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Purification and Characterization of α-*N*-Acetylgalactosaminidase from *Hilsha ilisha*

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Abstract

The objective of this study was to purify and characterize the alpha-*N*-acetylgalactosaminidase (α-GalNAcase) from hilsha fish, *Hilsha ilisha*. Digestive organ of hilsha fish was found to contain a large amount of α-GalNAcase in compared with the other tissues examined. α-GalNAcase was purified from the crude extract of hilsha fish by ammonium sulphate precipitation, Sephadex G-200 gel filtration, and SP-Sephadex C-50 column chromatography. The purified enzyme gave a single band on sodium dodecylsulphate-polyacrylamide gel electrophoresis and exhibited a molecular mass of 48 kDa. The final preparation of α-GalNAcase showed 3.02% α-galactosidase activity. The enzyme had an optimum pH of 4.0 and was found to be quiet heat stable at 37°C. Inhibition studies with metal ions demonstrated that the enzyme was highly inhibited by silver and mercury ions. Both *N*-acetylgalactosamine and galactose affect the enzyme activity. Kinetic studies with the enzyme showed that the K_M value for *p*-nitrophenyl-α-*N*-acetylgalactosaminide substrate was 3.31 mM and the *Vmax* value was 35.04 unit/mg.

Keywords: *Hilsha ilisha*; α-*N*-Acetylgalactosaminidase; α-Galactosidase; Purification.

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1. Introduction

Alpha-*N*-Acetylgalactosaminidase (α-GalNAcase) is a lysosomal glycosidase that cleaves the terminal α-linked *N*-acetylgalactosamine moiety from glycoprotein, glycolipid and polysaccharide, including those with O-linked carbohydrates attached to serine and

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threonine [1]. The wide spread occurrence of the substrates in human suggest its essential physiological function. Deficiency of α-GalNAcase resulting from the defect in α-GalNAcase gene leads to Schindler disease characterized by losing skills, blindness, seizures, loss of awareness [2,3], cardiomyopathy and hepatomegaly in infant [4] and Kanzaki disease characterized by hearing loss, weakness, loss of sensation, dark spot on the skin in adult [1,5] due to deposition of glycoconjugates. Despite the clinical importance, the structure and function of this enzyme are not completely understood.

Recently, α-GalNAcase has received attention due to its ability to convert type A to type O blood cell which is the universal donor blood group [6-10]. The blood group A antigen differ from blood group O antigen by a terminal α-GalNAc, whereas blood group B antigen differ from blood group O antigen by a terminal α-galactose. Thus, a α-GalNAcase without α-galactosidase activity is desirable to process blood group A to produce blood group O. Moreover, α-galactosaminyl moieties are known to be the common constituents of various glycoconjugates including Forssman glycolipid, bovine submaxillary mucin and complex carbohydrates [11]. For investigation of the structures and functions of these glycoconjugates, the enzyme is of great value.

Initially α-GalNAcase was thought to be an isoenzyme of α-galactosidase and thereafter called α-galactosidase B [12,13]. The two enzymes (α-galactosidase A and B) have similar physicochemical properties, homodimeric structure and similar amino acid composition. However, subsequent studies of their substrate specificities, kinetics, inhibitor, immunologic and gene mapping studies demonstrated that α-galactosidase A and B were distinct lysosomal enzymes and α-galactosidase B was really a α-GalNAcase [14,15]. α-GalNAcase had been purified from various animal tissues including human, bovine, pig and chicken. All the α-GalNAcases isolated from animal tissues contained α-galactosidase activity; whereas the enzyme from bacteria did not show such activity [8, 16, 17]. We first described the occurrence of two kinds of α-GalNAcases, α-GalNAcase I and α-GalNAcase II from squid liver and star fish [18-20]. α-GalNAcase I showed α-galactosidase activity; while α-GalNAcase II was absent from that activity. The diversity of α-GalNAcase in marine invertibrates generated interest to investigate further on α-GalNAcase from marine sources. Herein, we describe the purification and characterization of α-GalNAcase from hilsha fish, a marine vertebrate and popular fish food of Bangladesh.

2. Experimental

2.1. *Materials*

The fresh hilsha fish, originated from the Bay of Bengal, were collected from the Padma River, Chandpur, Bangladesh. Different tissues of hilsha fish, such as liver, digestive organ, egg, heart and fleshy mass were separated, immediately frozen and stored at -20°C until experimental work. *p*-Nitrophenyl(pNP)-α-D-galactopyranoside (pNP-α-Gal) and pNP-α-*N*-acetylgalactosaminide (pNP-α-GalNAc) substrates were purchased from Sigma Aldrich Chemical Co (Germany). Bovine serum albumin (BSA), Sephadex G-200 and SP

Sephadex C-50 were procured from Wako Pure Chemical Industries (Osaka, Japan). Redistilled water was used in the study. All buffer solutions were filtered through a 0.45 μm pore size membrane filter from Advantec^{R} (Japan). All other chemicals and reagents were obtained from commercial sources and were of the highest analytical grade available.

2.2. *Enzyme activity assay*

Glycosidase activities were determined by using pNP-glycoside as the substrates in 50 mM sodium citrate buffer, pH 4.0 as a modified procedure described previously [21]. Briefly, an aliquot of enzyme was incubated in 0.2 ml of 2.0 mMpNP-glycoside at 37°C. After incubation for an appropriate period, the reaction was terminated with 1.4 mL of stop solution (200 mM glycine-NaOH buffer, pH 9.8). The released *p*-nitrophenol was determined by UV method at 400 nm. One unit was defined as the amount of enzyme that catalyzed the formation of 1 μ mole *p*-nitrophenol per minute under the described conditions.

2.3. *Protein concentration assay*

Protein concentration was detected spectrophotometrically at 280 nm and measured by the simple modification of Lowry method [22]. Briefly, an aliquot of protein sample was mixed with 1.3 mL reagent mixture (2.0% w/v Na_2CO_3 solution in 0.1 N NaOH: 0.5% w/v copper sulphate in 1.0% sodium-potassium tartrate = 50:1) and incubated at room temperature. After 10 min., one mL of 10% v/v FCR was added and incubated for 30 min. at 37°C. The colour development for protein was measured spectrophotometrically at 750 nm. A standard calibration curve ($y=0.0076x+0.0091$, $R^2=0.9893$) was prepared using BSA in the concentration range 0 to 40 μg/mL in a 2.5 mL reaction mixture.

2.4. *Extraction and purification of α-GalNAcase enzyme*

Fresh-frozen tissue of hilsha fish was thawed. The tissue was diced and homogenized in a warring blender for 30 seconds at low speed and for 90 seconds at medium speed in 5 volumes (w/v tissue) of buffer A (50 mM sodium citrate buffer, pH 4.0, containing 1.0 mM phenyl-methyl-sulfonyl fluoride). The homogenate was filtered by cotton cloth and centrifuged at 4000 rpm for 20 minutes. The supernatant was saturated with 20% ammonium sulphate and stirred for 90 min. at 0°C. Then the 20% ammonium sulphate saturated solution was kept at 4°C for overnight and centrifuged at 4000 rpm for 20 min. The solid pellets were discarded and more ammonium sulphate was added up to 40% saturation. The precipitates containing α-GalNAcase enzyme obtained from 20-40% ammonium sulphate saturation were collected and dissolved in 50 mM sodium citrate buffer, pH 3.5. The α-GalNAcase enzyme fraction was purified by Sephadex G-200 gel filtration and SP Sephadex C-50 column chromatography (details given in legends to

Fig. 1). The glycosidase activities and protein contents were monitored in each step throughout the purification procedure.

2.5. *Determination of molecular mass*

Molecular mass of purified α-GalNAcase enzyme was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in denatured condition. Protein mixture were diluted with Laemmli sample buffer, heated at 95°C and subjected to electrophoresis on SDS-PAGE [23]. The electrophoresis was run on the gel at 80 volt for 15 min. and then at 120-150 volt for approximately 2-3 hr. The separated protein bands in gel were visualized by 0.25% Coomassie brilliant blue R-250 (CBB) in 50% methanol and 10% acetic acid, and destained with 25% methanol and 7.5% acetic acid [24]. A calibration curve (Y= - 1.4183x + 2.4187) was constructed by a semi logarithmic plot of relative mobility of protein on gel verses the log of molecular weight of standard protein (Fig. 2B). The molecular weight of α-GalNAcase was estimated from the standard calibration curve by comparing the relative mobility [25].

2.6. *Evaluation of kinetic parameters*

The kinetics of α-GalNAcase enzyme were measured by the hydrolysis of artificial substrate pNP-α-GalNAc at 37°C in 50 mM sodium citrate buffer, pH 4.0. The reaction was carried out at a substrate concentration 0 to 2.5 mM and quenched after 10 min. with stop solution. After hydrolysis the absorbance of released pNP was measured at 400 nm. The kinetic parameters, K_M and V_{max} were evaluated by using Linewever-Burk plot.

2.7. *Dose dependent activity*

The substrate pNP-α-GalNAc was subjected to hydrolysis in 50 mM sodium citrate buffer, pH 4.0 at 37°C with different enzyme concentrations for 10 min. and the reaction was terminated with the addition of stop solution.

3. Results and Discussion

3.1. *Purification of α-GalNAcase enzyme*

The α-GalNAcase activities of the different tissues, such as liver, digestive organ, egg, heart, and fleshy mass separated from hilsha fish, were measured and the results have been shown in Table 1. Among the tissues, digestive organ was found to contain the highest amount of α-GalNAcase (78.35 unit/20 gm) followed by liver (60.5 unit/20 gm). Therefore, digestive organ was subsequently used for isolation of α-GalNAcase from hilsha fish.

Table 1. Screening of various tissues (20 gm) of hilsha fish for α-GalNAcase enzyme.

Name of organ	α-GalNAcase (unit)[a]
Egg	1.92
Heart	2.52
Liver	60.5
Fleshy mass	2.57
Digestive organ	78.35

[a]Data are mean values of three determinations.

α-GalNAcase was purified from the crude extract of hilsha fish by means of ammonium sulphate precipitation, Sephadex G-200 gel filtration and SP-Sephadex C-50 column chromatography (Fig. 1). The specific activities, total protein content and percent recoveries during the whole purification procedures for α-GalNAcase from digestive organ of hilsha fish were summarized in Table 2. α-GalNAcase was purified to 90 fold from the crude homogenate with 21% recovery. Sephadex G-200 gel filtration and SP-Sephadex C-50 column chromatography are the two important steps that have previously been shown effective in the separation of α-GalNAcases and removal of major protein contaminants [18, 20]. As seen in Fig. 1A, the α-GalNAcase was eluted as a single peak from the Sephadex G-200 gel filtration, and a fraction of protein contaminants was eliminated. Further it was clearly observed that although the enzyme, α-GalNAcase was eluted as a single peak but the peak is slightly broad rather than sharp indicating that this fraction also contained other components. The fractions containing the enzyme activity (8.0 mL, Fig. 1A) were pooled together and subsequently purified by ion exchange chromatography. In the SP-Sephadex C-50 ion exchange chromatography (Fig. 1B), α-GalNAcase was eluted as a sharp peak with 50 mM sodium citrate buffer (pH 4.8) containing 200 mM sodium chloride. The final preparation of α-GalNAcase (specific activity 15.21 unit/mg) showed 3.02% α-galactosidase activities, which were co-eluted with α-GalNAcase activities during Sephadex G-200 gel filtration and SP Sephadex C-50 column chromatography.

Table 2. Summary of purification of α-GalNAcase from 160 g digestive organ of hilsha fish.

[a]Procedures	α-GalNAcase activity (Unit)	Total protein (mg)	S. A. (unit/mg)	Recovery (%)	Purification (fold)	α-Galactosidase/ α-GalNAcase
Homogenate	709	4189	0.169	100.0		0.328
Supernatant	626	2 337	0.268	88.3	1.6	0.252
20%$(NH_4)_2SO_4$ solution	554	1345	0.412	78.1	2.4	0.127
20-40% $(NH_4)_2SO_4$ ppt	324	185.5	1.747	45.7	10.3	0.051
Sephadex G-200	208	69.7	2.984	29.3	17.6	0.032
SP Sephadex C-50	149.7	9.84	15.21	21.1	90.0	0.030

[a]Purification was performed three times with similar results. S. A., Specific activity.

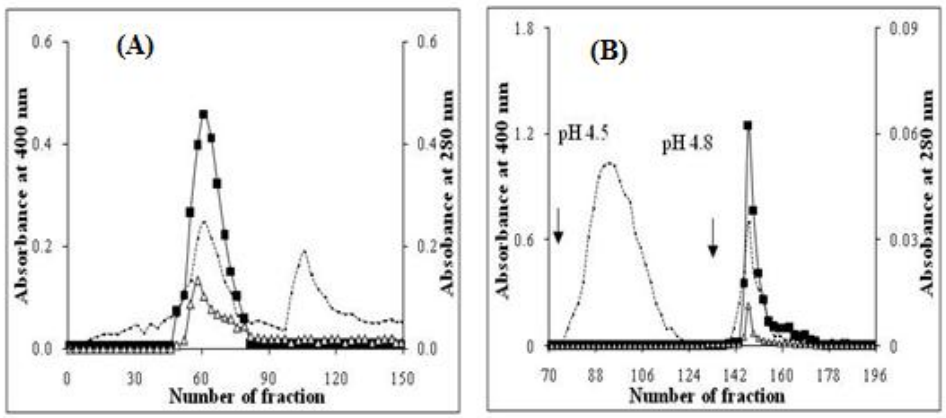

Fig. 1. Purification of α-GalNAcase enzyme by column chromatography.

(A) Sephadex G-200 gel filtration. The enzyme solution (10 mL) obtained from ammonium sulphate precipitation containing 60 mg protein was applied to a Sephadex G-200 column (3.0 cm × 75 cm) which had been equilibrated with 50 mM sodium citrate buffer, pH 4.2. The column was eluted with the same buffer at flow rate 8.0 ml per hour and 8.0 mL fractions were collected.

(B) SP Sephadex C-50 column chromatography. The enzyme solution obtained after Sephadex G-200 gel filtration containing 35.00 mg protein was applied to the column (2.0 cm × 20 cm) which had been equilibrated with 50 mM sodium citrate buffer, pH 4.0. The column was washed with the initial buffer (Fraction 1-70) and then with pH 4.5 (Fraction 71-130) and eluted with pH 4.8 of the same buffer containing 200 mMNaCl at a flow rate 10 mL/hour and 8.0 mL fractions were collected. --■--, α-GalNAcase activity; --Δ--, α-galactosidase activity; -----, absorbance for protein at 280 nm.

3.2. *Properties of α-GalNAcase enzyme*

3.2.1. *Purity and homogeneity of enzyme*

The purity of the α-GalNAcase isolated from hilsha fish was assessed on SDS-PAGE. The purified enzyme showed a clear band on SDS-PAGE (Fig. 2A) when stained with Coomassie brilliant blue. The enzyme is a monomer and its molecular mass is 48 kDa as estimated by comparing the relative mobility of α-GalNAcase protein band from the standard calibration curve (Fig. 2B).

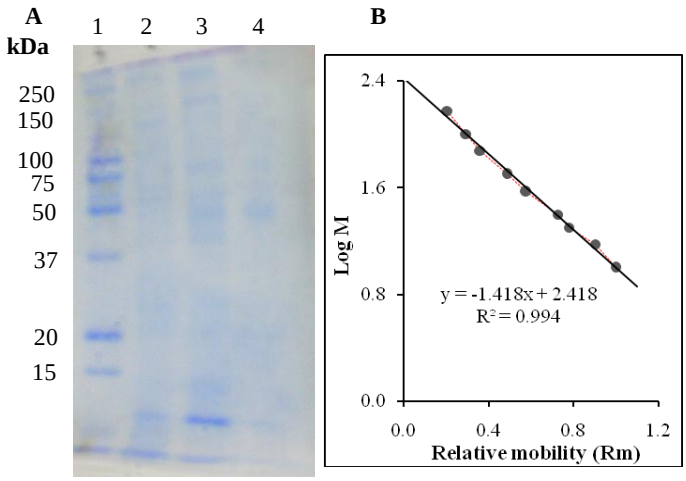

Fig. 2. Determination of molecular mass of α-GalNAcase enzyme by SDS-PAGE.

(A) SDS-PAGE of purified α-GalNAcase enzyme from hilsha fish. About 10 µg protein sample was denatured, reduced and then subjected to electrophoresis on 12.5% polyacrylamide gel using Tris-glycine buffer (pH 8.3) in presence of 0.1% SDS. The protein bands on SDS-PAGE were visualized by CBB staining method. 1, Standard marker; 2, α-GalNAcase enzyme fraction obtained from 20-40% ammonium sulphate precipitation, 3, Fraction obtained from Sephadex G-200 gel filtration and 4, Purified α-GalNAcase by SP Sephadex C-50 column. (B) Calibration curve to determine the subunit mass by SDS-PAGE. The relative mobility (Rm) of standard protein on SDS-PAGE was plotted against the respective log of molecular weight (LogM).

3.2.2. *Effect of pH and buffer on enzyme activity*

The effect of pH on the activity of α-GalNAcase was determined in 50 mM sodium citrate buffer using pNP-α-GalNAc as a substrate. The enzyme showed a pH optimum of 4.0 (Fig. 3A). To identify the best possible buffer to be used for characterization of enzyme, various buffers at pH 4.0 were used for assessing the activity. Among the tested buffers, sodium citrate and sodium phosphate buffers were found to be most suitable for analysis (Fig. 3B).

(A) Effect of pH on the enzyme activity. α-GalNAcase activity was determined by using pNP-α-GalNAc substrate in 50 mM citrate buffer at various pH (2.1 to 7.0). (B) Effect of various buffers on α-GalNAcase activity. An aliquot of enzyme solution was incubated in various buffers, pH 4.0 for 24 hr at 0°C and α-GalNAcase activity was determinedin 50 mM citrate buffer pH 4.0.

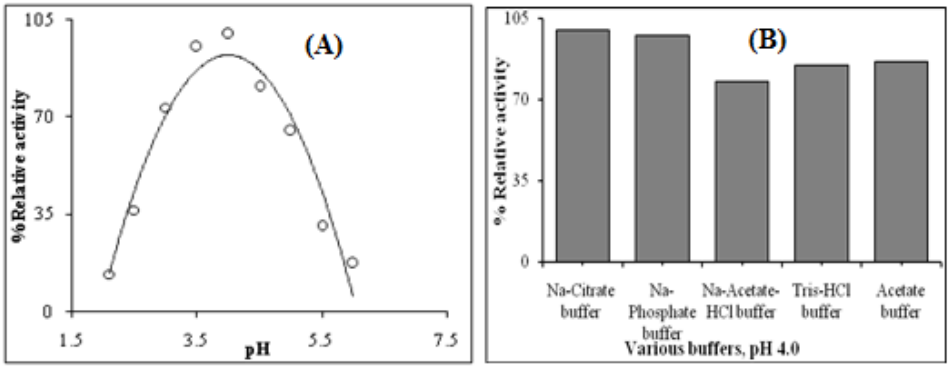

Fig. 3. Effect of pH and various buffers on α-GalNAcaseactivity.

3.2.3. *Stability studies of α-GalNAcase enzyme*

To know pH-stability of the purified enzyme α-GalNAcase, the activity at various pH values was investigated in 50 mM sodium citrate buffer, ranging in the pH 2.1 to 7.0 at 4°C for 24 h (Fig. 4A). The enzyme showed the highest activity at pH 4.0 and retained more than 80% of its activity in the pH range of 3.0 to 5.0. Moreover, heat stability test with α-GalNAcase revealed that the enzyme was quite stable as 80% activity was found to be retained on heating the enzyme at 37°C for one h (Fig. 4B).

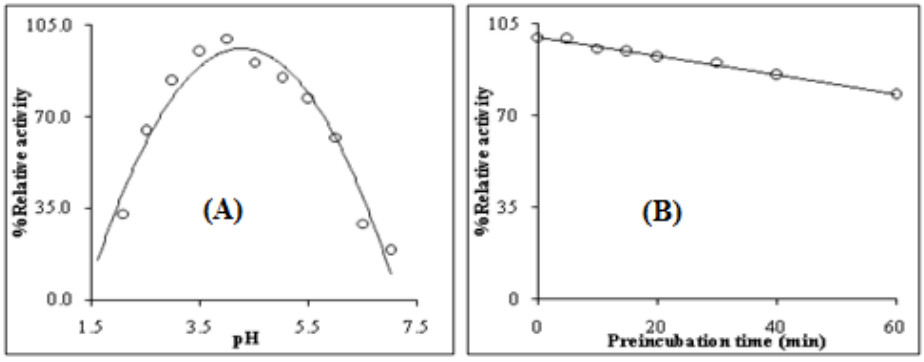

Fig. 4. Stability studies of α-GalNAcase enzyme at A) 4° and B) 37°C.

(A) pH Stability study. Aliquots of enzyme solution were incubated in 50 mM sodium citrate buffer in various pH (2.1 to 7.0) for 24 h at 0°C and residualα-GalNAcaseactivity was determined. (B) Heat stability study. Some portion of enzyme solution was heated at 37°C periodically at different period of time and the remaining α-GalNAcase activity was evaluated in 50 mM sodium citrate buffer, pH 4.0.

3.2.4. *Kinetic properties of α-GalNAcase enzyme*

To evaluate the enzyme kinetics, the initial velocities were determined against different concentration of substrate, pNP-α-GalNAc at 37°C in 50 mM sodium citrate buffer, pH 4.0. A straight line was obtained from the lineweaver Burk plot (1/[S] vs 1/[V]) indicating that α-GalNAcase activity was increased with the increase of substrate concentration and enzyme catalyzed hydrolysis of substrate followed the standard Michaelis-Menten kinetics (Fig. 5A). From the Fig. 5A, the K_M and V_{max} of α-GalNAcase against pNP-α-GalNAc were found to be 3.31 mM and 35.04 unit/mg, respectively. Moreover, in the experiment of "dose dependency of enzyme activity", we observed that at low enzyme concentration there was a linear relationship between enzyme concentration and its activity but at high dose the α-GalNAcase activity gradually slowed down as a result of the saturation of substrate (Fig. 5B). All the enzymatic properties of α-GalNAcase were summarized in the Table 3and compared with that from star fish [20]. The K_M value of α-GalNAcase from hilsha fish against pNP-α-GalNAc was very similar to that of α-GalNAcase II from starfish, while the *Vmax* value was similar to that of α-GalNAcase I.

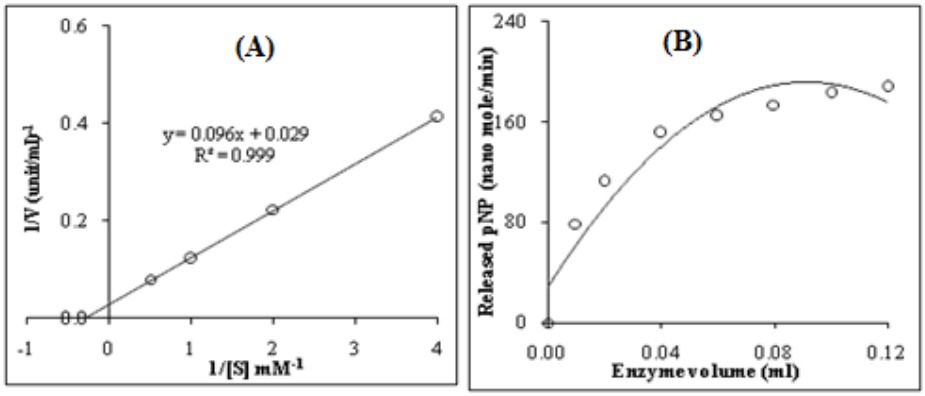

Fig. 5. Kinetic studies of α-GalNAcase enzyme from hilsha fish.

(A) Determination of K_M and V_{max}. Graph plotted for reciprocal of α-GalNAcase activities (1/V) against reciprocal of substrate concentrations (1/[S]). (B) Determination of dose dependent activity of α-GalNAcase enzyme.

Table 3. Summary of physicochemical characteristics of α-GalNAcase from digestive organ of hilsha fish and comparison with that of star fish.

Physicochemical parameter	[a]Hilsha fish	[b]Starfish	
	α-GalNAcase	α-GalNAcase-I	α-GalNAcase-II
Subunit mass (kDa)	48	47	43
Optimum pH	4.0	3.5	3.0
pH stability (4°C)	2.5-6.0	2.7-4.5	3.5-8.5

Physicochemical parameter	[a]Hilsha fish	[b]Starfish	
	α-GalNAcase	α-GalNAcase-I	α-GalNAcase-II
Heat stability (37°C, pH 4.0)			
Residual activity in 60 minutes	80%	50%	92%
% α-Galactosidase	3.02	6.74	0.29
K_M value (mM)	3.31	1.04	3.97
V_{max} value (μmole/min/mg)	35.04	49.5	126.6
Specific activity (unit/mg)	15.21	19.56	24.2

[a]Data are mean values of three determinations. [b][20]

3.2.5. *Effect of various Inhibitors on α-GalNAcase enzyme*

The effects of various metal ions and carbohydrates on α-GalNAcase from hilsha fish were investigated. Result (Table 4) showed that the activities of α-GalNAcase were significantly inhibited by silver (Ag^{+}) and mercury (Hg^{+2}) salts. The percent of inhibition of α-GalNAcase by silver and mercury was similar to that of α-GalNAcase I from starfish [20]. From this observation it can be suggested that sulfhydryl group (-SH) may be located at or near the active site of the enzyme and both Ag+ and Hg^{+2} salts can bind to the thiol (-SH) group of proteins forming stable complexes in a way to inhibit the activity of theenzyme. Among the monosaccharide tested as inhibitors, galactose and GalNAc inhibited α-GalNAcase activity 24% and 42%, respectively (Table 4). The result of this study (the product, GalNAc strongly inhibited α-GalNAcase activity) also agreed well with previous reports that product inhibition had profound effect on the regulation of α-GalNAcase activity [26,27].

Table 4. Inhibitory studies of α-GalNAcase enzyme from hilsha fish.

*Inhibitors		Inhibition of Activity (%)
Name	Conc.(mM)	
Control	0	0.0
$AgNO_3$	1.0	33.7
$HgCl_2$	1.0	26.9
$ZnSO_4$	1.0	5.1
$CdCl_2$	1.0	19.1
$CaCl_2$	1.0	0
$CuSO_4$	1.0	0
KCl	1.0	0
NaCl	1.0	1.2
Galactose	10.0	24
Glucose	10.0	1.4
GalNAc	10.0	42
GlcNAc	10.0	0.9
Mannose	10.0	0
Fructose	10.0	0

*An aliquot of enzyme solution was incubated in various inhibitor solutions at 0°C for 24 hr. The remaining α-GalNAcase activity was evaluated with pNP-α-GalNAc substrate in 50 mM sodium citrate buffer, pH 4.0. Data are mean values of three determinations.

4. Conclusion

In summary, we have purified a α-GalNAcase form hilsha fish that contained 3.02% α-galactosidase activity. The molecular mass and enzymatic properties such as optimum pH, pH stability, thermal stability and inhibition effect, and α-galctosidase activity of α-GalNAcase from hilsh fish were similar to that of α-GalNAcase I (α-galctosidase B) from starfish. Further studies on substrate specificity and protein structure of hilsha fish α-GalNAcase may prove useful in bioanalytical and biotechnological application.

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Abbreviations: α-GalNAcase, α-*N*-acetylgalactosaminidase; EC, Enzyme commission; FCR, Folin-ciocalteau reagent; Gal, galactose; K_M, Michaelis-Menten constant; V_{max}, maximum velocity; GalNAc, *N*-acetylgalactosamine.

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