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β-Hexosaminidase, an enzyme from ripening bell capsicum (*Capsicum annuum* var. *variata*)

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Abstract

β-Hexosaminidase activity increased significantly during fruit development and ripening of bell capsicum (*Capsicuum annuum* var. *variata*). Three isoforms of β-hexosaminidase from bell capsicum could be resolved upon ion exchange chromatography with step wise gradient (0.10, 0.15 and 0.20 M NaCl) having an abundance of 38, 47 and 15% for isoforms I, II and III respectively. Isoforms I and II were further purified on gel permeation chromatography. The pH optimum for these two isoforms was around 5. Isoform II exhibited higher thermal stability. Hg^{2+} and Zn^{2+} inhibited both, but isoform I showed a much higher inhibition by Cu^{2+} also. The K_m for isoforms I and II with pnp-β-D-*N*-acetyl glucosamine pyranoside was 3.00 and 1.75 mM, respectively. Isoform II on SDS–PAGE was found to be a monomer with a relative molecular mass of 85 kD. This isoform (the most major) appeared to be electrophoretically homogeneous. β-Hexosaminidase is novel in the context of fruit ripening. This enzyme has not been reported from fruits and studied hitherto.

Keywords: *Capsicum annum* var. *variata*; Solanaceae; Bell capsicum; β-Hexosaminidase

1. Introduction

β-Hexosaminidase, so far has been studied only in microbial and animal systems, where amino sugars are commonly present (Rankema et al., 1995; Sakamoto et al., 1998). Recently some microbial systems were reported to have a high activity of β-hexosaminidase, which were purified and studied (Keyhani and Roseman, 1996). β-Hexosaminidase is implicated in signal transduction, cell division and cell integrity in animal systems (Sokamoto et al., 1998; Roseman, 1991). In microbial system, this enzyme is known to play an important role in host-pathogen interaction (Chitlaru and Roseman, 1996; Roseman, 1991). Among higher plants, tomato fruit and leaves were shown to contain free *N*-glycans, which were absent in roots and stems (Priem et al., 1993).

Recently the profile of different glycosidases was checked in solanaceous fruits, interestingly β-hexosaminidase activity was found to be significantly higher than other glycosidases. Such a high activity of β-hexosaminidase in bell capsicum and other fruits tested (in our laboratory) and its increasing activity during fruit development followed by a further increase during ripening was noted to be striking. It was thought important to study this enzyme in the context of fruit ripening and to look into the nature of this enzyme in bell capsicum for a further study on its role in development and fruit ripening. This is the first report on β-hexosaminidase purification in fruit systems. Among other higher plant systems, the enzyme was purified and studied only in two tissues viz.: cabbage and lettuce (Chang et al., 1998; Pocsi et al., 1990). This enzyme is not well established and reported from higher plants.

2. Results and discussion

Fig. 1 shows an increased β-hexosaminidase activity during developmental stages followed by a significant rise in activity during ripening in bell capsicum. The purification data of β-hexosaminidase are detailed in Table 1. The purification profile of the same on DEAE-cellulose followed by Sephadex G-200 are depicted in Figs. 2 and 3, respectively. Ion exchange chromatography resolved the β-hexosaminidase activity into three distinct fractions

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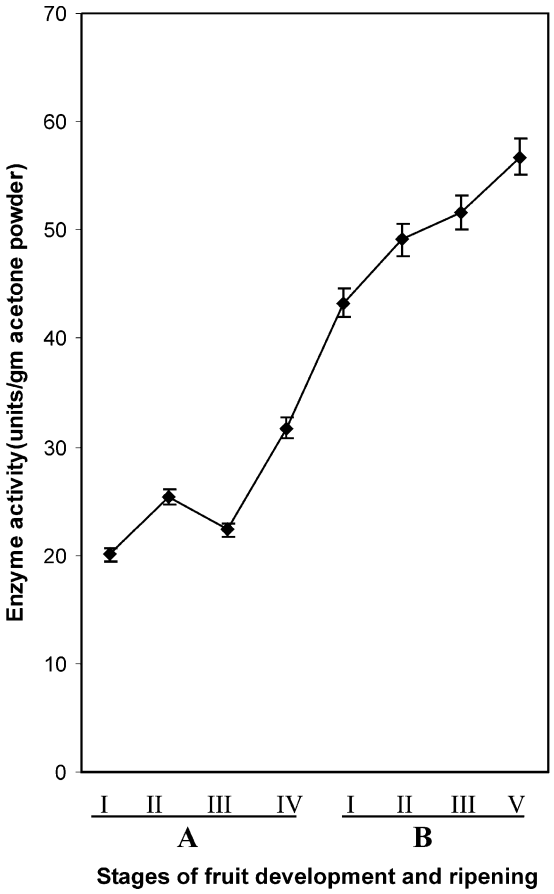

Fig. 1. β-Hexosaminidase activity during fruit development (A) and ripening (B) in bell capsicum.

elutable with 0.10, 0.15 and 0.20 M NaCl with a relative abundance of 38, 47 and 15% respectively. Based on elution profile, they were designated as isoform I, II and III. Further studies were conducted with isoforms I and II, after gel permeation chromatography (Fig. 3). The purity of isoforms I and II increased by 9 and 10 fold respectively with a recovery of 10 and 11%.

Some enzymatic properties of these isoforms are consolidated in Table 2. The optimum pH for β-hexosaminidase activity was 4.60 and 5.00 for isoforms I and II, respectively, their optimum temperature being 37–47 °C for isoform I and 47 °C for isoform II. β-Hexosaminidase II retained 100% activity at 57 °C for 15 min, while β-hexosaminidase I retained 71% of the activity under the same conditions. The K_m for pnp-β-D-*N*-acetyl glucosamine pyranoside was 3.00 and 1.75 mM for isoforms I and II, respectively. Among the metal ions tested for inhibitory action (Table 3), Hg^{2+} and Zn^{2+} showed higher inhibition, and the percentage inhibition being more for isoform I compared to isoform II. Also, Cu^{2+} showed a high inhibition for isoform I. At 1 mM concentration of Hg^{2+}, Cu^{2+} and Zn^{2+}, the enzyme showed 3, 9 and 14% residual activity over control for isoform I, while it was 5, 78 and 38% for isoform II. EDTA at 1 mM concentration exhibited 40% more activity for isoforms II. The substrate specificity of isoform I and II for pnp-β-D-*N*-acetyl galactosamine pyranoside was much less when compared to pnp-β-D-*N*-acetyl glucosamine pyranoside. The purified isoform II was checked for other glycosidase activities with pnp-substrates such as α- and β-D-glucopyranoside, α- and β-D-galactopyranoside, α-mannopyranoside and β-D-*N*-acetyl galactosamino pyranoside. This enzyme did not show any activity with these substrates except for β-D-*N*-acetyl galactosamino pyranoside. When compared to the β-D-*N*-acetyl glucosamino pyranoside(100%), enzyme with β-D-*N*-acetyl galactosamino pyranoside exhibited 33.5% activity.

The electrophoretic profiles (native and SDS–PAGE) for post ion exchange chromatographic fractions (a and b) are depicted in Fig. 4. The purified enzyme (isoform II) on non-denatured SDS–PAGE was homogeneous (Fig. 4c). This isoform showed a single band on SDS–PAGE with a relative molecular mass of 85 kD (Fig. 4d) and appeared to be a monomer.

β-Hexosaminidase enzyme from cabbage was recently reported to contain three subunits of 51, 57 and 64 kD on SDS–PAGE. Its K_m for pnp-β-D-*N*-acetylglucosamine was 0.94 mM, with an optimum pH of 4 and optimum

Table 1
Purification profile of β-hexosaminidase from bell capsicum

Purification steps	Total protein (mg)	Total activity[a] (EU)	Specific activity (EU/mg protein)	Purification fold	Recovery (%)
1. Crude extraction	256.00	268.92	1.05	1.00	100
2. Ammonium sulfate precipitation (35–70%)	136.40	174.54	1.27	1.20	64.9
3. DEAE-cellulose chromatography					
Isoform I	14.32	53.50	3.75	3.57	19.89
Isoform II	15.25	43.92	2.88	2.74	16.33
Isoform II	8.00	17.70	2.21	2.10	6.58
4. Gel permeation chromatography					
Isoform I	3.01	30.46	10.11	9.62	11.32
Isoform II	2.85	26.56	9.31	8.87	9.87

[a] 1EU is equivalent to 1 μmol pnp released min^{-1}.

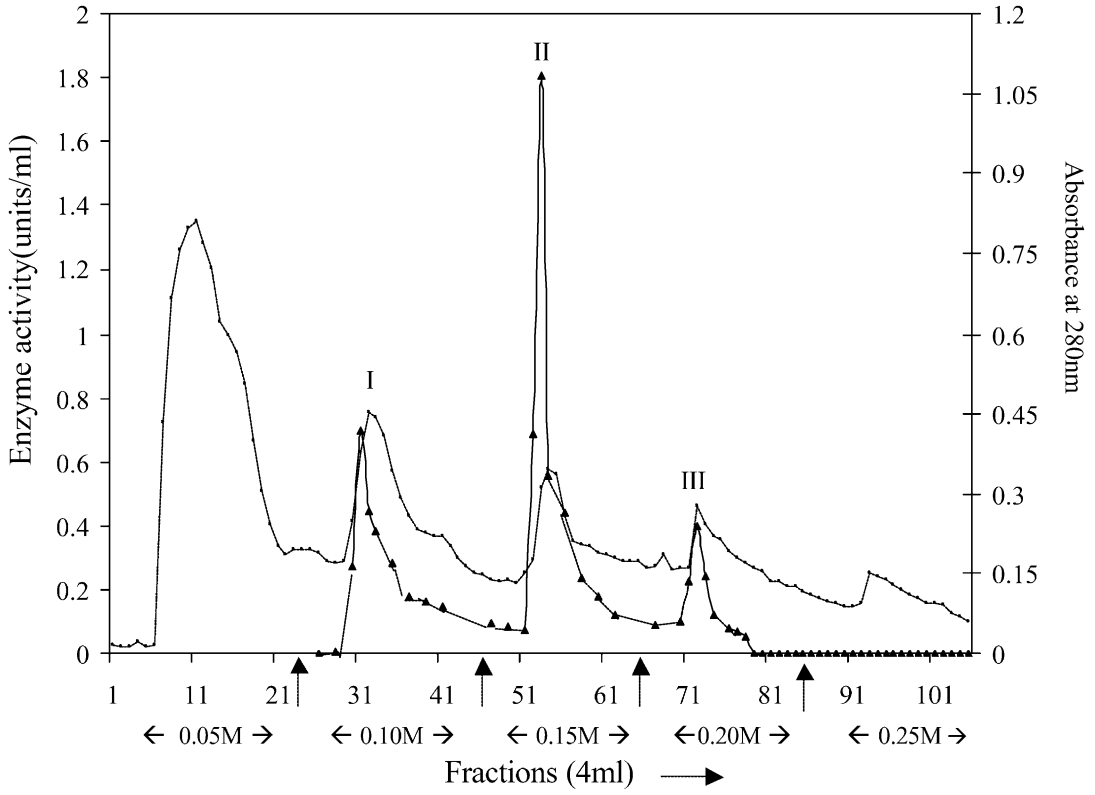

Fig. 2. Elution profiles of β-hexosaminidase isoforms from bell capsicum on DEAE-cellulose column equilibrated with double distilled water and eluted with increasing concentrations of 0.05, 0.10, 0.15, 0.20, and 0.25 M NaCl; pH 6.8, flow rate 0.66 ml min^{-1}. Isoforms I, II and III eluted with 0.10, 0.15 and 0.20 M NaCl, respectively. Arrows indicate the change of NaCl gradient (–●– is absorbance and -Δ- enzyme activity).

Table 2
Enzymatic properties of β-hexosaminidase isoforms from bell capsicum

Properties	Isoform I	Isoform II
1. Elution on IEC	0.10 M NaCl	0.15 M NaCl
2. Abundance	38%	47%
3. pH optimum	4.60	5.00
4. Temperature optimum	37–47 °C	47 °C
5. Thermal stability at 57 °C for 15 min	70%	100%
6. Inhibition	Hg^{2+}, Zn^{2+} and Cu^{2+}	Hg^{2+} and Zn^{2+}
7. K_m for pnp-β-D-*N*-acetyl glucosamine pyranoside	3.00 mM	1.75 mM
8. Specific activity (EU/mg protein) pnp-β-D-*N*-acetyl glucosamine	10.11	9.31

temperature of 60 °C (Chang et al., 1998). β-Hexosaminidase from lettuce was partially purified where its relative molecular mass was shown to be 69 kD on SDS–PAGE (Pocsi et al., 1990).

The abundant activity of β-hexosaminidase in bell capsicum and tomato when compared to other fruits is noted here (Table 4). This is the first report on β-hexosaminidase and its purification in fruits and also this enzyme is not established in higher plant systems. Though the functional role of β-hexosaminidase in signal transduction, cell multiplication, cellular integrity and host-pathogen interaction is studied in some animal and microbial systems, the implication of this enzyme in higher plants, particularly in fruits, is not known, which deserves further attention on the study of this enzyme in fruits in the context of fruit ripening. The increased activity of β-hexosaminidase during ripening is prominent. Besides, it was noticed in our earlier experiments (data not shown) that some new β-hexosaminidase band were developed during ripening. This also coincides with the increased β-hexosaminidase activity during ripening, when compared to developmental stages. It is thought that β-hexosaminidase may have an important function in fruit ripening by way of deglycosylation and generating free *N*-glycans. Free *N*-glycans were shown to be involved in fruit ripening in tomato (Priem et al., 1993).

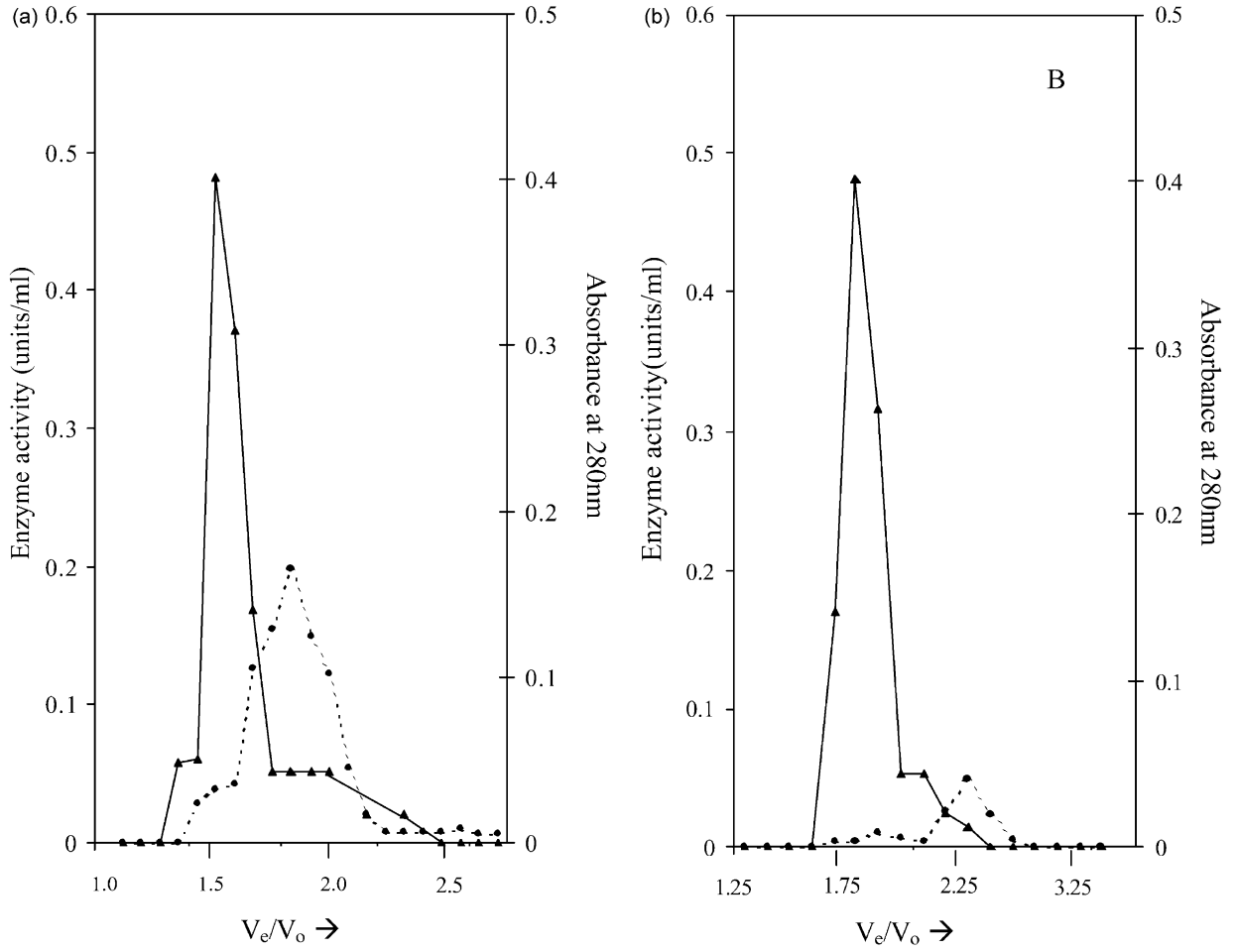

Fig. 3. Elution profiles of β-hexosaminidase isoforms from bell capsicum on Sephadex G-200 (—●— is absorbance and -Δ- enzyme activity). (A) Isoform I and (B) Isoform II.

Table 3
Effect of divalent metal ions on β-hexosaminidase activity

Inhibitors (1 mM)	Activity retained (%)±S.D.	
	Isoform I	Isoform II
Control	100.00 (±0.19)	100.00 (±0.30)
Cu^{2+}	9.00 (±0.34)	78.00 (±0.51)
Fe^{2+}	90.16 (±0.54)	119.35 (±0.63)
Hg^{2+}	2.78 (±0.30)	5.20 (±0.31)
Mg^{2+}	109.5 (±0.48)	67.33 (±0.40)
Ca^{2+}	110.80 (±0.86)	84.00 (±0.36)
Mn^{2+}	86.57 (±0.81)	101.45 (±0.61)
Zn^{2+}	14.57 (±0.33)	38.71 (±0.25)
EDTA	115.18 (±0.88)	139.96 (±0.81)

Table 4
Comparison of β-hexosaminidase activity in some important fruits

Fruits	EU[a]
1. Tomato	42.30
2. Capsicum	38.38
3. Papaya	18.19
4. Banana	3.75
5. Mango	1.05

[a] EU/g acetone powder.

3. Experimental

3.1. Materials

Freshly harvested bell capsicum was collected from a local farm. The four different developmental stages were selected based on size/weight and days (d) after fruit set. (I) 8-d, 2.5–3.5 g; (II) 16-d, 8.5–9.5 g; (III) 24-d, 60–62 g; and (IV) 32-d, 65–67 g. The four stages of ripening chosen were based on days after harvest. (I) Mature dark green, 3-d; (II) light green, 7-d; (III) orange green, 14-d and (IV) red ripe stage, 21-d. Acetone dried powders were prepared from the respective sample taken for the experiment. Acetone dried powders were also prepared from tomato, papaya, banana and mango from their climacteric stage. Post climacteric bell capsicum served as the source of enzyme for purification and characterization.

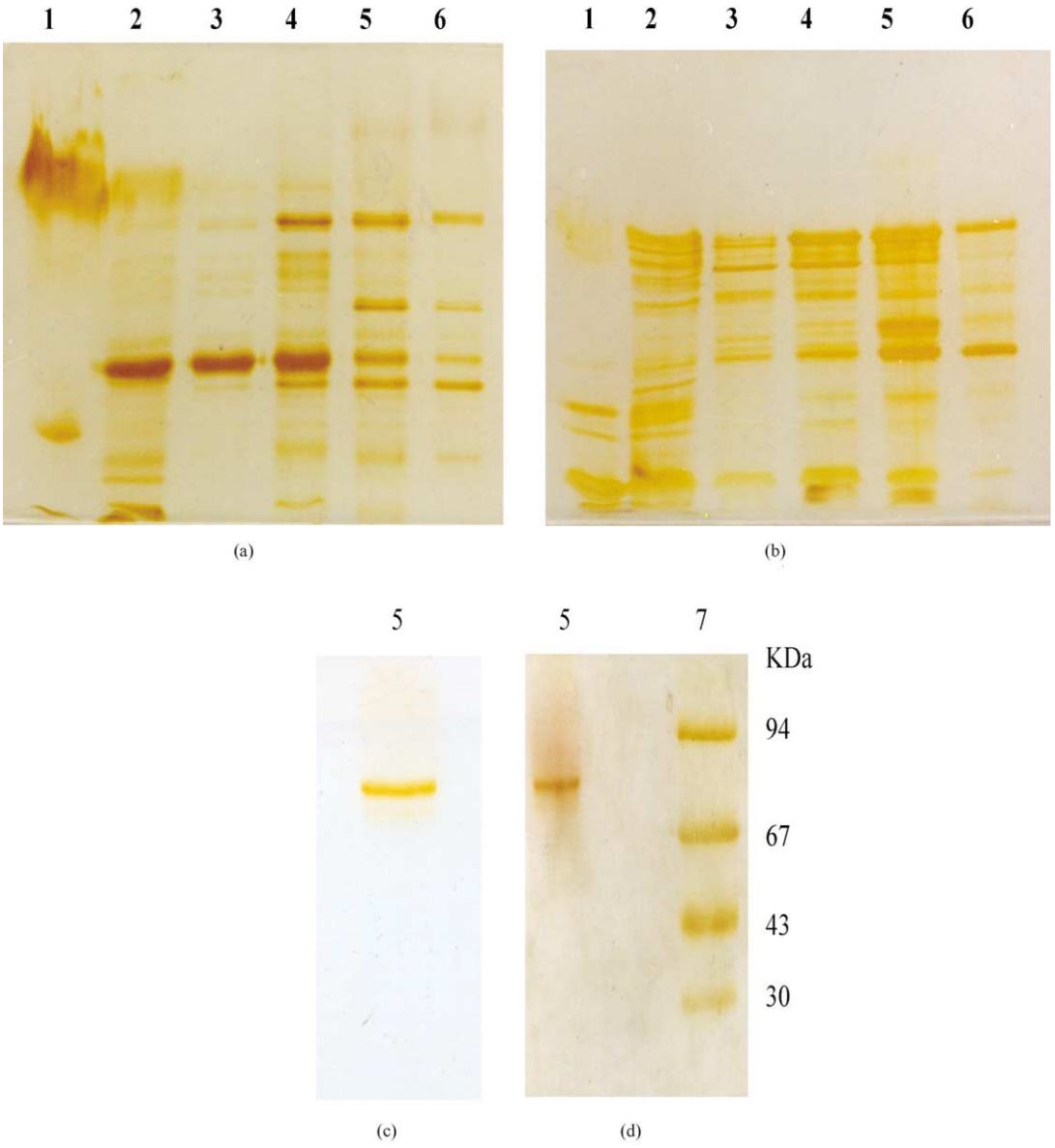

Fig. 4. Native (A and C) and SDS–PAGE (B and D) of β-hexosaminidase isoforms from bell capsicum. Lanes are as follows: 1, crude; 2, unbound fraction; 3, 0.05 M NaCl eluted; 4, Isoform I; 5, Isoform II; 6, Isoform III and 7, molecular mass standards (50 μg of protein was loaded per track for A and B and 10 μg were loaded for C and D).

3.2. Extraction, purification and electrophoresis

Acetone dried powder (10 g) was extracted (×3) with 0.05 M potassium phosphate buffer (pH: 6.60) containing 0.5 M sodium chloride and kept overnight for extraction at 4 °C. The enzyme extracts were pooled, filtered, clarified and dialyzed against double distilled water. The clear fraction was subjected to two-step ammonium sulphate precipitation (0–35% and 5–70% saturation). The fraction (35–70%) rich in β-hexosaminidase activity was subjected to ion exchange column chromatography (4×49 cm) on DEAE-cellulose after dialyzing against double distilled water (3×). The fractions were eluted with gradients of 0.05–0.25 M NaCl. The active fractions were collected individually and dialyzed. Distinctly separable fractions were designated as isoforms I, II and III. Isoforms I and II were suitably concentrated and further subjected to gel permeation chromatography using Sephadex G-200 (1.6×140 cm). The post gel filtration chromatographic fractions were used for further studies.

Native and SDS–PAGE were carried out according to the method of Laemmli (1970), and the proteins were visualized with silver staining according to the method (Porro et al., 1982).

3.3. Enzyme assay

The enzyme/substrate incubation was carried out for 15 min at 37 °C and the reaction mixture consisted of 1.25 mM pnp-β-D-*N*-acetyl glucosamine pyranoside,

100 mM sodium acetate buffer (pH: 5.00) and suitable aliquot of the enzyme. The activity was determined by measuring the liberated *p*-nitrophenol at 405 nm after addition of 500 mM sodium bicarbonate to the reaction mixture. One unit of the enzyme is defined as the amount of enzyme required to liberate one micro mol (μmol) of p-nitrophenol per minute. The specific activity was expressed as enzyme units per mg protein. Protein determination was done by the method of Sedmak and Grossberg (1977).

3.4. Enzyme properties

The experimental conditions for the study of various enzymic properties such has optimum pH, optimum temperature, thermal stability, K_m and divalent metal ion inhibition have been detailed previously (Priya Sethu and Prabha, 1997; Suvarnalatha and Prabha, 1999).

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