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Isolation, Purification and Characterization of β-Glucosidase from *Rauvolfia serpentina*

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Abstract

β-Glucosidase (EC 3.2.1.21) was extracted from different parts of *Rauvolfia serpentina* and was purified using ammonium sulphate fractional precipitation and Sephadex G-25 chromatography. The molecular weight of different bands in *Rauvolfia serpentina* was found in the range of 19.4KDa to 92.257KDa .The enzyme β-glucosidase has optimum pH 5.0 and the optimum temperature was found at 60ºC while the thermal stability of enzyme β-glucosidase was found to be 30ºC. The activation with $FeSO_4$ is due to Fe^{++} ions because $FeCl_3$ do not activate the enzyme.

Keywords: *Rauvolfia serpentine*; β-glucosidase; SDS PAGE; Enzyme activity

Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) comprises a heterogeneous group of enzymes that are able to cleave the β-glucosidic linkages of di- and/or oligosaccharides, or other glucose conjugates. β-Glucosidases are widely distributed and play pivotal roles in many biological processes, such as degradation of cellulosic biomass, hydrolysis of glycolipids, cyanogenesis, and modification of secondary metabolites [10]. In plants, β-glucosidase activity is involved in processes such as the compartmentalization and activity of phytohormones [17,18], defense mechanisms against microbes, insects, or parasitic plants [23], and they are also thought to have roles in lignification and cell wall decomposition. Several β-glucosidases of plant origin are highly substrate-specific, such as those having selective specificity toward saponin [16], hydrojuglone [9], cinnamyl alcohols [8], flavones [22] and isoflavones [15] β-Glucosidase occurs abundantly in young plants parts and localized in plastids [12].

Materials and Methods

The *Rauvolfia serpentina* was used as β -glucosidase source and was obtained from SHIATS, Allahabad. Plants were freshly harvested for use and processed immediately to avoid tissue breakage and loss of enzyme of activity. For the purification of β-glucosidase, ammonium sulfate, D-gluconolactone, imidazole, iodoacetate, EDTA, FCC reagent, TCA, paranitrophenol, β-D-glucopyranoside, standard BSA, acrylmide, bisacryl and glycine, TEMED, ammonium per sulfate were used.

Sephadex G-25 column chromatography

Pellets obtained after ammonium sulphate precipitation (30-70%) were dissolved in elution buffer and loaded on sephadex G. 25 column and fractions of volume 2 ml each were collected. The reading of protein was measured by spectrophotometer at 280 nm and activity was also estimated at 405 nm.

Electrophoretic methods

Crude enzyme preparations and chromatographic fractions were subjected to native PAGE. Samples were electrophoresed through 0.75 mm thick gel slabs (2cm 4% staking gel, pH 8.5 and 13.5 cm 10% resolving gel pH 8.8) using the alkaline gel system. The running buffer was 3.5 gm Tris and 14 gm Glycine, pH 8.3. For SDS PAGE, enzyme SDS buffer were mixed in ratio (4:1) v/v. Samples were heated in boiled water for 5 min. and were chilled in liquid nitrogen and. Thereafter, surface was loaded and electrophoresis was performed as described by [19].

Results and Discussion

The enzyme from each part of *Rauvolfia serpentina* was extracted and activity was estimated using spectrophotometer at wave length 405 nm. For the reaction, $_p$NPG was used as substrate. The reaction mixture was incubated for 15 min at room temperature. Change in OD was calculated and the activity was estimated (Table 1).

The activity of crude enzyme in *Rauvolfia serpentina* was observed in per gram. From data shown in Figure 1, it was observed that mature leaf 1 has the highest activity of crude enzyme in *Rauvolfia serpentina*. This indicates the higher requirement of β-glucosidase mediated reaction in corresponding plant parts.

Purification of β-glucosidase from crude protein extract

β-glucosidase was purified from crude protein extract by Ammonium sulfate precipitation and Sephadex G-25 column chromatographic procedure.

1. **Ammonium sulfate Precipitation:** $(NH_4)_2SO_4$ was mixed to crude enzyme under chilled conditions and kept for 2 hrs in refrigerator to develop the precipitate. There after centrifugation was done at 12,000 rpm, 4ºC for 10 minutes and precipitate was discarded and supernatant processed further. For 0 to 30 % precipitation, weight of $(NH_4)_2SO_4$ = 7.79 gm, Volume of supernatant = 49 ml, for 30 to 70% precipitation,

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weight of $(NH_4)_2SO_4$ =12.201 gm, after centrifugation, pellet obtained was used for further process study.

2. Sephadex G -25 Column Chromatography: Pellet was dissolved in elution buffer and after loading protein on pre-equilibrated column was run and fractions were collected of volume 2 ml each, measured absorbance of protein at 280 nm and enzyme activity at 405 nm was estimated to be 60° C.

Characterization of enzyme

Molecular weight estimation of proteins in *Rauvolfia serpentina* from SDS gel: Total number of bands were recorded as per their distance migrated and Molecular weight determined from SDS gels of *Rauvolfia*. Graph was plotted for *Rauvolfia serpentina* (Figure 2).

In each plant, distance of marker was used as x-axis while log molecular weight was used as y-axis. Plant parts were selected in which there were a maximum number of bands. For each band distance was measured and logs molecular weight and molecular weight for each band was calculated. Total number of bands for each parts of plant was also calculated (Figure 3).

The SDS PAGE developed was analyzed to the protein bands and their molecular weight was determined in comparison to standard molecular weight marker. In *Rauvolfia serpentina* the maximum number of bands of protein was found to be five in fruit, and the minimum number of bands of protein was found one in old leaf and stem. The molecular weight of different bands in *Rauvolfia serpentina* was found in the range of 19.48 KDa to 92.257 KDa. (Table 2).

S. No.	Sample	Change in OD/10 µl	Units/ml/min	Activity (%)
1.	Fruit	0.03	200	118
2.	Flower	0.165	1100	64.7
3.	Flower stem	0.061	400	23.5
4.	Very young leaf	0.240	1600	94.1
5.	Young leaf	0.076	500	29.4
6.	Mature leaf 1	0.256	1700	100
7.	Mature leaf 2	0.095	633	37.2
8.	Old leaf	0.132	880	51.8
9.	Stem	0.035	230	13.5
10.	Root	0.025	160	9.4

Table 1: β glucosidase activity profile in *Rauvolfia Serpentina*

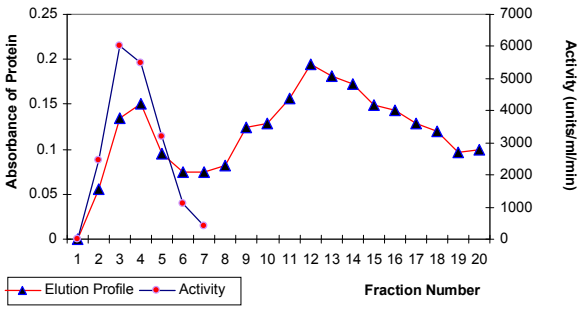

Figure 1: Elution profile and activity of enzyme in sephadex G-25 column chromatography.

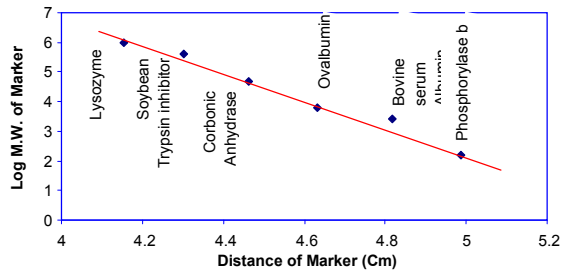

Figure 2: Molecular weight estimation of proteins in *Rauvolfia serpentina* from SDS gel.

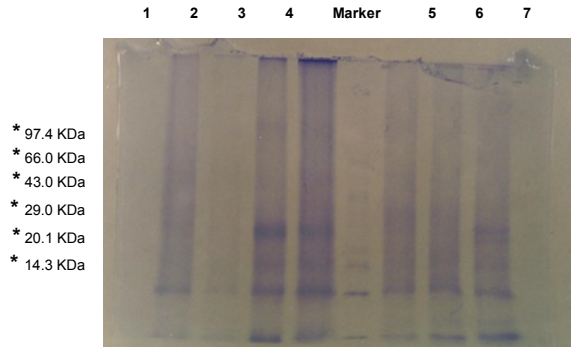

Figure 3: SDS PAGE gel of *Rauvolfia serpentina* in the presence of β mercaptoethanol.
Lane1 - stem, Lane2 - old leaf, Lane3 - mature leaf, Lane4 - young leaf, Lane5 - flower stem, Lane6 - flower, Lane7 - fruit.

S. No.	Log M.W. of marker	M.W. of marker (KDa)	Name of marker	Distance of marker (Cm)	Distance of protein band (Cm)	Log M.W. of protein	M.W. of protein (Da)
1.	4.988	97.4	Phosphorylase b	2.2	2.3	4.965	92,257.14
2.	4.819	66	Bovine serum Albumin	3.4	3.5	4.720	52,480.75
3.	4.633	43	Ovalbumin	3.8	4.1	4.595	39,355.01
4.	4.462	29	Corbonic Anhydrase	4.7	4.4	4.535	34,276.78
5.	4.303	20.1	Soybean Trypsin inhibitor	5.6	5.1	4.39	24,547.09
6.	4.155	14.3	Lysozyme	6.0	5.6	4.29	19,498.45

Table 2: Molecular Weight from SDS (Coomasie Staining) gel of *Rauvolfia serpentina*

Determination of optimum temperature

The pH 5.0 buffer was used for activity measurement using 20 mM substrate at temperature range 0 to 80°C. Thus optimum temperature was estimated. It is interesting to note that in case of *Rauvolfia serpentina* flower the β- glucosidase is active even beyond 60°C temperature (Figure 4). It is not so rare observation yet quite intriguing as it reflects its much stronger regulation at the cellular level, where increase in temperature at such a high level seems unlikely. On the other hand, it makes the reaction quite selective, if we extrapolate these results to in-vivo scenario. The optimum temperature in different

plants were as Strawberry 60°C [24], *Leuconostoc mesenteroides* 50°C [6], Grape 45°C [20], Soybean 30°C [25] and maize 50°C [10].

Determination of optimum pH

After studying the kinetics, it was concluded that optimum pH was found in range of 4.5 to 5.0 as shown in Figure 5. The optimum pH for catalyzing β-glucosidase action of different plants ranged as strawberry – 4.0 [3], Lodgepole Pine - 5 to 6 in variety of substrate [8], *Leuconostoc mesenteroides* – 6.0 [2], *Dalbergia cochinchinensis* Thai Rosewood –5.0 [27], Grape –5.0 [1], soybean -6.0 [14], Maize –5.8 [10].

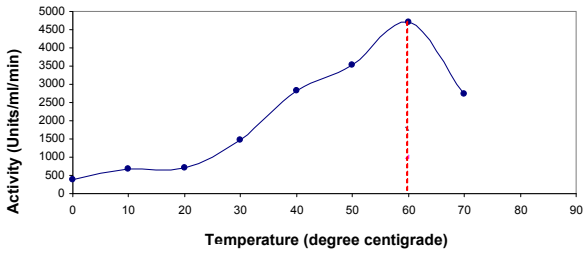

Figure 4: Determination of temperature optima for β-glucosidase.

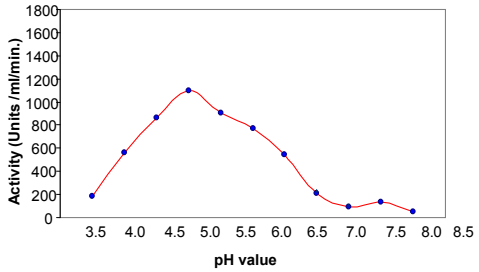

Figure 5: Determination of pH optima for β-glucosidase.

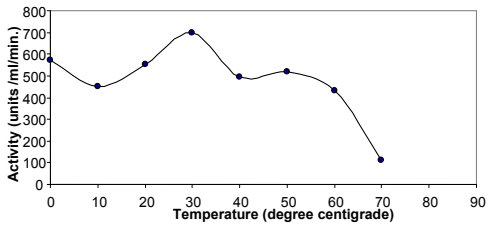

Figure 6: Thermal stability of enzyme.

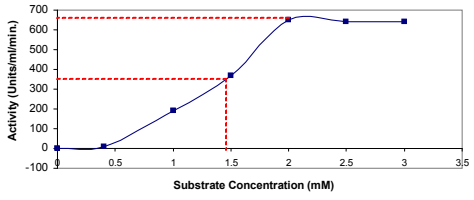

Figure 7: Effect of substrate concentration on β-glucosidase.

Determination of thermal stability

Thermal stability of β-glucosidase was determined. It showed highest activity display at temperature 60°C, while the activity was approximately 50% at temperatures below and above it. Interestingly, a substantial proportion (60%) remained even at 60°C, and around 20% even at 70°C as shown in Figure 6. It was clear that enzyme β-glucosidase has pH optima 5.0. The optimum temperature was found at 60°C (Figure 6). Thermal stability of enzyme β-glucosidase was found to be 30°C.

Effect of varying concentration of the substrate

The substrate para nitro phenyl β-D glycoside was used for measuring the effect of varying concentration of substrate. It was observed that β-glucosidase has V_{max} = 680 units / ml /min. and Km = 1.4 mM as shown in Figure 7. From the Michaelis - Menten curve, it was observed that β-glucosidase has Km= 1.4 mM and V_{max} = 680 units /ml/min (Figure 7). In *Catharanthus roseus* Km< 20mm and V_{max} = 243 nkatal per mg protein [21] In *Leuconostic mesenteroides* Km values were 3.7m $mole^{-1}$ using linamarin as substrate while it was 0.07 m $mole^{-1}$ using pN glycopyranoside substrate [13], while Km were 5.4 mM with pNPG substrate in *Dalbergia cochinchinensis* Thai Rosewood [27] In some other plants species, Km and V_{max} were known as following strawberry – Km = 18.5 mM, V_{max} = $474x10^{-6}$ IU/ml [7], papaya –Km = 0.11mM, V_{max} =$9.51x10^{-6}$ IU/ml. [26], grape –Km= 1.81 mM, V_{max} = $43.9x10^{-6}$ IU/ml [5] and *Rauvolfia serpentina*, for Raucaffricine glucosidase Km = 1.3 mM, V_{max} =0.5n Kat /µg protein and for storictosidine glucosidase –Km= 1.8mM, V_{max}= 2.6p Kat/ µg protein [11]. In our study of kinetics, it was observed that D-glucono lactone highly inhibited the enzyme. The same result was concluded from some other plants *Dalbergia cochinchinensis* Thai Rose wood [27], grape [20]. In present study it was also found that D-glucono lactone is a strong inhibitor for glucosidase activity. It also implies that D-gluconolactone could be a completive inhibitor of the enzyme i.e., occupying the active site in place of substrates.

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