International Journal of Trend in Scientific Research and Development (IJTSRD)
International Open Access Journal
ISSN No: 2456 - 6470 | www.ijtsrd.com | Volume - 2 | Issue – 5

Prunin Production From Orange Peel Naringin Hydrolyzed By a-L-rhamnosidase from *Aspergillus Flavipus* MTCC- 4644

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ABSTRACT:
An orange (*Citrus sinensis*) is the most common fruits in the world. The wastes generated from the orange fruit needs to be put in to beneficial use. In this study the primary wastes (peel) of orange is use for preparation of prunin. α-L-Rhamnosidase (EC 3.2.1.40) secreted by *Aspergillus flavipus* MTCC-4644 are potential catalysis in hydrolysis of naringin content present in orange peels. α-L-rhamnosidase from the culture filtrate of a fungal strain, Aspergillus flavipus MTCC-4446 has been purified to homogeneity. The procedure involved concentration by ultra filtration and cation-exchange chromatography on carboxymethyl cellulose. The purified enzyme gave a single protein band corresponding to molecular mass of 40.0 kDa in SDS-PAGE analysis showing that the enzyme preparation was pure. The native PAGE analysis of the purified enzyme also gave single protein band confirming the purity of the enzyme preparation. Using p-nitro phenyl -α-L-rhamnopyranoside as substrate, *Km* and *kcat* values of the enzyme were 0.48 mM and 28. 4 s-1 respectively. The pH and temperature optima of the enzyme were 5.0 and 50 °C, respectively. The enzyme is stable below10ºC and at pH 4.5. The energy of activation for thermal denaturation of enzyme determined by Arrhenius plot was 32.06 k J mol-1.The enzyme hydrolyzed naringin content of orange peel to L-rhamnose and prunin.

Keyword: *Citrus sinansis, Naringin, α-L-Rhamnosidase, Prunin, Aspergillus flavipus, L-rhamnose*

1. INTRODUCTION

India is the second largest producers of the fruits and third rank in respect of production of citrus fruits in the world. The citrus fruit juice has high therapeutic value as antispasmodic, sedative, cytophylactic, digestive, ant carcinogenic, anti inflametory and anti allergic. The health benefits of citrus fruit juices have been attributed due to the presence of bioactive and antioxidant compounds. Naringin (flavonoid) is major component in citrus fruit juice with very bitter test.

α-L-rhamnosidase [3.2.1.40] derhamnosylates terminal α-L-rhamnose specifically from naringin to prunin and L-rhamnose[1-2]. The derhamnosylation of natural glycoside naringin gives prunin (quercetin-3-o-ß-glucoside) which is a rare product with several biological activities[3]. It is an antithrombic drug to treat myocardial ischemia, cerebral hypoxia and ischemic disease due to its nonoxidizable, anti-inflammatory, anti-mutagenetic, anti-viral properties and other pharmacological effects [4-6]. Prunin is rarely occurs in nature, its extraction yield is low, and therefore it is expensive. The transformation of naringin to prunin has been investigated using acid hydrolysis, heating, microbial transformation and enzymatic hydrolysis process under suitable conditions [7-10]. These studies have prompted the authors to initiate the search for an α-L-rhamnosidase specific for the debittering of citrus fruit juice and hydrolyzed naringin content in orange fruit peel in to prunin and L-rhamnose. Orange fruit is a natural source of naringin. Microorganisms growing on orange fruit peel surface may secrete enzyme specific for the conversion of naringin to prunin. Tempted by this hypothesis, an α-L-rhamnosidase secreting fungal strain has been isolated from the decaying orange fruit peel by the authors. The fungal strain has been identified as *Aspergillus flavipus* MTCC-4644. Its α-L-rhamnosidase has been purified and characterized.

It specifically converts naringin to prunin. The results of the studies are reported in this communication.

2. MATERIALS AND METHODS

2.1 `Chemicals

P-Nitro phenyl-α-L-rhamnopyranoside,, naringin, L-rhamnose, and CM cellulose were purchased from Sigma Chemical Company ,St. Louis, (USA). Manganous sulphate, sodium chloride, sodium acetate were from Merck Ltd., Mumbai, (India) and acetic acid, tartaric acids, citric acids succinic acids and other chemicals were from s.d. fine chem. Ltd., Mumbai, (India) and were used without further purifications. The chemicals for electrophoresis including the protein molecular weight markers used in the SDS-PAGE and native-PAGE analysis were procured from Bangalore GENEI Pvt. Limited Bangalore (India).

2.2 α-L-rhamnosidase purification

For the purification of α-L-rhamnosidase, the fungal strain was grown in ten sterilized 100-mL culture flasks each containing 20 mL liquid culture medium amended with 0.5% (w/v) naringin and 2.0% (w/v) orange peel. The maximum activity of the enzyme was achieved on the sixth day of the growth. On the 6th day, fungal cultures from all the culture flasks were pooled and the mycelia were removed by filtering the culture through four layers of cheese cloth. The culture filtrate (150mL) was centrifuged using Sigma refrigerated centrifuge model 3K30 at 4000 g for 20 min at 4 °C to remove the particles, and was concentrated by ultrafiltration to 10 mL using Amicon concentration cell model 8200 and PM10 membrane. The concentrated crude enzyme was dialyzed against 0.01 M sod. acetate/acetic acid buffer of pH 5.0. Seven milliliters of the dialyzed enzyme solution was loaded on CM-cellulose column of size 10 mm x 170 mm equilibrated with 0.01 M sod. acetate/acetic acid buffer of pH 5.0. The column was washed with the same buffer, and α-L- rhamnosidase activity was eluted using the linear NaCl gradient of 0–1 M in the same buffer (50 mL of the buffer + 50 mL buffer containing 1 M NaCl). The fractions of 4.5 mL were collected and were analyzed for α-L-rhamnosidase activity and protein concentration using the respective methods reported in the literature [11-12]. The active fractions of the enzyme were combined and concentrated by ultra filtration. The purified enzyme sample was stored in the refrigerator at 4°C and was used whenever required.

2.3 SDS- PAGE and Native PAGE analysis of the purified enzyme

The purity of the purified enzyme was checked by SDS-PAGE analysis. The molecular weight markers used were phosphorylase B (97.4 kDa), bovine serum albumin (66.0 k Da), ovalbumin (43.0 kDa), carbonic anhydrase (29.0) and soyabean trypsin inhibitor (20.1 kDa). The resolving gel was 12% acryl amide in 1.5 M Tris-HCl buffer of pH 8.8 and stacking gel was 5% acryl amide in 0. 5 M Tris-HCl buffer of pH 6.8. The electrophoresis buffer was 0.025 M Tris - glycine buffer of pH 8.5. The gel was run at constant current of 20mA. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. The molecular mass of the purified enzyme was determined by the reported method [13].

The native polyacryl amide gel electrophoresis was done using the reagent kit supplied by Bangalore GENEI Pvt. Limited Bangalore (India). The resolving gel was 10% acryl amide in1.5 M Tris – HCl buffer of pH 8.8 and the stacking gel was 5% acryl amide in 0.5 M Tris – HCl buffer of pH 6.8. Bovine serum albumin (66.0 k Da) was run as reference protein. The proteins were located by silver staining.

2.4 Determination of enzymatic characteristics

The K_m and V_{max} values of the purified enzyme for the substrate p-nitrophenyl-α-L-rhamnopyranoside were determined by measuring the steady-state velocity of the enzyme catalyzed reaction at different concentrations of the substrates (0.1–2.0 mM) using the reported method[11]. The K_m and V_{max} values were calculated by linear regression analysis of the data points (average of triplicate measurements) of the double reciprocal plots. The pH 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 in the range 3.0-7.0 using 0.2 M Sodium acetate/ acetic acid buffer. The steady-state velocity was plotted against pH of the reaction solution, and pH optimum was calculated from the graph. The temperature optimum was determined by measuring the steady-state velocity of the enzyme catalyzed reaction in solutions of varying temperatures (30–70°C) using p-nitrophenyl-α-L-rhamnopyranoside as the substrate. The steady-state velocity of the enzyme catalyzed reaction was plotted against the temperature of the reaction solution, and temperature optimum was calculated from the graph. The pH stability of the

enzyme was determined by incubating the enzyme in the buffers of different pHs for 24 h at 25°C. The residual activities were assayed and plotted in the form of steady state velocity versus pH to which the enzyme was exposed for 24 h. The thermal stability was determined by incubating the aliquots of the enzyme at different temperatures (in the range 10-60°C) for one hr and assaying the residual activity. The residual steady state velocity was plotted against the temperature at which the enzyme aliquot was exposed for one hr. For the determination of the energy of activation for the thermal denaturation of the enzyme, the enzyme sample was kept at a particular temperature, its activity was determined at regular interval of 10 minutes for one hr and the steady state velocity was plotted against time. The rate constant k for the thermal denaturation of the enzyme at a particular temperature was calculated by determining the half life time of denaturation of the enzyme, $t_{1/2}$ using the equation $k=0.692 / t_{1/2}$. The energy of activation for the thermal denaturation of the enzyme was determined by Arrhenius plot of log k vs 1/T where T is the temperature in °K.

2.5 Studies on derhamnosylation of naringin by the enzyme

To a 10.0-mL solution of 0.5 mM naringin in 0.2 M sodium acetate /acetic acid buffer pH 4.5 at 50 °C, 200 µL of α-L-rhamnosidase (0.52 IU/mL) was added. The reaction solution was left overnight and the release of prunin was detected by thin-layer chromatography using silica gel on glass plates using butanol: acetic acid: water (40:11:29) (v/v) solution as the mobile phase. Starting compound naringin and naringenin were used as reference compounds. The spots were developed by keeping the glass plate in iodine chamber.

Another experiment was performed using orange peel shocked in ethanol at overnight. pH of ethanolic solution of orange peel was maintained at pH 4.5 by sodium acetate acetic acid buffer and treated by α-L-rhamnosidase (0.82 IU/mL) at 50ºC. The reaction solution was left overnight and the release of prunin was detected by thin-layer chromatography process describe above. The release of L-rhamnose was detected by thin-layer chromatography using silica gel on glass plates. The mobile phase used was chloroform: methanol mixture 70:30 (v/v). The detection was made using the iodine chamber.

3. RESULTS AND DISCUSSION

3.1. Purification of the α-L-rhamnosidase

The purification procedure of the α-L-rhamnosidase from the culture filtrates of *Aspergillus flavours MTCC*-4644 is summarized in table1. The purification involved concentration of the culture filtrate by ultra filtration, dialysis of the concentrated filtrate and cation exchange chromatography on CM cellulose. The enzyme bound on CM cellulose in 10 mM sodium acetate/acetic acid buffer of pH 5.0 and was eluted in the linear gradient of NaCl in the same buffer in the range 0.68–0.76 M sodium chloride. (The elution profile is shown Fig, 1). About 24 fold purification with 24% yield of the enzyme has been achieved, using a relatively simple procedure. The purity of the enzyme preparation was checked by SDS-PAGE and native –PAGE analysis. The results are shown in Fig.2. Figure 2(a) shows the results of SDS-PAGE analysis in which the purified enzyme was applied in lane 1 and the molecular weight markers were applied in lane 2. The purified enzyme sample gave a single protein band indicating that the enzyme preparation was pure. The results of native –PAGE analysis are shown in Fig.2 (b) in which reference proteins ware applied in lane 1 and the purified enzyme was applied in lane 2. The presence of a single protein band in the native-PAGE analysis confirmed the purity of the enzyme preparation. The molecular mass of the purified enzyme calculated from the SDS-PAGE analysis was 40.0 kDa which is in the same range as the molecular masses of other fungal α-L-rhamnosidases reported in the literature [1].

3.2. Enzymatic characterization

The Michaelis–Menten behaviour of the purified enzyme using p-nitrophenyl-α-L-rhamnosidase as the substrate was determined (the results not shown). The calculated K_m value for this enzyme using p-nitrophenyl- α-L-rhamnopyranoside as the substrate was 0.48 mM at 50°C in 0.2 M Sodium acetate / acetic acid buffer pH 5.0. The K_m values for α-L-rhamnosidases purified from *Pseudomonas paucimobilis* FP 2001 [14], from *Fusobacterium* K-60 [15], from *Aspergillus aculeatus* RhaA and RhaB [16], from *Aspergillus flavus* MTCC-9606 [17] and from *Penicillium citrinum* MTCC-8897 [18] have been reported to be 1.18, 0.06, 0.30 and 2.80, 1.89 and 0.36 mM, respectively. Thus, the purified α-L-rhamnosidase has intermediate affinity for p-nitrophenyl-α-L-rhamnopyranoside as compared to reported α-L-rhamnosidases [1]. The calculated k_{cat} value for the purified α-L-rhamnosidase was 28. 4 s^{-1}.

The k_{cat} values for some α-L-rhamnosidases using p-nitrophenyl-α-L-rhamnopyranoside as the substrate reported in the literature are 17.1 $s^{-1}$of *A. flavus* MTCC-9606, 20.1 $s^{-1}$of *P. citrinum* MTCC-3565, 39.9 $s^{-1}$of *A. awamori* MTCC-2879, 35.7 $s^{-1}$of *A. corylopholum* MTCC-2011 [17, 19-21]. Thus the k_{cat} value of the purified α-L-rhamnosidase using p-nitrophenyl-α-L-rhamnopyranoside as the substrate is in the same range as reported in the literature for other fungal α-L-rhamnosidases. The k_{cat}/K_m value of the purified α-L-rhamnosidase is 5.91 $x10^4$ $M^{-1}s^{-1}$.

3.3. pH and temperature stabilities

The pH optimum of the purified enzyme was 5.0 using p-nitrophenyl- α-L-rhamnopyranoside as the substrate. Most of the α-L-rhamnosidases reported so far have pH optima in the acidic pH range [1]. Only α-L-rhamnosidases of pig liver [22] and *P. citrinum* MTCC-8897 [18] have pH optima in the neutral pH range. The acidic pH range of this enzyme makes it suitable for specific applications in the systems. In order to find out the conditions for the preservation of the purified enzyme, pH stability of the enzyme studies shows that the enzyme is most stable at pH 5.0 if left for 24 hr. The temperature optimum of the purified enzyme was 50°C, which lies in the range of temperature optima reported for the other fungal α-L-rhamnosidases[1]. The results of studies on testing the thermal stability of the purified enzyme in which the activity left after 1 h exposure of enzyme aliquots at a particular temperature has been plotted against the temperature. It is obvious that the enzyme starts losing its activity very slowly even at 10 °C but appreciable rate of loss of activity starts above 20 °C. The activation energy for the thermal denaturation of the pure enzyme calculated from Arrhenius plot was 32.06 kJ/mol. On the basis of these studies, the purified enzyme can be stored at pH 5.0 in the refrigerator.

3.4. Hydrolysis of orange peel naringin to prunin

The reaction process of hydrolysis of orange peel naringin to prunin are shown in scheme 1.The results of studies on the enzymatic de-rhamnosylation of naringin, shown in Figure 3A. The spots in lane 1 and lane 2 are due to the standard sample of naringin and its aglycon naringenin, respectively; where as the spot in lane 3 is due to the enzymatic de-rhamnosylated product of naringin. The spot in lane 4 and 5 are due to naringin and derhamnoslated product of naringin from orange peel. Thr R_f value of the derhamnosylated product of naringin neither coincides with the R_f value of naringin nor with the value of aglycon naringenin. It has been inferred that the enzymatically derhamnosylated product of naringin is prunin. Further confirmation of the identity of the product prunin was done by purifying it by preparative TLC and analyzing by HPLC-Mass spectrometry. The hydrolysis product of orange fruit peel was also analyzed in the same way as the enzymatic hydrolytic product of naringin. The results are given in Fig. 3B.

The enzymatically released L-rhamnose from orange peel naringin was analyzed by TLC and HPLC –mass spectrometry studies. The results are shown in figure 4.In figure 4(A). The spot in lane 1 due to standard sample of L-rhamnose and spot of lane 2- due to L-rhamnose released by enzymatic hydrolysis of orange peel naringin. In lane 3 no spot shows that no hydrolysis takes place in solution of orange peel without enzyme. The L-rhamnose confirmed by purifying it by preparative TLC analyzing by HPLC –mass spectrometry. The results are shown in Figure 4 (B). The result inferred that agro waste material i.e. orange peel is valuable for production of bioactive compounds when treated with α-L-rhamnosidase.

4. CONCLUSION

In conclusion, this communication reports the purification of an α-L-rhamnosidase from the culture filtrate of a new fungal strain *A. flavipus MTCC-4446*, isolated from decayed orange fruit peel, using a simpler procedure compared to the procedures reported for the purifications of the other fungal α-L-rhamnosidases reported in the literature [1]. The enzyme is active in acidic pH range and can be used for the preparation of L-rhamnose and prunin from ethanolic solution of orange fruit peel. The enzyme is specific for the conversion of naringin to prunin which is a pharmaceutically important bio available, rare compound of medicinal and food values.

Disclosure Statement

No potential conflict of interest was reported by the authors.

ACKNOWLEDGEMENTS

Dr. Sarita Yadav acknowledges the financial support of UGC, New Delhi in the form of a Post Doctoral Fellowship for Women no. PDFWM-2014-15-OB-UTT-23606.

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Table 1 Summary of purification procedure

Steps	Total Activity (IU)	Total Protein (mg)	Specific activity IU/mg	Purification fold	% yield
Culture filtrate	68.00	52.00	1.30	1	100
Concentration by Ultra filtration	42.09	4.58	9.20	7	62
Dialysis	41.80	4.03	10.40	8	61
Anion exchange chromatography on DEAE	16.58	0.52	31.88	24	24

Legends to the figures:

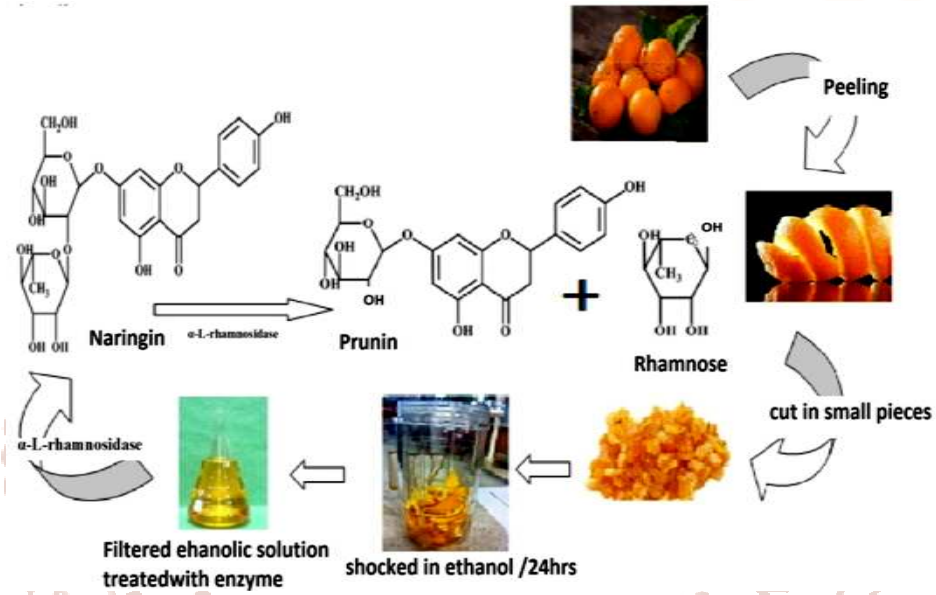
Scheme1: Enzymatic hydrolysis of naringin to pruning from orange peel.

Figure1. Elution profile of α-L-rhamnosidase from CM-cellulose column. Protein (●); activity (○); NaCl gradient (----).

Figure2. Results of (a) SDS-PAGE and (b) Native PAGE analysis of the purified enzyme:
A. Lane 1 purified enzyme 2.0µ g and lane 2 molecular weight markers.
B. Lane 1 ovalbumin (43.0 kDa) and tripsin (20.0 kDa). Lane 2 purified enzyme 2.0µ g.

Figure3. Results of the enzymatic hydrolysis of naringin to prunin by the purified enzyme:
A. lane 1 pure sample of narngin, lane 2 pure sample of naringenin and lane 3 product of enzymatic hydrolysis of naringin, Lane 4 orange peel naringin and lane 5 product of enzymatic hydrolysis of orange peel naringin purified by preparative TLC.
B. HPLC-Mass analysis result of the product prunin.

Figure4. Results of the release of *L*-rhamnose from enzyme treated orange peel sample.
A. Lane 1 pure L-rhamnose sample, lane 2 L-rhamnose purified from orange peel sample treated with pure enzyme by preparative TLC.
B. HPLC-Mass analysis result of the product L-rhamnose.



Scheme.1. Enzymatic hydrolysis of naringin to prunin from orange peel

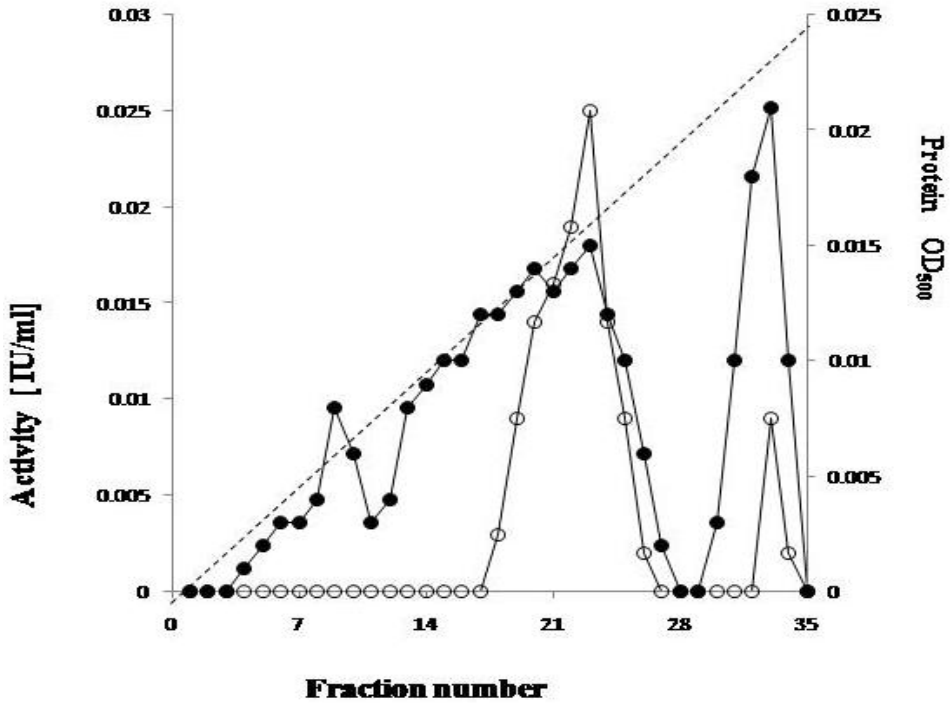
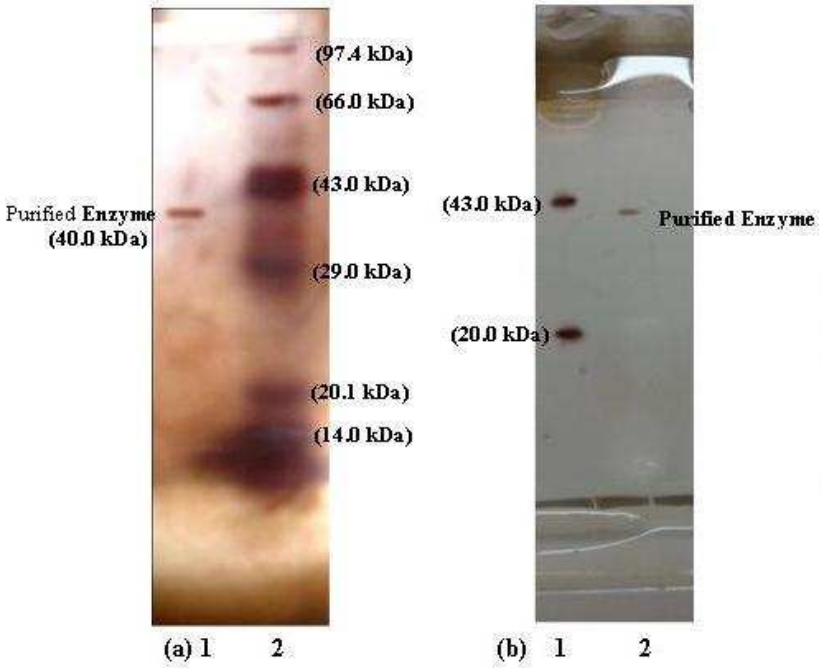


Fig.1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(66.0 kDa)
(43.0 kDa)
Purified Enzyme
(40.0 kDa)
(29.0 kDa)
(20.1 kDa)
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(a) 1
2
(43.0 kDa)
Purified Enzyme
(20.0 kDa)
(b) 1
2

Fig. 2

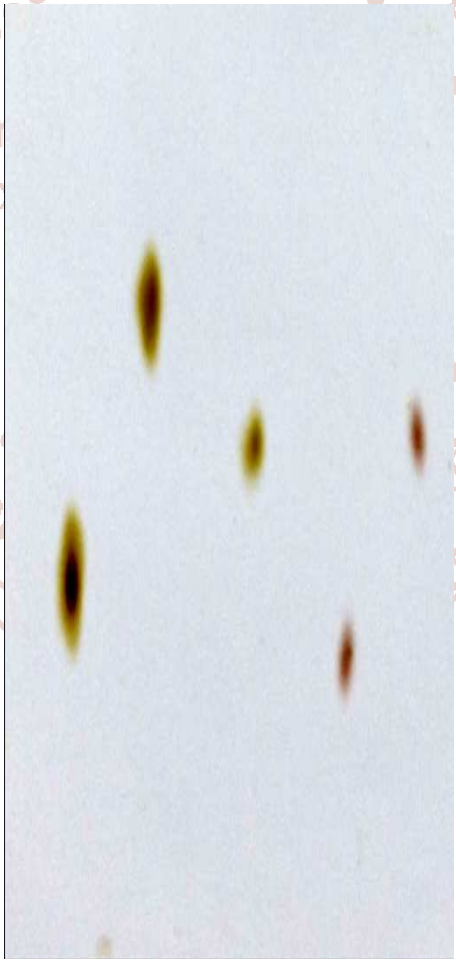
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Fig. 3 (A)

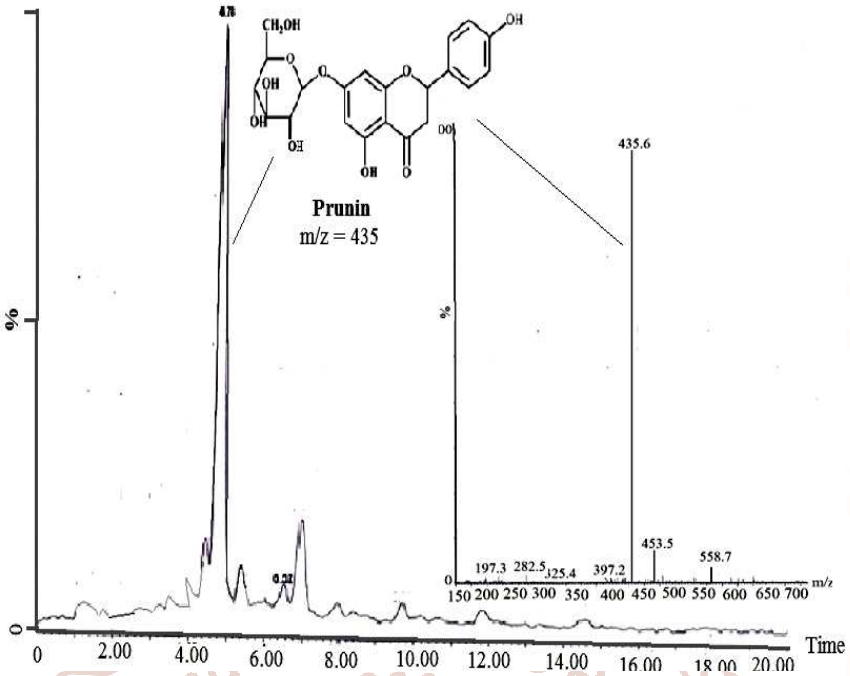
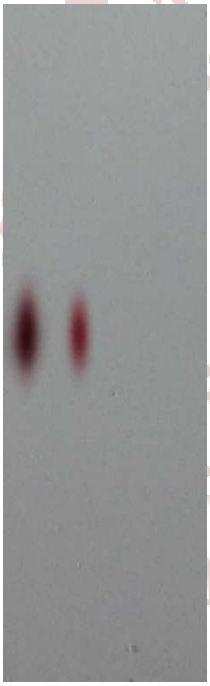


Fig 3 (B)



1 2 3

Fig4 (A)

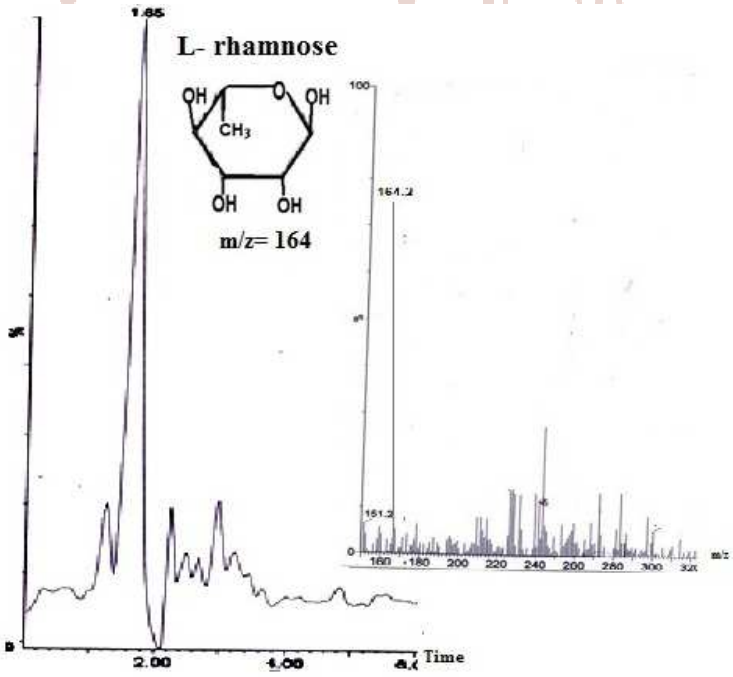


Fig.4 (B)