substrate and measuring the steady state velocity of the enzyme catalyzed reaction in solutions of varying pH from 2 to 13 and temperature from 20 to 70°C. The buffers used were 0.1 M citric acid-$NaH_2PO_4$ (McIlvaine buffer, pH 3–7), Clarks and Lubs solution buffer (pH 8–10) and 0.1 M Na-phosphate buffer (pH 11–13).

**Studies on the enzymatic hydrolysis of naringin to prunin.** One ml of 1.0 mM naringin in Na-phosphate buffer (pH 10.0) was treated with 200 μl of the crude α-L-rhamnosidase containing $3.2 \times 10^{-2}$ IU/ml. The reaction was allowed to occur at 30°C for 1 h. The reaction solution was extracted thrice with 1.0 ml of ethyl acetate. The extract was analyzed using HPLC with 4.6 × 250 mm column, model M-600E with Spherisob, $C_{18}$, 5*U* (Waters, India Private Ltd). The solvent used was methanol–water 50 : 50 by volume at the flow rate of 2 ml/min. The peaks were detected at 254 nm.

## RESULTS AND DISCUSSION

The purification procedure of the α-L-rhamnosidase of *A. clavato-nanicus* MTCC-9611 is summarized in Table and the elution profile of the enzyme from the CM-cellulose column is shown in Fig. 2. The activity peak of the enzyme coincides with the major protein peak indicating that the eluted enzyme is relatively pure. The results of SDS-PAGE analysis are shown in Fig. 3a. The presence of single protein band in lane 2 in which purified enzyme has been loaded indicates that the enzyme is homogenous. The molecular weight calculated from the SDS-PAGE data was 82 kDa. The results of native PAGE analysis of the purified enzyme are shown in Fig. 3b in which catalase has been loaded in lane 1 and the purified enzyme has been loaded in lane 2. In the native PAGE, the purified α-L-rhamnosidase gives single protein band which supports the results of SDS-PAGE.

The $K_m$ values determined for the purified enzyme at 50°C in 0.1 mM sodium phosphate buffer pH 10.0 using p-nirophenyl-α-L-rhamnopyranoside and naringin as the substrate from double reciprocal plots (data not shown) were 0.65 mM and 0.95 mM respectively. The $K_m$ values using the same substrate for p-nitrophenil-α-L-rhamnopyranoside purified from *Fagopyrum esculeutum* [31], *Bacteroides* JY-6 [13], *Pseudomonas paucimobilis* FP 2001 [32], *Fusobacterium* K-60 [14], *Penicillium decumbens* [29] and *Aspergillus aculeatus* RhaA and RhaB [11] have been reported to be 0.33, 0.29, 1.18 0.057, 1.52 and 0.3 and 2.8 mM respectively. Similarly, the reported $K_m$ values using naringin as the substrate for α-L-rhamnosidases from *Bacteroides* JY-6 [13], *Pseudomonas paucimobilis* FP 2001 [32], *Fusobacterium* K-60 [14] and *Penicillium decumbens* [29] were 0.89, 0.17, 0.021 and 7.0 mM respectively. Thus, the purified α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611 had intermediate affinities for both p-nitrophenyl-α-L-rhamnopyranoside and naringin as compared to the reported α-L-rhamnosidases.

The results of the dependence of activity of the purified enzyme from *A. clavato-nanicus* MTCC-9611 on the variation of the pH of the reaction mixture are shown in Fig. 4a. The enzyme was active in a broad pH range 4–12 but the maximum activity was at pH 10.0. The most of the α-L-rhamnosidases reported so far have pH optima either in the acidic [3, 11, 24, 25] or

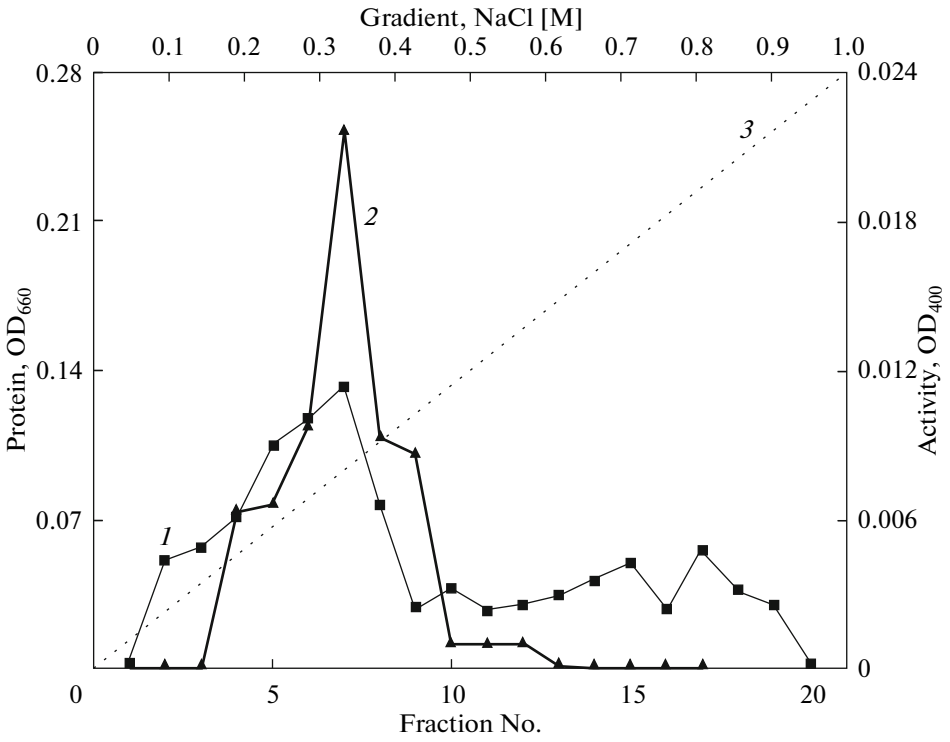



**Fig. 2.** Elution profile of the α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611 from CM-cellulose. *1* – protein; *2* – enzyme activity; *3* – NaCl gradient.

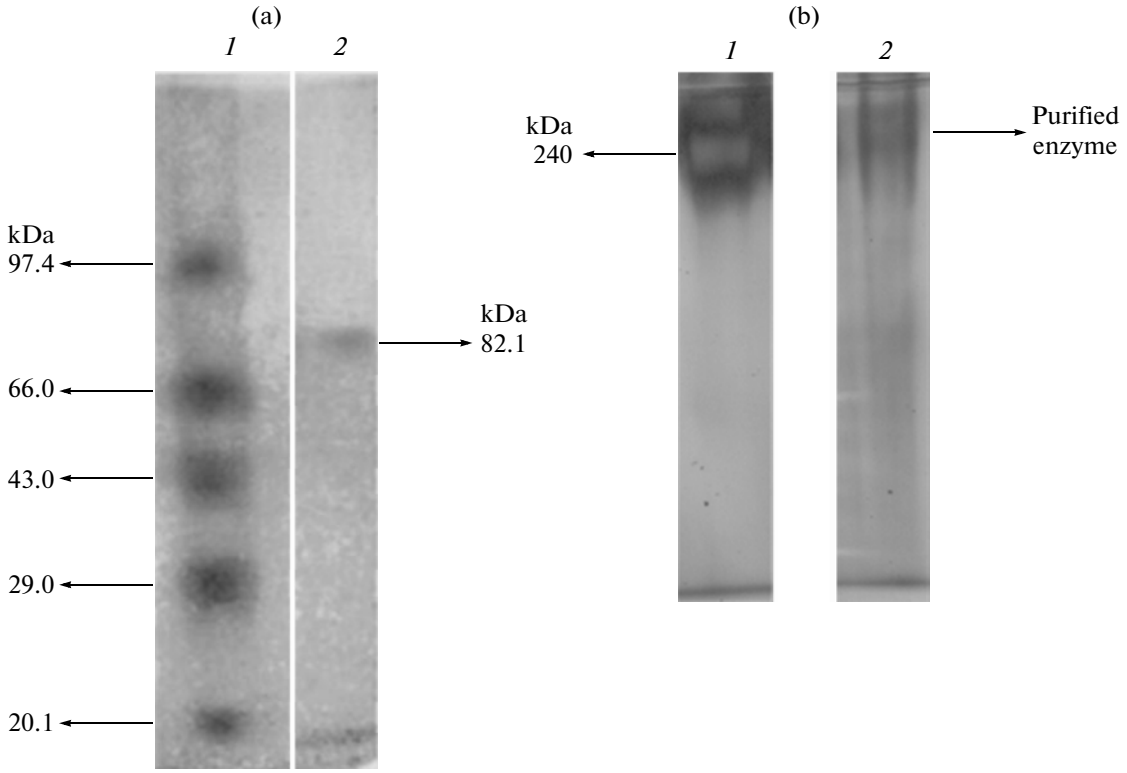



**Fig. 3.** PAGE of the purified α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611. a – SDS-PAGE: lane *1* – molecular weight markers; lane *2* – purified enzyme (0.63 μg); b – native PAGE: lane *1* – molecular weight marker; lane *2* – purified enzyme (1.2 μg). An arrow shows the position of α-L-rhamnosidase.

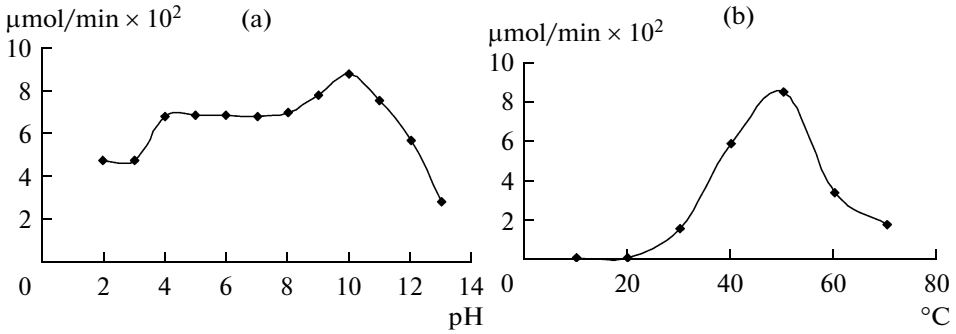



**Fig. 4.** Determination of pH (a) and temperature optima (b) for α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611. The dependence of the enzyme activity on pH of the reaction mixture at 50°C. The dependence of the enzyme activity on temperature of the reaction mixture in 0.1 M Na-phosphate buffer (pH 10.0).

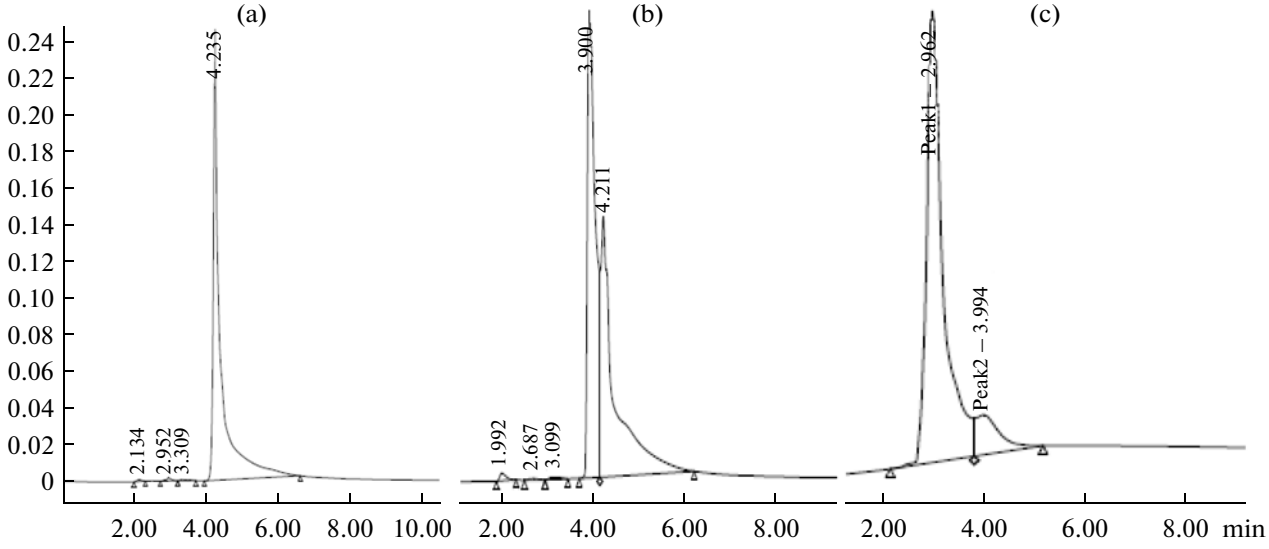



**Fig. 5.** HPLC of the ethyl acetate extract of the hydrolysis of naringin by α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611. a – control – the ethyl acetate extract of 1 mM naringin in 0.1 M Na-phosphate buffer (pH 10.0); b – the ethyl acetate extract of the enzyme ($3 \times 10^{-3}$ IU) treated naringin after 30 min at 30°C; c – the ethyl acetate extract of the enzyme ($3 \times 10^{-3}$ IU) treated naringin after 1 h at 30°C.

neutral [4, 13, 26] pH ranges. Only α-L-rhamnosidases of *Clostridium stercorarium* [15], *Sphingomonas paucimobilis* [33], *Pseudomonas paucimobilis* FP2001 [32] and bacterium PRI-1686 [34] have their pH optima values of 7.5, 8.0, 7.9 and 7.8 respectively. Thus, the purified enzyme from *A. clavato-nanicus* MTCC-9611 is the only α-L-rhamnosidase having pH optimum at 10.0. This makes it more suitable for the enzymatic conversion of naringin to prunin, a pharmaceutically important rare compound, because at this pH, β-glucosidase activity even if present in the α-L-rhamnosidase preparation will not be able to further hydrolyze prunin to naringenin.

We detected also the pH stability of the purified enzyme in the pH range (2–12) and found that the enzyme was the most stable at pH 4.0.

The variation of the activity of the purified enzyme with temperature of the reaction solution is shown in the Fig. 4b. The temperature optimum of the enzyme was 50°C. The temperature optima of the α-L-rhamnosidases reported in the literature [7, 9, 10, 34, 35] were in the range 40–80°C.

One of the applications of α-L-rhamnosidase is in the conversion of naringin which is 4′,5,7-trihydroxyflavanone 7-rhamnoglucoside to prunin which is 4′,5,7-trihydroxyflavanone 7-glucoside. If β-glucosidase activity is present in the α-L-rhamnosidase preparation, the glucose unit of prunin is further cleaved giving naringenin which is 4′,5,7-trihydroxyflavanone. To make the α-L-rhamnosidase preparation suitable for the conversion of naringin to prunin, it must not contain β-glucosidase activity or it must be active in alkaline pH range where β-glucosidase

should have no activity. Keeping these points in view, crude preparation of α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611 was tested at pH 10.0 for the conversion of naringin to prunin. The results of HPLC analysis of the ethyl acetate extract of the enzymatic hydrolysis products are shown in Fig. 5b, c. The peak at retention time 3.99 min is due to naringin and the peak with retention time 2.99 min is due to prunin. Pure samples of naringin and naringenin gave peaks at retention time 4.0 and 7.4 min respectively under identical conditions. In Fig. 5, there is no peak corresponding to retention time 7.4 min indicating that there is no naringenin present in the enzymatic hydrolysis products of naringin showing that prunin is not hydrolyzed to naringenin by crude enzyme preparation. In the ethyl acetate extract 76% of the enzymatic reaction products was prunin indicating that the purified α-L-rhamnosidase from *A. clavato-nanicus* MTCC-9611 can be used for the conversion of naringin to prunin and prunin is not hydrolyzed to naringenin under these conditions.

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## REFERENCES

1. Puri, M. and Banerjee, U.C., *Biotechnol. Adv.*, 2000, vol. 18, no. 3, pp. 207–217.
2. Roitner, M., Schalkhammer, T., and Pittner, F., *Monatsh. Chem.*, 1984, vol. 115, no. 10, pp. 1255–1267.
3. Kurosawa, Y., Ikeda, K., and Egami, F., *J. Biochem.* 1973, vol. 73, no. 1, pp. 31–37.
4. Qian, S., Yu, H., Zhang, C., Lu, M., Wang, H., and Jin, F., *Chem. Pharm. Bull.*, 2005, vol. 53, no. 8, pp. 911–914.
5. Suzuki H., *Arch. Biochem. Biophys.*, 1962, vol. 99, no. 3, pp. 476–483.
6. Bourbouze, R., Pratviel-Sosa, F., and Percheron, F., *Phytochemistry,* 1975, vol. 14, no. 6, pp. 1279–1282.
7. Yanai, T. and Sato, M., *Biosci. Biotechnol. Biochem.*, 2000, vol. 64, no. 10, pp. 2179–2185.
8. Manzanares, P., Orejas, M., Ibanez, E., Valles, S., and Ramon, D., *Lett. Appl. Microbiol.,* 2000, vol. 31, no. 3, pp. 198–202.
9. Scaroni, E., Cuevas, C., Carrillo, L., and Ellenrieder, G., *Lett. Appl. Microbiol.*, 2002, vol. 34, no. 6, pp. 461–65.
10. Puri, M. and Kalra, S., *World J. Microbiol. Biotechnol.* 2005, vol. 21, no. 5, pp. 753–58.
11. Manzanares, P., Broeck, H.C.V., Graaff, L.H.D., and Visser, J., *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 5, pp. 2230–34.
12. Yu, H., Gong, J., Zhang, C., and Jin, F., *Chem. Pharm. Bull.*, 2002, vol. 52, no. 2, pp. 175–178.
13. Jang, I.S. and Kim, D.H., *Biol. Pharm. Bull.,* 1996, vol. 19, no. 12, pp. 1546–1549.
14. Park, S., Kim, J., and Kim, D., *J. Microbiol. Biotechnol.*, 2005, vol. 15, no. 3, pp. 519–24.
15. Zverlov, V.V., Hertel, C., Bronnenmeier, K., Hroch, A., Kellermann, J., and Schwarz, W.H., *Mol. Microbiol.*, 2000, vol. 35, no. 1, pp. 173–79.
16. Gray, G.M. and Olson, A.C., *J. Agric. Food. Chem.*, 1981, vol. 29, no. 6, pp. 1299–1301.
17. Manjon, A., Bastida, J., Romero, C., Jimeno, A., and Iborra, J.L., *Biotechnol. Lett.*, 1985, vol. 7, no. 7, pp. 477–482.
18. Tsen, H.Y., Tsai, S.Y., and Yu, G.K., *J. Ferment. Bioeng.*, 1989, vol. 67, no. 3, pp. 186–189.
19. Prakash, S., Singhal, R.S., and Kulkarni, P.R., *J. Sci. Food Agric.*, 2002, vol. 82, no. 4, pp. 394–397.
20. Caldini, C., Bonomi, F., Pifferi, P.G., Lanzarini, G., and Galante, Y.M., *Enzyme Microb. Technol.*, 1994, vol. 16, no. 4, pp. 286–291.
21. Elujoba, A.A. and Hardman, R., *Fitoterapia,* 1987, vol. 58, pp. 299–303.
22. Monti, D., Pisvejcova, A., Kren, V., Lama, M., and Riva, S., *Biosci. Biotechnol. Bioeng.*, 2004, vol. 87, no. 6, pp. 763–771.
23. Feng, B., Ma, B., Kang, L., Xiong, C., and Wang S., *Tetrahedron*, 2005, vol. 61, no. 49, pp. 11758–63.
24. Kaji, A. and Ichimi, T., *Agric. Biol. Chem.*, 1973, vol. 37, no. 2, pp. 431–432.
25. Feng, B., Kang, L., Ma, B., Quan, B., Zhou, W., Wang, Y., Zhao, Y., Liu, Y., and Wang, S., *Tetrahedron,* 2007, vol. 63, no. 29, pp. 6796–6812.
26. Hashimoto, W., Nankai, H., Sato, N., Kawai, S., and Murata, K., *Arch. Biochem. Biophys.*, 1999, vol. 368, no. 1, pp. 56–60.
27. Seeley, J.H.W., Vandemark, P.J., and Lee, J.J., *Microbes in Action, A Laboratory Manual of Microbiology.* 4th Ed. New York: W.H. Freeman and Co., 1991, pp. 37–55.
28. Davis D.W., *Anal. Chem.*, 1947, vol. 19, no. 7, pp. 476–478.
29. Romero, C., Manjon, A., Bastida, J., and Iborra, J.L., *Anal. Biochem.,* 1985, vol. 149, no. 2, pp. 566–71.
30. Pharmacia Fine Chemicals AB. Ion Exchange Chromatography: principles and Methods. BOX 175, S-75104 Uppsala 1, Sweden 1983.
31. Bourbouze, R., Perchekon, F., and Courtois, J.E., *Eur. J. Biochem.,* 1976, vol. 63, no. 2, pp. 331–37.
32. Miake, F., Satho T., Takesue, H., Yanagida, F., Kashige, N., and Watanabe, K., *Arch. Microbiol.,* 2000, vol. 173, no. 1, pp. 65–70.
33. Hashimoto, W. and Murata, K., *Biosci. Biotechnol. Biochem.,* 1998, vol. 62, no. 6, pp. 1068–1074.
34. Birgisson, H., Hreggvidsson., G.O., Fridjónsson, O.H., Mort, A., Kristjánsson, J.K., and Mattiasson, B., *Enzyme Microb. Technol.,* 2004, vol. 34, no. 6, pp. 561–571.
35. Busto, M.D., Meza, V., and Mateos, N.P., *Food Chem.,* 2007, vol. 104, no. 3, pp. 1177–1182.