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SCREENING AND ISOLATION OF b - GLUCOSIDASES FROM SELECTED MEDICINAL AND AROMATIC PLANTS

Siddhartha Kumar Mishra[1,2] , Swati Tripathi[3], Neelam Singh Sangwan[1], Rajender Singh Sangwan[1, 4]

1. Metabolic and Structural Biology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O.-CIMAP, Lucknow - 226015 (U.P.) India. 2. School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar - 470003 (M.P.) India. 3.Vegetable Research Division, National Institute of Horticultural & Herbal Science, Rural Development Administration, Wanju-gun, Jeolabuk-do - 565852, Republic of Korea (4. Centre for Innovative and Applied Bioprocessing (an Autonomous Institute under Department of Biotechnology, Govt. of India), Mohali -140306 (PB), India.)

E-mail: siddharthakm@yahoo.com ; sangwan.lab@gmail.com

Glucosidase catalyzes the hydrolysis of a wide range of β-glucosides and complex β-1,4 linked gluco-conjugates. The enzyme is involved in various cellular metabolic processes and other biochemical and biotechnological processes. We screened a wide variety of medicinal and aromatic plants (MAPs) for β-glucosidase activity and comparatively reviewed the kinetic attributes of the enzyme. The enzyme was isolated from young leaves of the plants and subjected to enzyme assay using p-nitrophenyl -D-glucopyranoside (PNPG) as substrate. The isozyme profiling of enzyme was performed on native-PAGE and band scoring was analyzed. Results demonstrate that *Rauvolfia serpentina* showed highest enzyme activity (348 IU/gm f.w.t.) as compared to other MAPs screened in the study. Other five plants with high activity were *Lycopersicum esculentum, Withania somnifera, Physalis alkekengi, Mentha arvensis and Andrographis paniculata* with 192, 174, 142, 138 and 136 IU/gm f.w.t., respectively. *Ocimum basium* showed no enzyme activity. *Zea mays* showed highest specific activity (51.91) with 107 IU enzyme activity. The *in situ* localization of enzyme demonstrated a diverse distribution of β-glucosidase isozymes in MAPs. Total eight isozymes of β-glucosidase (GS-iso-I through VIII) were observed. GS-iso-I and VII were exclusively present one plant each. GS-iso-IV was diversely present in five MAPs, GS-iso-II and V present in four MAPs, GS-iso-VI was present in three aromatic plants, and GS-iso-III and VIII were present only in two plants each. Results of the study are highly important for biochemical and enzymatic studies in plant secondary metabolism. β-Glucosidases identified were found to be novel members of the family in terms of enzyme kinetics.

Keywords : β-glucosidase, glycosides, aglycone, medicinal and aromatic plants, enzyme kinetics, secondary metabolism

b-Glucosidase glucohydrolase (E.C. 3.2.1.21) commonly known as b-glucosidase catalyzes the hydrolysis of a wide range of b-glucosides including alkyl- & aryl-b -glucosides, as well as diglucosides and oligosaccharides (Esen 1993). The enzyme is localized in the lysosome of the cell, cleaves a glucose moiety from a substrate at b-1,4 linkage. The enzyme is widely used in biochemical and biotechnological processes such as degradation of cellulosic biomass, hydrolysis of glycolipids, defense against microbes by cyanogenesis (Dharmawardhana *et al*. 1995, Esen 1993, Jin *et al*. 2011, Poulton 1990). b-Glucosidase is also associated with important developmental functions such as floral development and pigmentation (Koes *et al*. 1994) and ABA metabolism (Matsuzaki and Koiwai 1986). b-Glucosidase also parts various roles in modification of secondary metabolites such as production of fuel ethanol from cellulosic agricultural residues (Xin *et al*. 1993), release of a wide variety of volatile compounds from their glucosidic precursors in fruit and vegetables (Estibalitz *et al*. 2001, Gueguen *et al*. 1996), conversion of storage form of cytokinin to its active form (Smith and van Staden 1978), development of some flavor compounds like monoterpenols, C-13 norisoprenoids and shikimate derived compounds accumulates in fruits as non-flavor precursor linked to mono- or di-glycosides before enzymatic hydrolysis (Vasserot *et al*. 1995, Winterhalter and Skouroumounis 1997). These monoterpenols, linalol, geraniol, nerol, citronellol, α-terpeneol, monoterpenol and linalol oxide are found linked to their diglycosides, contributed significantly to the flavor and fragrance in wine, which are the products of a series of enzymatic hydrolysis by an α-L-rhamnosidase, α-L-arabinosidase or a b-D-apiofuranosidase cleaves the (1 → 6) osidic linkage, and then, the flavor compounds are liberated from the monoglucosides by the action of a b-glucosidase (Gunata *et al*. 1988,

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Williams and Allen 1995).

Plant secondary metabolites are abundant in nature with wide varieties and functions. These metabolites occur in glycosylated (mainly glucosylated) form for vacuolar storage due to their enhanced water solubility. The aglycone part can be obtained from their gluco-conjugates by enzymatic process in various circumstantial and specific plant functions quickly. An overwhelming number of plant metabolites from various chemical and functional groups are obtained in gluconjugated forms such as terpenoids, flavanoids, coumarins, quinones, hydroxamic acid, rotenoids, alkaloids etc. (Akiyama *et al*. 1998, Poulton 1990, Svasti *et al*. 1999).

Therefore, understanding the catalytic and kinetic features of various β-glucosidases from medicinal and aromatic plants may elaborate the interesting functions in biochemical process in the plants. It can also be utilized for biotechnological applications in biotransformation and biocatalytic conversions. Glycosides and aglycones of secondary products occur in large number of plant tissues through enzymatic modifications. In this comparative study, we screened a wide variety of medicinal and aromatic plants (MAPs) for β-glucosidase activity and comparatively reviewed the kinetic attributes of the enzyme. Characterization of enzymes in plant secondary metabolism has pharmaceutical importance as well, thus, we performed this study on screening, isolation and enzymatic characterization of β-glucosidases from selected important MAPs.

MATERIALS AND METHODS

Plant Materials

MAPs studied in this study were grown at the experimental agricultural fields of CIMAP, Lucknow. Selected known MAPs with glycosides as secondary metabolites were screened for b-glucosidase activity. MAPs used in this study are: *Andrographis paniculata, Azadirachta indica, Bacopa monnieri, Boerhavia diffusa, Capsicum annum, Catharanthus roseus, Chlorophytum borivillianum, Chrysanthemum cinerariifolium, Citrus limon, Coriandrum sativum, Cymbopogon flexuosus, Cymbopogon martini, Cymbopogon winteranius, Eucalyptus citriodora, Lipia alba, Lycopersicon esculentum, Mentha arvensis, Mentha citrate, Ocimum basium, Physalis alkekengi, Rauvolfia serpentine, Solanum melongena, Stevia rebaudiana, Vetiveria zizanoides, Withania somnifera, Zea mays*.

Chemicals and Reagents

Chemicals used in the study were of highest purity grade. Sodium carbonate, citric acid, potassium dihydrogen orthophosphate, sodium chloride and ethylene diamine tetraacetic acid (EDTA) were obtained from Merck. Solutions and additives for enzyme extraction, assay substrates, protein separation were procured from Sigma (MO, U.S.A.) and Hi-Media laboratories.

Enzyme Isolation

Young leaves of the plants were harvested, washed with distilled water, dried on blotting paper and ground to fine powder in liquid nitrogen. The powder was homogenized in 100 mM potassium-phosphate buffer (pH 6.0) containing 10 mM b-mercaptoethanol, 2 mM EDTA with addition of 10% PVPP powder in homogenate prior to extraction. The tissue homogenate was centrifuged at 12,000×g for 30 min at 4°C and the clear supernatant was collected. All the enzymatic procedures were performed at 4°C unless specified otherwise.

b-Glucosidase Assay

Enzyme activity was assayed using the method as described earlier with minor modifications (Stevens *et al*. 1993). The assay mixture in a total volume of 200 L contained 2.5 mM p-nitrophenyl b-D-glucopyranoside (PNPG) substrate in 100 mM citrate-phosphate buffer (pH 5.0) with 20 mL of enzyme extract at 37°C. After 15 min of incubation time the reaction was stopped by adding 800 mL of 1 M sodium carbonate and the absorbance of p-nitrophenol (the product of reaction) was measured at wavelength 405 nm using spectrophotometer.

Calculation of Enzyme Units

The enzyme activity was expressed in terms of mole of p-nitrophenol formed per minute using the molar extinction coefficient (ε°) 18,350 m^{-1} cm^{-1}. In accordance with the Nomenclature Committee of the International Union of Biochemistry, "µmole substrate formed per minute" was represented as international unit (IU) in this study.

***In situ* localization of b-glucosidase on native-PAGE**

Native-PAGE was performed on continuous gel (7.5cm x 10cm x 0.75mm) on Mini Protean system II (BioRad). The composition of gel was 10% acrylamide and 0.1% bisacrylamide (ratio 100:1) in 375 mM Tris buffer (pH 8.8) without addition of SDS. To achieve polymerization, the mixture was made 0.1% ammonium persulfate and 0.1% TEMED prior to pouring the solution in casting cassettes. Total 25µg protein was loaded on the gel and run in electrode buffer (24 mM Tris and 186 mM glycine) at 90 v for 2 h at 4°C, using bromophenol blue as tracking dye in a lane. The staining of gel was carried out by the method as described earlier with minor modifications (Esen 1992, Fieldes and Gerhardt 1994). Briefly, the native-gel was washed with 50 mM citrate-phosphate buffer (pH 5.5) and transferred to a staining system containing 1.3 mM 6-bromo-2-naphthyl -D-glucopyranoside as chromogenic substrate and 1.9 mM Fast Blue BB salt (coupling dye) in 100 mM citrate-phosphate buffer (pH 5.5). Placed the gel in covered glass tray in dark at 37°C for 18 hour, then gel was placed in fixing solution containing acetic acid : methanol : water (1:1:5) for 24 h.

Protein Estimation

Protein estimation in samples was done by modified Lowry method using bovine serum albumin as reference standard.

RESULTS AND DISCUSSION

This study aimed to elucidate the catalytic and kinetic features of β-glucosidases from a wide variety of medicinal and aromatic plants. We performed the screening, isolation and enzymatic characterization of β-glucosidases from selected MAPs. The enzyme has metabolic relevance thus a comparative review of the enzyme characteristics and secondary metabolites of the screened plants was performed.

Screening and isolation of β-glucosidases from selected MAPs

A large number of plants with medicinal and aromatic values were screened and total 26 plants were selected for isolation of β-glucosidase. We isolated β-glucosidase from young leaves of MAPs by homogenization in isolation buffer followed by centrifugation and collection of supernatant, as described in Materials and Methods. The supernatant was used to assay β-glucosidase enzyme activity by the method as described earlier (Stevens *et al*. 1993). The estimated enzyme units of β-glucosidase in MAPs showed interesting activity profile, as described in Table 1.

Rauvolfia serpentina, Lycopersicon esculentum, Withania somnifera, Physalis alkekengi, Mentha arvensis and *Andrographis paniculata* showed high range of enzyme activity. *Zea mays, Chrysanthemum cinerariifolium, Solanum melongena, Coriandrum sativum, Catharanthus roseus, Capsicum annum* and *Chlorophytum borivillianum* showed middle range of enzyme activity. A very low activity range was found in *Cymbopogon martinii, Stevia rebaudiana, Azadirachta indica, Eucalyptus citriodora, Cymbopogon winteranius, Mentha citrata, Boerhavia diffusa, Cymbopogon flexuosus, Vetiveria zizanoides, Bacopa monnieri, Lipia alba* and *Citrus limon. Ocimum basium* showed no enzymatic activity. Observations based on specific activity made our results more interesting; *Zea mays, Andrographis paniculata, Rauvolfia serpentina, Chlorophytum borivillianum* and *Withania somnifera* showed very high specific activity. A medium higher range of specific activity was observed in *Chrysanthemum cinerariifolium, Capsicum annum, Stevia rebaudiana, Physalis alkekengi, Mentha arvensis, Cymbopogon*

Table 1. β-glucosidase activity profile in selected medicinal and aromatic plants.

S. N.	Plant	Activity (IU/ml)	Activity (IU/gm f.w.t.)	Protein (mg/gm f.w.t.)	Specific Activity
1	*Andrographis paniculata*	272	136	3.306	41.13
2	*Azadirachta indica*	23	19	6.222	3.05
3	*Bacopa monnieri*	11	4	0.621	6.44
4	*Boerhavia diffusa*	20	12	2.072	5.79
5	*Capsicum annum*	218	66	2.362	27.94
6	*Catharanthus roseus*	120	72	4.603	15.64
7	*Chlorophytum borivillianum*	101	62	2.004	30.93
8	*Chrysanthemum cinerariifolium*	265	106	3.766	28.14
9	*Citrus limon*	7	4	0.307	13.02
10	*Coriandrum sativum*	78	86	10.34	8.21
11	*Cymbopogon flexuosus*	14	11	1.407	7.81
12	*Cymbopogon martinii*	33	25	1.384	18.06
13	*Cymbopogon winteranius*	29	16	0.793	20.17
14	*Eucalyptus citriodora*	34	17	5.592	3.04
15	*Lipia alba*	7	4	2.868	1.39
16	*Lycopersicon esculentum*	160	192	13.36	14.37
17	*Mentha arvensis*	138	138	5.938	23.24
18	*Mentha citrata*	20	15	4.672	3.21
19	*Ocimum basium*	00	00	3.802	00
20	*Physalis alkekengi*	158	142	6.099	23.28
21	*Rauvolfia serpentina*	290	348	8.917	39.02
22	*Solanum melongena*	149	102	6.184	16.49
23	*Stevia rebaudiana*	23	19	0.760	25.00
24	*Vetiveria zizanoides*	36	11	0.840	13.06
25	*Withania somnifera*	290	174	5.967	29.16
26	*Zea mays*	158	107	2.061	51.91

f.w.t., fresh weight tissue

winteranius, Cymbopogon martinii, Solanum melongena and *Catharanthus roseus*. The lowest specific activity range was observed in *Lycopersicon esculentum, Vetiveria zizanoides, Citrus limon, Coriandrum sativum, Cymbopogon flexuosus, Bacopa monnieri, Boerhavia diffusa, Mentha citrata, Azadirachta indica, Eucalyptus citriodora* and *Lipia alba* (Table 1).

According to the assay conditions, *R. serpentina* showed highest enzyme activity (348 IU/gm f.w.t.) as compared to other screened MAPs. Then five plants with highest enzyme activity were *L. esculentum, W. somnifera, P. alkekengi, M. arvensis* and *A. paniculata* with 192, 174, 142, 138 and 136 IU/gm f.w.t., respectively. These values represent the enzyme activity in plants and any direct correlation may not be drawn because of their diverse secondary metabolites. The secondary metabolites profile of screened MAPs containing the list of glycosides and aglycone moieties is described in Table 2. Among screened MAPs, *O. basium* showed no activity which may be due to either low abundance or inhibition of enzyme activity in isolates (Table 1). *O. basium* contains high composition of oily molecules mainly monoterpenes yet glycosides are non-specified in the plant which may another reason of low enzyme activity (Table 2.).

The comparative account of β-glucosidase activity (IU/gm f.w.t) and specific activity (IU/mg f.w.t. protein) show that *R. serpentina* contained highest enzyme activity (348 IU) with slightly lower specific activity (39.02). *Z. mays* showed highest specific activity (51.91) with 107 IU enzyme activity (Table 1). Again, these enzymatic and proteomic attributes are not comparable within plants due their different ecological, physiological and functional properties. However, these results are highly important for understanding the kinetic characteristics of β-glucosidase and biochemical and enzymatic studies in plant secondary metabolism. The MAPs with high enzymatic activities may be of great importance in industrial application of enzyme for synthesis and modification of glycosides of secondary metabolites.

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***In situ* localization of β-Glucosidase and isozyme profiling**

The *in situ* screening of b-glucosidase activity on native-PAGE performed from selected MAPs showed very interesting and diverse results with reference to the isozymic profiling (Fig. 1). A constant activity of enzyme was observed on repeated gel staining with low and high substrate concentrations from 18 medicinal and aromatic plants out of selected 26 plants. We observed only one isozyme band in 14 plants : *W. somnifera, R. serpentina, C. borivillianum, P. alkekengi, C. roseus, C. martinii, M. citrata, C. limon, S. rebaudiana, S. melongena, L. esculentum, C. annum, C. cinerariifolium* and *Z. mays*. Four plants contained two bands: A. paniculata, *V. zizanoides, E. esculentum* and *O. basium*. It's

Table 2. Glycone and aglycone pairs of screened medicinal and aromatic plants.

S.N.	Plant	Glycone Moieties	Aglycone Moieties
1	*Andrographis paniculata*	Andrographiside	Andrographolide & Neoandrographolide
2	*Azadirachta indica*	Flavonol-*O*-glycosides	Azadirachtin
3	*Bacopa monnieri*	Bacosides-A, -B	Brahmine, Herpestatine & Bacopin
4	*Boerhavia diffusa*	Nonspecific glycosides	Flavonoids, Tannins & Saponins
5	*Capsicum annum*	Steroidal glycosides	Capsicine & Capsanthine
6	*Catharanthus roseus*	Strictosidine	Vincristine, Vinblastine & Ajmalicine
7	*Chlorophytum borivillianum*	Spirostanol glycoside	Hecogenin & Tigogenin
8	*Chrysanthemum cinerariifolium*	Triterpene glycosides	Pyrethrins-I, II & Cenerins-I, II
9	*Citrus limon*	Flavonoid glycosides	Citral & Limonene
10	*Coriandrum sativum*	Monoterpenoid glycosides	Decanal & Linalool
11	*Cymbopogon flexuosus*	Flavonoid glycosides	Citral & Geraniol
12	*Cymbopogon martinii*	Flavonoid glycosides	Geraniol, Geranyl acetate
13	*Cymbopogon winteranius*	Flavonoid glycosides	Citronellol, Citronellyl acetate, Geraniol & Geranyl acetate
14	*Eucalyptus citriodora*	Cynogenic phenol glycosides	Citronellol, Citronellal, Geraniol & Geranial
15	*Lipia alba*	Flavonoid glycosides	Linalool
16	*Lycopersicon esculentum*	Tomatine (steroid alkaloid glycoside)	Lignin & Lycopene
17	*Mentha arvensis*	Flavonoid glycosides	Menthol, Menthyl acetate & Menthone
18	*Mentha citrata*	Flavonoid glycosides	Linalool & Linalyl acetate
19	*Physalis alkekengi*	Steroid and alkaloid glycoside	Physalin-A,B,C
20	*Rauwolfia serpentina*	Strictosidine	Reserpine, Raucaffricine & Ajmaline
21	*Solanum melongena*	Steroidal sapogenins	Polyphenols, Tannins & Alkaloids
22	*Stevia rebaudiana*	Stevioside & Rebaudioside A–E	Sterols, Flavonoids, Di & Tri-terpenes
23	*Vetiveria zizanoides*	Flavonoid glycosides	Citronella & Laurel
24	*Withania somnifera*	Sitoindosides VII-X & Glycowithanolides	Withaferin & Withanolides
25	*Zea mays*	Flavone *C*-glycoside	Fatty oils
26	*Ocimum basium*	Nonspecific Glycosides	Linalool & Eugenol

interesting that *O. basium* showed two bands even though no enzyme activity detected by spectrometric assay. This indicates that *O. basium* might have different conditions of enzyme kinetics than utilized in this study.

The band intensity analysis was performed and a plot was drawn from the measurement of bands on gel. The bands visualized were sharp and prominently marked except in some samples. The migration distance of bands was measured from top to bottom on a 7 cm gel (Fig. 1). First isozymic band designated as GS-iso-I, located at least migration distance (0.5 cm), was present only in *A. paniculata*. The second isozyme band, GS-iso-II, was prominently marked in four plants *W. somnifera, R. serpentina, C. borivillianum* and *P. alkekengi* at migration distance of 1.2 cm. Third isozyme band, GS-iso-III, was located only in two plants *P. alkekengi* and *C. annum* at 1.6 cm migration distance. Fourth isozyme band GS-iso-IV was located at distance 2.2 cm was diversely marked in *C. martinii, S. rebaudiana, S. melongena, L. esculentum* and *C. cinerariifolium*. Fifth isozyme band GS-iso-V was also diversely present in *M. citrata, C. limon, C. roseus* and *Z. mays* at 2.5 cm migration distance. Sixth isozyme band, GS-iso-VI, was visualized in three aromatic plants only *V. zizanoides, O. basium* and *E. citriodora*. Seventh isozymic band, GS-iso-VII, was uniquely present only in aromatic plant *E. citriodora* at 3.2 cm migration distance. Eighth isozymic band, GS-iso-VIII, was present only in two aromatic plants *V. zizanoides* and *O. basium* at longest migration distance (3.5 cm). Although some of GS-isozyme bands were different in intensity, their correlation could not be drawn yet. Also some plants showed no GS-isozyme band: *B. diffusa, B. monnieri, C. flexuosus, C. winteranius, M. arvensis and L. alba; and A. indica* and *C. sativum* showed severe streaking on gel.

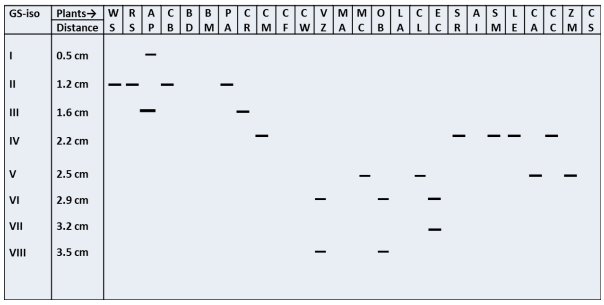

Figure 1. *In situ* β-glucosidase activity localization on native-PAGE and scoring of bands for isozyme profiling.

Based on above observation, a diversity analysis of GS-isozymes was performed which indicates that eight isoforms of -glucosidase (GS-iso-I through VIII) were diversely localized in MAPs. Among them, GS-iso-I and VII was exclusively present one plant each, which might have specific genotype of β-glucosidase gene expression. GS-iso-IV was most diverse isozyme (present in five MAPs) followed by GS-iso-II and V (diversely present in four plants). GS-iso-VI was diversely present in three aromatic plants and Gs-iso-III and VIII were present only in two plants each. The diversity of GS-isozymes indicate a wide range of molecular weight of enzyme distribute in MAPs. This information has high relevance in biochemical and proteomic studies for deciphering the structural and functional characteristics of the enzyme.

A comparative account of β-glucosidase from MAPs is represented in Table 3. We have earlier isolated, purified and characterized β-glucosidase from *Rauvolfia serpentina* and the process of extraction was patented (Sangwan *et al*. 2007). Strictosidine β-glucosidase from *R. serpentina* is important in generation of other aglycones such as synthesis of vomilenin from raucaffricine glucoside. We have recently performed isolation, purification, and kinetic characterization of β-glucosidases from medicinal plants: *Withania somnifera* (Solanaceae) (Mishra *et al*. 2013a), *Silybum marianum* (Asteraceae) (Mishra *et al*. 2013b),

and *Andrographis paniculata* (Acanthaceae) (Mishra *et al*. 2013c). The catalytic attributes of the enzyme were apparently novel with respect to its activity and preferences from a medicinal plant resource. A comparative account of structural and kinetic properties of β-glucosidases is discussed in Table 3.

Table 3. Comparative enzyme kinetics of β-glucosidase from medicinal and aromatic plants

Plant	Subunit MW (kDa)	pH optima	Temp. optima	*Km* (mM)	Reference
Withania somnifera	52	4.8	40	0.19	(Mishra et al 2013a)
Silybum marianum	67.6 (homodimer)	5.5	40	0.25	(Mishra et al 2013b)
Andrographis paniculata	60	5.5	55	0.25	(Mishra et al 2013c)
Dalbergia cochinchinensis	66 (homo-pentamer)	5.0	Nd	5.4	(Srisomsap et al 1996)
Catharanthus roseus	63	6.0	50	0.02	(Luijendijk et al 1998)
Citrus sinensis	55/65	4.5	60	0.27	(Barbagallo et al 2007)
Vigna radiate	95	6.5	37	Nd	(Zeng & Elbein 1998)
Manihot glaziovii	63	6-6.5	55	0.3	(Eksittikul & Chulavatnatol 1988, Yeoh & Wee 1994)
Passiflora foetida	nd	5.5	57	3.89	(Yeoh & Wee 1994)
Prunus avium	68	5.5	60	3.3	(Gerardi et al 2001, Yeoh & Wee 1994)
Malus domestica	60 (homodimer)	6.0	70	1.2	(Yu et al 2007)
Secale cereale	60 (homo-pentamer)	5.0-5.5	25-30	0.9	(Sue et al 2000)
Dalbergia nigrescens	62-63 (hetero-tetramer)	5.0-6.0	65	14.7	(Chuankhayan et al 2005)
Zea mays	60 (monomer)	5.8	50	0.64	(Esen 1992)

nd, not determined

CONCLUSIONS

This study on the screening, isolation and profiling of β-glucosidases from medicinal and aromatic plants demonstrates the catalytic and functional features of the enzyme as well as distribution of isozymes. A comparative review of the data from previous reports on the line emphasizes the importance of the enzyme in transformation of secondary metabolites in plants. Results demonstrate that *R. serpentina* showed highest enzyme activity (348 IU/gm f.w.t.) as compared to other plants screened. Five other plants with high activity were *L. esculentum, W. somnifera, P. alkekengi, M. arvensis* and *A. paniculata* with 192, 174, 142, 138 and 136 IU/gm f.w.t., respectively. The isozyme profiling of β-glucosidase was performed which showed total eight isozymes diversely distributed in selected MAPs. Among screened MAPs, *O. basium* showed no enzyme activity in assay but showed isozyme profile. The enzyme characteristic attributes hold potential to develop a library of catalytically active members of the hydrolase family from plants. The enzyme has potential to be used in biotransformation applications for

modification of metabolites.

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