Journal of Biochemistry and Molecular Biology, Vol. 35, No. 3, May 2002, pp. 313-319

JOURNAL OF
Biochemistry and Molecular Biology

Purification and Characterization of β-*N*-Acetylhexosaminidase from Rice Seeds

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Received 16 January 2002, Accepted 5 March 2002

***N*-Acetyl-β-D-hexosaminidase (β-HexNAc'ase) (EC 3.2.1.52) was purified from rice seeds (*Oryza sativa* L. var. Dongjin) using ammonium sulfate (80%) precipitation, Sephadex G-150, CM-Sephadex, and DEAE-Sephadex chromatography, sequentially. The activities were separated into 7 fractions (F_1-F_7) by CM-Sephadex chromatography. Among them, F6 was further purified to homogeneity with a 13.0% yield and 123.3 purification-fold. The molecular mass was estimated to be about 52 kDa on SDS-PAGE and 37.4 kDa on Sephacryl S-300 gel filtration. The enzyme catalyzed the hydrolysis of both *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc) and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (pNP-GalNAc) as substrates, which are typical properties of β-HexNAc'ase. The ratio of the pNP-GlcNAc'ase activity to the pNP-GalNAc'ase activity was 4.0. However, it could not hydrolyze chitin, chitosan, pNP-β-glucopyranoside, or pNP-β-galactopyranoside. The enzyme showed K_m, V_{max} and K_{cat} for pNP-GlcNAc of 1.65 mM, 79.49 mM min^{-1}, and 4.79×10^6 min^{-1}, respectively. The comparison of kinetic values for pNP-GlcNAc and pNP-GalNAc revealed that the two enzyme activities are associated with a single binding site. The purified enzyme exhibited optimum pH and temperature for pNP-GlcNAc of 5.0 and 50°C, respectively. The enzyme activity for pNP-GlcNAc was stable at pH 5.0-5.5 and 20-40°C. The enzyme activity was completely inhibited at a concentration of 0.1 mM $HgCl_2$ and $AgNO_3$, suggesting that the intact thiol group is essential for activity. Chloramine T completely inhibited the activity, indicating the possible involvement of methionines in the mechanism of the enzyme.**

Keywords: Enzyme purification, *N*-Acetyl-β-D-galactosaminidase, *N*-Acetyl-β-D-glucosaminidase, *N*-Acetyl-β-D-hexosaminidase, Rice

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Introduction

N-Acetyl-β-D-hexosaminidase (*N*-acetyl-β-D-hexosaminide *N*-acetylhexosaminohydrolase, EC 3.2.1.52) (β-HexNAc'ase) has been detected in a wide variety of plant cells (Boller and Kende, 1979). In particular, high activities of β-HexNAc'ase are found in the aleurone grains of germinating seeds, suggesting a probable physiological function in the degradation of reserve glycoproteins (Harris and Chrispeels, 1975). β-HexNAc'ase also reportedly occurred in tissues that contain no reserve proteins or protein bodies (Matile, 1975), in vacuoles isolated from tulip petals, pineapple leaves, and suspension cultured tobacco cells (Boller and Kende, 1979), as well as in the latex of *Hevea brasiliensis* (Giordani *et al.*, 1992). Krisha and Murray (1988) demonstrated that the acid glycosidase activities in imbibing cotyledons of several leguminous seeds could be attributed to the rehydration of enzyme molecules that are formed during seed development, rather than to the *de novo* synthesis.

On the other hand, some plant β-HexNAc'ases also degrade chitin and chitin oligomers (Li and Li, 1970; Yi, 1981; Barber and Ride, 1989). Therefore, their participation in the process of chitin-elicited lignification has been suggested. In wheat leaves, β-HexNAc'ase may be involved in the metabolism of lignification-eliciting chitin oligosaccharides (Barber *et al.*, 1989). Choi and Gross (1994) discussed the significance of this enzyme in cleaving terminal non-reducing *N*-acetylglucosaminyl residues from *N*-glycans in relation to fruit ripening. However, the role of this enzyme is not yet fully understood.

Beyond their physiological significance, plant β-HexNAc'ases are very effective tools in the investigation of the oligosaccharide moieties of different glycoproteins and glycolipids (Li and Li, 1977). Promising analytical applicability prompted research groups to isolate more effective β-hexosaminidase preparations from various plant species, and to test their substrate specificity on a wide variety

of biological materials (Li and Li, 1970; Yi, 1981; Agrawal and Bahl, 1968). But, in comparison with the intensively investigated fungal and animal β-HecNAc'ases (Jones and Kosman, 1980; Koga *et al.*, 1982; Bedi *et al.*, 1984), only a limited amount of information is available on the higher plant enzymes. β-HexNAc'ase from rice has never been purified to homogeneity. In this paper, we describe the purification and molecular properties of a β-HexNAc'ase from rice seeds.

Materials and Methods

Chemicals Unless otherwise stated, all of the chemicals were obtained from Sigma (St. Louis, USA). Protein molecular weight standards were purchased from Amersham-Pharmacia Biotech Korea Ltd. (Seoul, Korea). Chitin oligomers $(GlcNAc)_{2\text{-}6}$ and deacetylated chitin oligomers $(GlcN)_{2\text{-}4}$ were from Wako Pure Chemical (Osaka, Japan). Chitin (DA 99.8%) and chitosan (DA 15.3%) were used as received from Taehoon Bio (Seoul, Korea).

Enzyme purification Throughout the following purification procedure, the enzyme activity was monitored by the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNP-GlcNAc) at pH 5.0, and protein by the Bradford assay kit (from Bio-Rad), using BSA as the standard. Unless otherwise stated, all of the steps were carried out at 4°C.

Rice seeds (*Oryza sativa* L. var. Dongjin, 500 g) were soaked in distilled water overnight and ground in mortar and pestle. The enzyme was extracted with 500 ml of McIlvaine's buffer (pH 5.0). The extract was centrifuged at $10,000 \times g$ for 30 min. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$ and held at 4°C overnight. After centrifugation at $10,000 \times g$ for 30 min, the pellet was resuspended in 30 ml of McIlvaine's buffer (pH 5.0), dialyzed against the same buffer, and centrifuged $10,000 \times g$ for 1 h. The supernatant from the centrifugation was concentrated by ultrafiltration (PM 10 membrane, Amicon) to 5 ml, centrifuged at $11,600 \times g$ for 10 min to remove insoluble material, and applied to a Sephadex G-150 gel filtration column (2.8×60 cm). The eluent was McIlvaine's buffer (pH 5.0) at a flow rate of 1 ml · min^{-1}. The components were monitored by measuring the absorbance at 280 nm. The fractions (total 135 ml) that contained β-GlcNAc'ase activity were pooled. The pooled fractions from the gel filtration step were reduced to 2.5 ml by ultrafiltration. After centrifugation at $11,600 \times g$ for 10 min to remove insoluble material, the sample was applied to a CM-Sephadex column (2.8×60 cm) that was pre-washed with McIlvaine's buffer. The column was eluted with a step-wise gradient of 0, 0.05, 0.1, 0.25, and 0.5% NaCl (200 ml each) in McIlvaine's buffer (1 ml · min^{-1}). The components were monitored by measuring the absorbance at 280 nm. The fractions that contained β-GlcNAc'ase activity were pooled. The pooled fractions from the CM-Sephadex column were reduced to 4.5 ml by ultrafiltration in McIlvaine's buffer. This was applied to a DEAE-Sephadex column (1.5×33 cm) and eluted with the same buffer (2.5 ml/tube).

Enzyme assay Enzyme activity towards pNP-glycosides was assayed by incubating a suitably diluted sample of the enzyme (50 μl) with 50 μl of a 5 mM substrate and 100 μl McIlvaine's buffer at 37°C for 15 min (Chang *et al.*, 1998). The reaction was stopped by the addition of 1,000 μl of 0.2 M Na_2CO_3. The absorbance was read at 405 nm. The concentration of *p*-nitrophenol was determined from a standard curve. One unit of β-HexNAc'ase activity is equal to the release of 1 μmol *p*-nitrophenol per min at 37°C. *p*-Nitrophenyl-*N*-acetyl-β-D-galactosaminide (pNP-GalNAc) was also used as substrate. Activity towards chitin, chitin oligomers, and their deacetylated derivatives was assessed by incubating a suitably diluted sample of the enzyme (10 μl) with 50 μl of 1.0 mM oligomer or 4 mg/ml chitin, or their deacetylated derivatives in distilled water and 40 μl of McIlvaine's buffer at 30°C for 2 h. Release of GlcNAc and GlcN was determined colorimetrically (Baek *et al.*, 2001).

Characterization of the β-HexNAc'ase The purity of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970). Native gel electrophoresis was also performed, according to Bollag *et al.* (1996). The protein band was stained with Coomassie brilliant blue R-250. The molecular weight of the native enzyme was determined by Sephacryl S-300 gel filtration (Bollag *et al.*, 1996). The pH optima for the β-HexNAc'ase were determined using pNP-GlcNAc or pNP-GalNAc in McIlvaine's buffer (pH 3.0-7.0), 0.1 M sodium phosphate buffer (pH 7.5-8.0), and 0.1 M carbonate buffer (pH 9.0-11.0). For the pH stability test, the enzyme was incubated in various pH buffers at 37°C for 1 h, and the remaining activity was measured. The temperature optima were also determined for the pH optima using pNP-GlcNAc or pNP-GalNAc in McIlvaine's buffer (pH 5.0) in the range of 20-70°C for 30 min incubation. For the temperature stability test, the enzyme was incubated at 20-70°C for 1 h, and the remaining activity was measured. The effects of cations (1-10 mM) and chemical modifiers (0.1-1.0 mM) were measured in 1.25 mM pNP-GlcNAc as the substrate.

Results and Discussion

Purification A purification of approximately 123 fold and a recovery of 13% were achieved using by a series of chromatographic procedures (Table 1). The enzyme was stable throughout the purification steps. The enzyme was eluted from the CM-Sephadex column as 7 isoenzyme peaks of activity (Fig. 1). Among them, one isoenzyme (F_6) showed the highest activity and was further purified by DEAE-Sephadex chromatography in order to give a single peak of activity (Fig. 2). The specific activity of the purified enzyme was 58,000 U/mg protein. SDS-PAGE revealed a single band of 52 kDa (Fig. 3).

Resolution of 7 isozymes by CM-Sephadex chromatography was unexpected. Four isozymes from fenugreek cotyledons (Bouquelet and Spik, 1978), three from lupin seeds (Póci *et al.*, 1990), and two from pea leaf (Gaudreault and Beevers, 1983) were resolved by DEAE chromatography. Five isoenzymes were separated from wheat leaf by isoelectric focusing (Barber and Ride, 1989). Two isozymes with molecular weights (*Mr*) of 45 and 90 kDa from mung bean seeds were resolved by gel filtration (Dey, 1984).

Table 1. Purification of β-HexNAc'ase from rice seeds

Step	Protein content (mg)	Total activity (units)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude	322.7	152,038	471	100	1
AS precipitate	175.1	34,886	199	22.9	0.42
Sephadex G-150	27.1	34,460	1,271	22.7	2.70
CM-Sephadex	0.82	29,515	35,994	19.4	76.4
DEAE-Sephadex	0.34	19,742	58,065	13.0	123.3

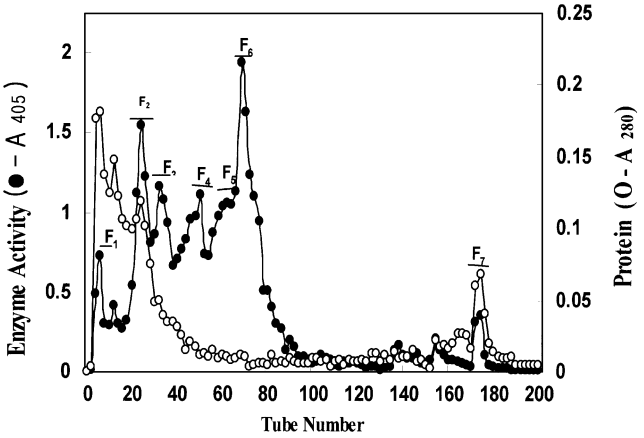

Fig. 1. CM-Sephadex chromatography. The enzyme was loaded on the column that was equilibrated in McIlvaine's buffer (pH 5.0). It was eluted with the same buffer (pH 5.0) that contained 0.05-0.5% NaCl.

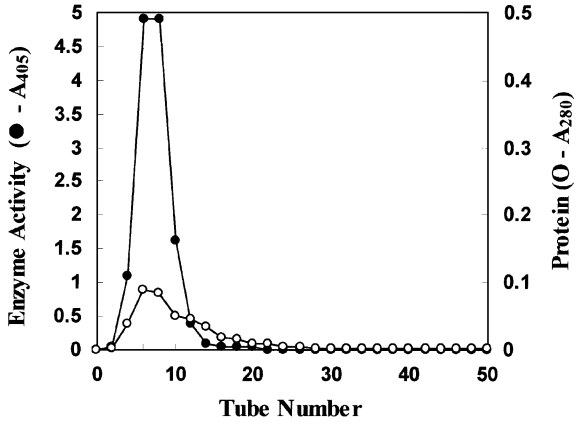

Fig. 2. DEAE-Sephadex column chromatography.

The *Mr* of rice β-HexNAc'ase (52 kDa) is approximately half of the *Mr* that is reported for pinto beans (Agrawal and Bahl, 1972), caster beans (Harley and Beevers, 1985), jack beans (Li and Li, 1970), malted barley (Mitchell *et al.*, 1976), and cotton (Yi, 1981), which are in the range of 90-125 kDa. The *Mr* determined for the β-HexNAc'ase isozymes could be inaccurate, apparently due to the glycoprotein nature of the isoenzymes. The *Mr* of the β-HexNAc'ase that was purified from lupin seeds is 69 kDa, as determined by SDS-PAGE, and

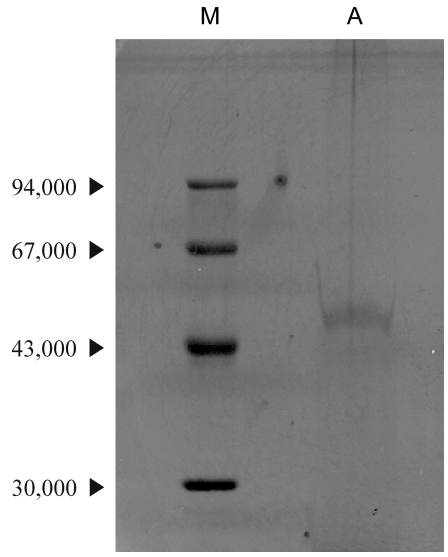

Fig. 3. SDS-PAGE of β-HexNAc'ase purified from rice seeds. M, molecular markers; A, purified enzyme. Sample was reduced with 2-mercaptoethanol.

62.5 kDa by Bio-Gel P-60 filtration. On a Sephadex G-75 column, however, a significant retardation (apparent *Mr* 15.3 kDa) could be observed (Posi *et al.*, 1990). This is the case for rice β-HexNAc'ase. The *Mr* of the enzyme appeared to be 37.4 kDa, due to a substantial retardation on a Sephacryl S-300 (data not shown). Rice β-HexNAc'ase appears to be monomeric since there are no differences in the SDS-PAGE profiles of the enzyme under reducing and nonreducing conditions, as well as in the SDS-PAGE profiles of the enzyme that were treated and nontreated with glutaldehyde (data not shown),.

Substrate specificity The β-HexNAc'ase from rice seeds released *p*-nitrophenol from both pNP-GlcNAc and pNP-GalNAc (Table 2). This is the same as β-HexNAc'ases that are described from pinto beans (Agrawal and Bahl, 1972), fenugreek cotyledons (Bouquelet and Spik, 1978), wheat aleurone (Carratu *et al.*, 1985), castor beans (Harley and Beevers, 1985), jack beans (Li and Li, 1970), lupin seeds (McFarlane *et al.*, 1984), *Ficus* latex (Orlacchio *et al.*, 1985), and cotton seeds (Yi, 1981). The ability of the enzyme to utilize a number of potential substrates was assessed in

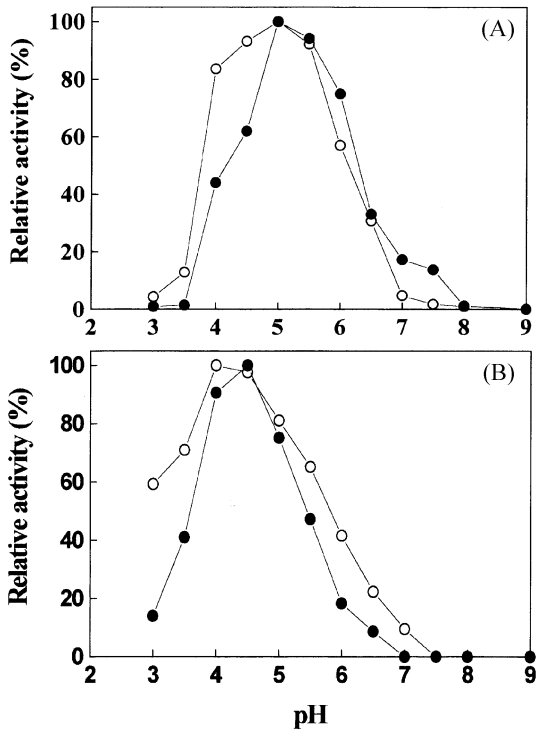

Fig. 4. Effect of pH on the activity of rice β-HexNAc'ase. Each was assayed using pNP-GlcNAc (A) and pNP-β-D-GalNAc (B) as the substrates. ● - Optimal pH, ○ - pH stability.

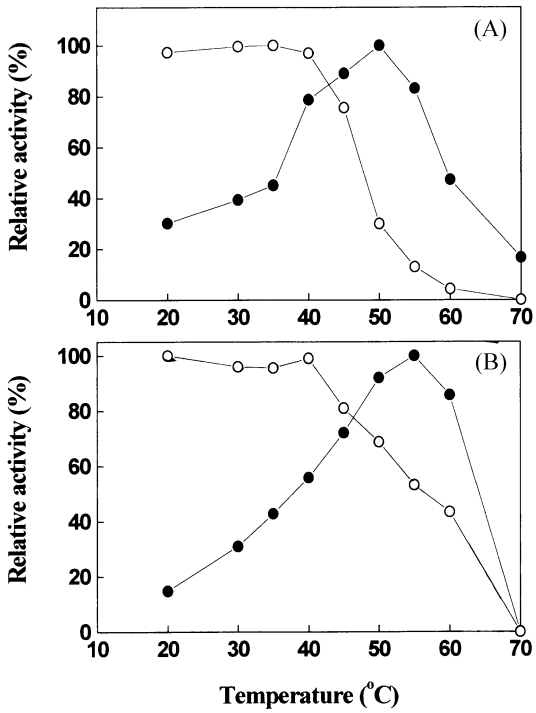

Fig. 5. Effect of temperature on the activity of rice β-HexNAc'ase. Each was assayed using pNP-GlcNAc (A) and pNP-β-D-GalNAc (B) as the substrates. ● - Optimal temperature, ○ - Temperature stability.

Table 2. The substrate specificity of rice β-HexNAc'ase

Substrate	Relative activity (%)
pNP-GlcNAc	100
pNP-β-D-GalNAc	25.2
pNP-β-glucopyranoside	0
pNP-β-galactopyranoside	0
Glycol chitin	0
Swollen chitin	0

McIlvaine's buffer (pH 5.0). The enzyme readily hydrolyzed pNP-GlcNAc and pNP-GalNAc, but was virtually inactive towards pNP-β-D-glucopyranosaminide and pNP-β-D-galactopyranosaminide. The enzyme was also actually inactive toward swollen chitin, glycol chitin, and soluble chitosan under the experimental conditions. This result is consistent with wheat β-HexNAc'ase, which was unable to release GlcN from deacetylated chitin oligomers $(GlcN)_{2\text{-}4}$ or GlcNAc from chitin (Barber and Ride, 1989).

β-HexNAc'ase from wheat was significantly less active in releasing pNP from pNP-β-D-$(GlcNAc)_{2\text{-}6}$, in contrast to the relatively high activity towards pNP-GlcNAc and pNP-GalNAc (Barber and Ride, 1989). The data from experiments with pNP-glycosides is consistent with the enzymes that release terminal GlcNAc residues from the non-reducing end of the substrates. β-HexNAc'ase from fenugreek (Bouquelet and Spik, 1978) have similarly shown lower activity towards $(GlcNAc)_2$ compared to pNP-GlcNAc. Interestingly, fenugreek also possesses a β-HexNAc'ase isoenzyme with a high activity towards $(GlcNAc)_2$ and a low activity towards the pNP-GlcNAc.

pH dependence The pH dependence of the enzyme was assessed using a McIlvaine's buffer (pH 3.0-7.0), phosphate buffer (pH 7.5-8.0), and carbonate buffer (pH 9.0-11.0) (Fig. 4). The pH optima of the β-HexNAc'ase towards pNP-GlcNAc and pNP-GalNAc were 5.0 and 4.0 with half-widths of 2.0 and 3.0, respectively. The optimum pH for hydrolysis of pNP-GlcNAc was higher than that for pNP-GalNAc, as seen in soybeans (Gers-Barlag, *et al.*, 1988), castor beans (Harley and Beevers, 1985), jack beans (Li and Li, 1970), and cotton (Yi, 1981). The rate of hydrolysis of both substrates at pH 6.5 is 20 to 30% the maximal rate. The activities toward pNP-GlcNAc and pNP-GalNAc were stable at pH 5.0-5.5 and 4.5-5.0, respectively.

The activity ratio of pNP-GlcNAc'ase to pNP-GalNAc'ase was 4.0 (Table 2). The β-HexNAc'ase isozymes also have different hydrolysis ratios towards pNP-GlcNAc and pNP-GalNAc, ranging from 1.25 to 18.0 for the four isoenzymes from fenugreek (Bouquelet and Spik, 1976), 1.9 to 4.8 for the three from lupin (McFarlane *et al.*, 1984), and 4.0-12.9 for the four from pea seeds (Harley and Beevers, 1987). It is reported that the activity ratio of pNP-GlcNAc'ase to pNP-GalNAc'ase is pH dependent due to different pH optima for the substrates (Barber and Ride, 1989; Harley and Beevers, 1987).

Table 3. Kinetic data of β-HexNAc'ase form rice seeds

Parameters	Substrate		Ratio (pNP-GlcNAc/pNP-GalNAc)
	pNP-GlcNAc	pNP-GalNAc	
K_m (mM)	1.65	0.17	9.7
V_{max} (nmol/min)	79.5	7.46	10.6
k_{cat} (min^{-1})	4.79×10^6	4.50×10^5	10.6
k_{cat}/K_m ($min^{-1}mM^{-1}$)	2.90×10^6	2.65×10^6	1.1
V_{max}/K_m (L/min)	4.82×10^{-5}	4.39×10^{-5}	1.1

Temperature dependence The optimal temperature of the enzyme towards pNP-GlcNAc and pNP-GalNAc were 50°C and 55°C, respectively (Fig. 5). The profiles of temperature stability toward pNP-GlcNAc and pNP-GalNAc were quite similar, and the enzyme was stable under 40°C. The β-GalNAc'ase activity was more persistent (compared to β-GlcNAc'ase above 40°C), and maintained 40% maximal activity at 60°C, while β-GlcNAc'ase maintained only 5%. *Hevea* latex β-HexNAc'ase showed optimum temperature of 50°C and was stable under 50°C (Giordani *et al.*, 1992). Chinese cabbage β-HexNAc'ase showed optimum temperature of 60°C and was stable under 30°C (Chang *et al.*, 1998).

Effect of substrate concentration The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) for the enzyme that acted on pNP-GlcNAc and pNP-GalNAc were calculated from Lineweaver-Burk plots that were determined at the pH optimum for each activity (Table 3). The following values were obtained: K_m 1.65 mM, V_{max} 79.5 nmol/min, K_{cat} 4.79×10^6 min^{-1} for pNP-GlcNAc, and K_m 0.17 mM, V_{max} 7.26 nmol/min, K_{cat} 4.50×10^5 min^{-1} for pNP-GalNAc. The ratio of kinetic values for pNP-GlcNAc and pNP-GalNAc remained constant, suggesting that the two enzyme activities are associated with a single binding site. These values are comparable with those for the lupin enzyme (Póci *et al.*, 1990). The K_m of the enzyme for pNP-GlcNAc from various other plant sources varied from 40 μM to 1.13 mM (Bouquelet and Spik, 1978; Chang *et al.*, 1998; Giordani *et al.*, 1992) and from 40 μM to 1.71 mM for pNP-GalNAc (Bouquelet and Spik, 1978; Harley and Beevers, 1987; Choi and Gross, 1994).

It was reported that pNP-GlcNAc and pNP-GalNAc are bound to a single active site (Choi and Gross, 1994; Li and Li, 1970). The ratios of K_m and V_{max} of pNP-GlcNAc'ase to pNP-GalNAc'ase were 9.7 and 1.06 for rice β-HexNAc'ase, respectively. With jack bean enzymes, Li and Li (1970) also found that K_m and V_{max} values were lower for the GalNAc derivatives than for those of GlcNAc. The ratio V_{max}/K_m were 4.82×10^{-5} for pNP-GlcNAc and 4.39×10^{-5} for pNP-GalNAc, which showed that pNP-GlcNAc is the preferred substrate. The pNP-GlcNAc bound less tightly (higher K_m) to the active site than did pNP-GalNAc, while the V_{max} value and the catalytic efficiency were higher for pNP-GlcNAc.

Inhibition studies Inhibition of β-HexNAc'ases was studied by incorporating potential inhibitors into the enzyme assay by measuring pNP-GlcNAc'ase activity (Table 4). Of the various metal ions tested, Hg^{2+}, Ag^+ and to a lesser extent Cu^{2+}, Pb^{2+}, and Sn^{2+} ions were inhibitory. This indicates that the sulfhydryl groups are important for catalysis and/or conformation stability. The inhibition was blocked by sulfhydryl that contained compounds such as β-mercaptoethanol (data not shown). β-HexNAc'ases from fenugreek (Bouquelet and Spik, 1978), wheat leaf (Barber and Ride, 1989), soybeans (Gers-Barlag *et al.*, 1988), and jack beans (Li and Li, 1970) are strongly inhibited by Ag^+. β-HexNAc'ases from pinto beans (Agrawal and Bahl, 1972), fenugreek (Bouquelet and Spik, 1978), wheat grain (Carratu *et al.*, 1985), jack beans (Li and Li, 1970), *Ficus* latex (Orlacchio *et al.*, 1985), cotton (Yi, 1981), pea seeds (Harley and Beever, 1987), and *Hevea* latex (Giordani *et al.*, 1992) are also strongly inhibited by $HgCl_2$. The caster bean enzyme, however, is not (Harley and Beevers, 1985). β-HexNAc'ases from jack beans (Li and Li, 1970), *Ficus* latex (Orlacchio *et*

Table 4. Effect of cations and anions on the activity of rice β-HexNAc'ase

Inhibitor	Concentration (mM)	Relative activity (%)
Control	-	100
$AlCl_3$	1	99.5
	10	79.7
KCl	1	100.9
	10	100
$ZnSO_4$	1	99.5
	10	81.9
$Pb(NO_3)_2$	1	97.1
	10	50.2
$MgSO_4$	1	90.1
	10	77.6
$CuSO_4$	1	88.8
	10	25.1
$HgCl_2$	1	8.4
	10	0
$SnCl_2$	1	74.4
	10	56.1
$AgNO_3$	1	0
	10	0

Table 5. Effect of inhibitors on the activity of rice β-HexNAc'ase.

Inhibitor	Concentration (mM)	Relative activity (%)
Control	-	100
N-Acetylimidazole	0.1	95.9
	1	69.0
N-Bromosuccinimide	0.1	102.2
	1	93.7
Chloramine T	0.1	0
	1	0
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	0.1	95.3
	1	86.5
N-Ethylmaleimide	0.1	98.3
	1	91.0
p-Hydroxy-mecuribenzoic acid	0.1	97.6
	1	77.8
Iodoacetamide	0.1	95.4
	1	84.0
Iodoacetic acid	0.1	98.8
	1	84.4
Phenylglyoxal	0.1	94.5
	1	79.5
Phenylmethylsulfonyl fluoride	0.1	101.0
	1	98.9
Glucosamine	0.1	98.6
	1	77.5

al., 1985), cotton (Yi, 1981) and wheat leaf (Barber and Ride, 1989) are also inhibited by $CuSO_4$ and $FeSO_4$. Activation of rice β-HexNAc'ase was not observed by cations. One isoenzyme of pea β-HexNAc'ases was activated by Mn^{2+}, Mg^{2+}, and Ca^{2+} (Harley and Beever, 1987).

Complete inhibition of the activity was observed when the enzyme was treated with chloramine T, a potential chemical modifier of methionine residue on proteins. This suggests a possible location of methionine residues at the active site (Table 5). *N*-Acetylimidazole, a tyrosine residue modifier, also inhibited to the extent of 30% at the 1.0 mM concentration. There is not much reported information on the enzyme inhibition by chemical modifiers. Fenugreek β-HexNAc'ases were inhibited by *p*-chloromercuribenzoate (Bouquelet and Spik, 1978). Glucosamine was a very poor inhibitor; the residual activity was 77% at a concentration of 1 mM. Some β-HexNAc'ases reportedly are inhibited by acetate (Li and Li, 1970; Harley and Beevers, 1985); however, the soybean enzyme is not (Gers-Barlag *et al.*, 1988). As shown in Table 6, rice β-HexNAc'ase was inhibited by 40% with 0.1 M acetate buffers (pH 5.0), compared with the McIlvaine's buffer (pH 5.0). Generally inhibitors of β-GlcNAc'ase activity are also inhibitory to the β-GalNAc'ase activity, possibly due to their shared active site of the enzyme (Gers-Barlag *et al.*, 1988; Horsch *et al.*, 1997).

It has been suggested that β-HexNAc'ase could function as a post-translational processing enzyme (Vitale and Chrispeels, 1984), in addition to a role in the mobilization of seed storage reserves during germination (Neely and Beevers, 1980). In regard to rice β-HexNAc'ase as a processing enzyme, an interesting observation is that the enzyme levels of the seeds (per fresh weight) peak around 42s day post-anthesis in the developing seeds, then they hold steady (data not shown). During rice seed germination, the initial β-HexNAc'ase activity increased for the first week, held, and then decreased after 2 weeks (data not shown). Treatment of cotyledons of germinating pea seeds with cycloheximide or 6-methylpurine had no effect on β-HexNAc'ase levels. This indicates that all of the β-HexNAc'ase that are present were deposited during seed development and not synthesized *de novo* (Neely and Beevers, 1980). To date, this is the first report on the purification and characterization of β-HexNAc'ases from rice. The physiological significance of rice β-HexNAc'ases and other chitin-oligomer metabolizing enzymes in defence-related functions are currently being studied.

Table 6. Effect of buffers (0.1 M, pH 5.0) on the activity of rice β-HexNAc'ase

Buffer	Relative activity (%)
McIlvaine	100
Na-acetate	60.8
K-acetate	59.2
Phosphate	91.8

Acknowledgment The Chonnam National University (1999) financially supported this work.

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