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Isolation and Properties of β-N-Acetyl-D-glucosaminidase B from Rat Uterus

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β-N-Acetyl-D-glucosaminidase B was highly purified with the following sequence of steps; DEAE-cellulose, CM-cellulose, and Sephadex G-200 gel filtration chromatograpies. The specific activity of the purified β-N-acetyl-D-glucosaminidase B was 2.2 units/mg protein with 12.9 % yield and 196.2 fold purity. The purified β-N-acetyl-D-glucosaminidase B showed single band on polyacrylamide gel electrophoresis. The final preparation of β-N-acetyl-D-glucosaminidase B was completely free from arylsulfatase and β-glucuronidase. β-N-Acetyl-D-glucosaminidase B had pH optimum of 4.5 in 0.5 *M* sodium citrate buffer. The molecular weight of β-N-acetyl-D-glucosaminidase B was 133,000 by Sephadex G-200 gel filtration. The K_m value of β-N-acetyl-D-glucosaminidase B using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate was 1.0 m*M* and V_{max} was 0.014 μ mole/min.. β-N-Acetyl-D-glucosaminidase B was stable at 55°C for 70 minutes. The crude β-N-acetyl-D-glucosaminidase in 70 % ammonium sulfate retained 93 % activity after 7 months storage at −26°C. Bovine serum albumin, sodium chloride, and phosphate activated β-N-acetyl-D-glucosaminidase B. N-Acetyl-D-glucosamine, α-methyl-D-mannoside, and acetate inhibited β-N-acetyl-D-glucosaminidase B.

Introduction

β-N-Acetyl-D-glucosaminidase is widely distributed in mammalian tissues and has been found in many reproductive organs[1-4]. Uterus and epididymis are known to be the richest source of β-N-acetyl-D-glucosaminidase[5]. This enzyme exists in two major forms, β-N-acetyl-D-glucosaminidase A and B[6]. Heating at 50°C destroys the activity of the acid form, β-N-acetyl-D-glucosaminidase A, while the basic from, β-N-acetyl-D-glucosaminidase B, is stable[7]. β-N-Acetyl-D-glucosaminidase B is considered to be a precursor of β-N-acetyl-D-glucosaminidase A[8]. Carrol and Robinson[9] have demonstrated that β-N-acetyl-D-glucosaminidase A and B are related to each other, despite differences in heat stability and electrophoretic mobility. The absence of both β-N-acetyl-D-glucosaminidase A and B activity in Sandhoff's disease suggests the existence of a structural and genetic relationship between two isozymes[10].

It has been found that uterus has a high ability to develop sperm capacitation, a process by which sperm achieve the capacity to penetrate the ovum in the female reproductive tract[11]. Capacitation of sperm was investigated by injection of sperm into the uterus and the sperm found within cumulus after mating showed acrosomal changes characteristic of capacitation[12]. Dukelow[13] suggested that capacitation was an enzymatic removal of seminal plasma membranes on sperm. The biochemical mechanism of sperm capacitation in the female reproductive organ is still undefined and the exact role of enzymes involved in that process is also uncertain.

In the present paper, we report the purification and some properties of β-N-acetyl-D-glucosaminidase B from rat uterus as an initial step of research in sperm capacitation.

Materials and Methods

Materials. Rat (Sprague-Dowley, about 9 months old) was obtained from the Animal Breeding Center of Seoul National University. p-Nitrophenyl-N-acetyl-β-D-glucosaminide, DEAE-cellulose, *p*-nitrophenyl-β-D-glucuronide, and *p*-nitrocatechol sulfate dipotassium salt were purchased from Sigma Chemical Co.. Sephadex G-200 was obtained from Pharmacia Fine Chemicals. CM-cellulose was obtained from Whatman Ltd.. All other reagents used were the highest purity available.

Assays. β-N-Acetyl-D-glucosaminidase activity was determined using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate by the method of Tarentino and Maley[14]. The assay mixture consists of 0.3 m*l* of 0.5 *M* sodium citrate buffer

(pH 4.5), 0.1 m*l* of 0.012 *M* substrate and 0.1 m*l* of enzyme. The mixture was incubated for 30 minutes at 38°C. After reaction, add 2.5 m*l* of 0.1 *M* Na_2CO_3 and read O. D. at 400 nm. One unit is defined as the amount enzyme that hydrolyzes 1μ mole of substrate per minute. β-Glucuronidase activity was determined using *p*-nitrophenyl-β-D-glucuronide as substrate by the method of Harris *et al.*[15], and read O. D. at 400 nm. Arylsulfatase activity was determined using *p*-nitrocatechol sulfate as substrate by the method of Yang and Srivastava[16], and read O. D. at 515 nm. Protein was estimated by the method of Lowry *et al.*[17] using bovine serum albumin as reference.

Preparation of crude extract. The matured rat uterus was weighed and minced with scissors. These tissues were solubilized in 3 volumes of 0.05 *M* Tris-HCl buffer (pH 7.2). The suspension was homogenized for 20 minutes at 40 rpm using Talboys Prestle, and sonicated for 15 minutes (output 6, dutycycle 50 %) using Ultrasonic Sonicator at 4 °C. The mixture was centrifuged at 20,000 rpm for 30 minutes and the precipitates were removed.

Purification

Step I. Ammonium sulfate fractionation. The centrifuged supernatants were brought to 70 % saturation with $(NH_4)_2SO_4$ at 4°C and allowed to stand for 2 hours. The solution was centrifuged at 20,000 rpm for 30 minutes and the supernatants were removed. The precipitate was solubilized in the 0.05 *M* Tris-HCl buffer (pH 7.2), and dialyzed overnight at 4 °C against the Tris-HCl buffer. The nondialyzable solution (21 m*l*)was centrifuged and the precipitates were removed.

Step II. DEAE-cellulose chromatography. The dialyzed material was applied to a column of DEAE-cellulose (2.8×20 cm) at 4°C equilbrated with 0.05 *M* Tris-HCl buffer (pH 7.2). The column was washed with the same buffer and developed with a linear gradient of 0.5 *M* NaCl at the rate of 42 m*l* per hour. The fractions containing β-N-acetyl-D-glucosaminidase B activity were collected and concentrated to 10 m*l* with Amicon Diaflo ultrafiltrater using PM-10 filter. The concentrated enzyme was dialyzed against 0.02 *M* sodium phosphate buffer (pH 6.0) at 4 °C.

Step III. CM-cellulose chromatography. The dialyzed enzyme was applied to a column of CM-cellulose (2.8×12 cm) at 4°C equilibrated with 0.02 *M* sodium phosphate buffer (pH 6.0). The column was washed with the same buffer and developed with a linear gradient of NaCl at the rate of 42 m*l* per hour. The fractions containing β-N-acetyl-D-glucosaminidase B activity were collected and concentrated to 2 m*l*.

Step IV. Sephadex G-200 chromatography. The concentrated enzyme was applied to a column of Sephadex G-200 chromatography (2.0×45 cm) which had been equilibrated with the 0.02 *M* sodium phosphate buffer (pH 6.0). The column was washed with the same buffer at the rate of 14 m*l* per hour.

Molecular weight determination. The molecular weight was estimated by chromatography on a column of Sephadex G-200 by the procedure of Andrews[18]. The void volume of the column was determined with Blue Dextran 2000. The column was eluted with 0.05 *M* Tris-HCl buffer, pH 7.2 containing 0.05*M* NaCl. Catalase, human γ-globulin, bovine serum albumin, ovalbumin, α-chymotrypsinogen A were used as reference proteins.

Disc gel electrophoresis. Polyacrylamide-gel electrophoresis was performed at pH 8.3 on a 7.5 % gel at room temperature by the method of Brewer and Ashworth[19].

Results and Discussion

The crude homogenate from rat uterus was ammonium sulfate fractionated with 2.9 fold purification. The first protein peak of DEAE-cellulose column fractions showed β-N-acetyl-D-glucosaminidase B activity associated with arylsulfatase activity, and the second protein peak showed β-N-acetyl-D-glucosaminidase A activity associated with arylsulfatase and β-glucuronidase activities (Figure 1). In this work, the fractions containing β-N-acetyl-D-glucosaminidase B activity were collected and further purified. From CM-cellulose column, most protein was removed from β-N-acetyl-D-glucosaminidase B activity (Figure 2). β-N-Acetyl-D-glu-

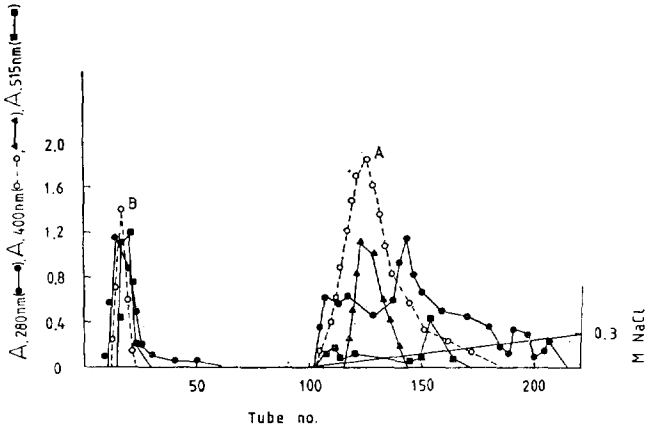

Figure 1. Elution profile of rat uterus homogenate by DEAE-Cellulose column chromatography. Protein was eluted with 0.05 *M* Tris-HCl buffer, pH 7.2 followed by a NaCl gradient. The column dimension was 2.8×20 cm and 5 ml fractions were collected. ●, Protein; ○, β-N-Acetyl-D-glucosaminidase; ■, Arylsulfatase; ▲, β-Glucuronidase.

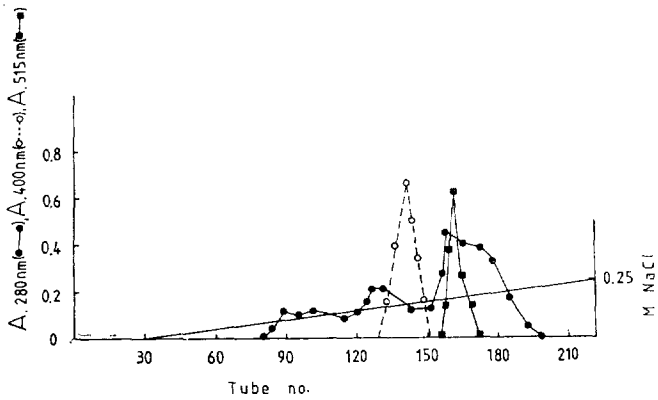

Figure 2. CM-cellulose column chromatograhy of β-N-acetyl-D-glucosaminidase B active fractions from DEAE-cellulose column. The column was eluted with 0.02 *M* sodium phosphate buffer, pH 6.0, followed by a NaCl gradient. The column dimension was 2.8×12 cm and 3 m*l* fractions were collected. ●, Protein; ○, β-N-Acetyl-D-glucosaminidase B; ▩, Arylsulfatase.

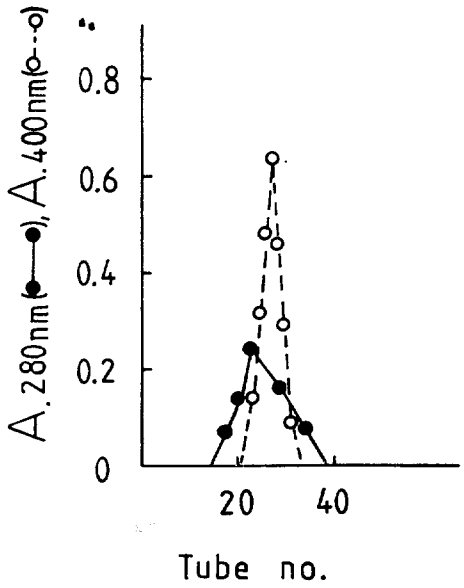

Figure 3. Sephadex G-200 column chromatograhy of β-N-acetyl-D-glucosaminidase B active fractions from CM-cellulose column. The column was eluted with 0.02 *M* sodium phosphate buffer, pH 6.0. The column dimension was 2.0×45 cm and 3 mf fractions were collected. ●, Protein; ○, β-N-Acetyl-D-glucosaminidase B.

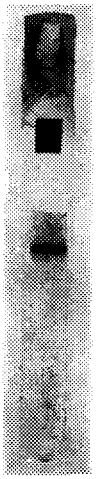

Figure 4. Polyacrylamide gel electrophoresis of finally purified β-N-acetyl-D-glucosaminidase B. Sixty micrograms of protein were used. The electrophoresis was performed at pH 8.3 on a 7.5 % gel at room temperature.

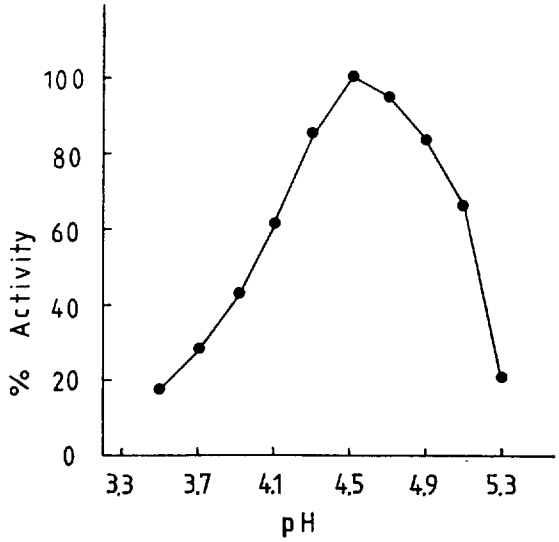

Figure 5. Effects of pH on the hydrolysis of *p*-nitrophenyl-N-acetyl-β-D-glucosaminide by β-N-acetyl-D-glucosaminidase B.

TABLE 1: Purification Table of β-N-Acetyl-glucosaminidase B from Rat Uterus

Step	Total unit (units)	Specific activity (units/mg)	Yield (%)	Purificatoin (fold)
Homogenate	31.9	0.011	100	1.2
Ammonium sulfate fractionation	28.1	0.032	88.1	2.9
DEAE-celluose	8.5	0.098	26.7	8.9
CM-cellulose	5.9	0.983	18.5	89.4
Sephadex G-200	4.1	2.158	12.9	196.2

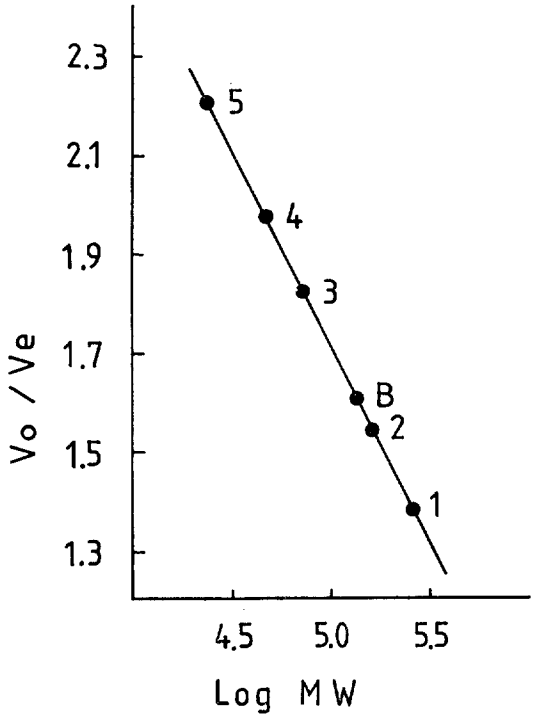

Figure 6. Estimation of molecular weight by Sephadex G-200 gel filtration. The experimental conditions are described in the text. 1, catalase; 2, γ-globulin; 3, bovine serum albumin; 4, ovalbumin; 5, α-chymotrypsinogen A; B, β-N-acetyl-D-glucosaminidase B.

cosaminidase B activity was in sodium phosphate buffer containing NaCl, and it was free of arylsulfatase activity. From Sephadex G-200 column, β-N-acetyl-D-glucosaminidase B was completely purified with 196.2 fold purification (Figure 3). The high degree of purity of rat uterus β-N-acetyl-D-glu-glucosaminidase B obtained by the above procedures was shown in Table 1. The highly purified β-N-acetyl-D-glucosaminidase B showed single band on polyacrylamide gel electrophoresis (Figure 4). Rat uterus β-N-acetyl-D-glucosaminidase B showed its maximum activity at pH 4.5 in 0.5 *M* sodium citrate buffer (Figure 5). The optimum pH of β-N-acetyl-D-glucosaminidase from pig epididymis was previously reported at pH 4.3 [20]. The molecular weight of rat uterus β-N-acetyl-D-glucosaminidase B estimated by Sephadex G-200 column (2.1×88 cm) was about 133,000 similar with the values of around 130,000 obtained by gel filtration [21,22] (Figure 6). The K_m of β-N-acetyl-D-glucosaminidase B using *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate was found to be 1.0 m*M* and V_{max} was 0.014 μmole per minute on Lineweaver-Burk plot (Figure 7). Rat uterus

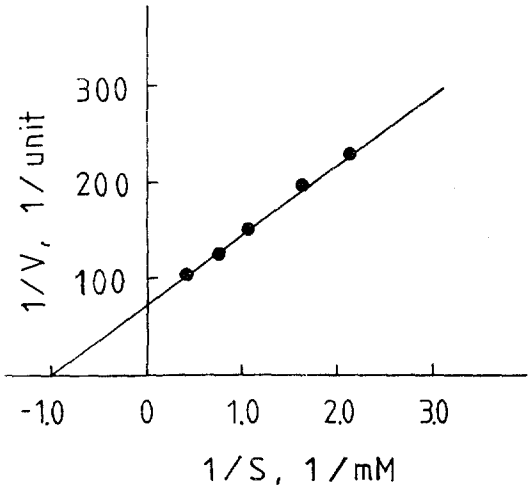

Figure 7. Lineweaver-Burk plot for the determination of K_m and V_{max}. The action of rat uterus β-N-acetyl-β-D-glucosaminidase on *p*-nitrophenyl-N-acetyl-β-D-glucosaminide was assayed using highly purified enzyme at 38°C in 0.5*M* sodium citrate buffer, pH 4.5.

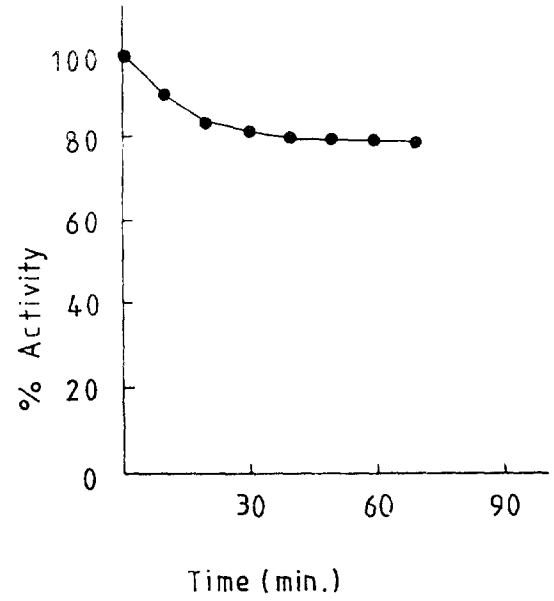

Figure 8. Heat stability of rat uterus β-N-acetyl-D-glucosaminidase B. The enzyme was incubated at 55°C for various time and assayed remained activity.

β-N-acetyl-D-glucosaminidase B was stable at 55°C for 70 minutes incubation in accord with the description by Robinson and Stirling [6]. After incubation it showed 78 % activity (Figure 8). Crude rat uterus β-N-acetyl-D-glucosaminidase in Tris-HCl buffer (pH 7.2) was stable at room temperature for about a week, and ammonium sulfate fractionated enzyme retained 93 % activity after 7 months storage at −26°C. N-Acetyl-D-glucosamine has been known as the most effective inhibitor of β-N-acetyl-D-glucosaminidase [23]. N-Acetyl-D-glucosamine, α-methyl-D-mannoside, and acetate inhibited rat uterus β-N-acetyl-D-glucosaminidase B. Several cations tested didn't have much effect on rat uterus β-N-acetyl-D-glucosaminidase B (Table 2). However, Verpoorte[24] reported that Hg^{2+} and Ag^{+} strongly inhibited the enzyme. Sodium chloride, bovine serum albumin and phosphate activated rat uterus β-N-acetyl-D-glucosaminidase B.

TABLE 2: Effects of Various Substances

Substance	Concentration (m*M*)	Relative activity (%)	Remark
N-acety-D-glucosamine	10	34	Inhibition
α-Methyl-D-mannoside	10	75	Inhibition
NaCl	10	151	Activation
Co^{2+}	10	100	
Cu^{2+}	10	100	
K^{+}	10	95	
Mg^{2+}	10	98	
Ca^{2+}	10	109	
Zn^{2+}	10	101	
Mn^{2+}	10	106	
PO_4^{2-}	10	158	Activiaton
SO_3^{2-}	10	95	
SO_4^{2-}	10	100	
CH_3COO^{-}	10	74	Inhibition
Bovine serum albumin	0.1 %	155	Activation
Control		100	

Human β-N-acetyl-D-glucosaminidase has been interestingly studied mainly due to its relevance to several genetic disorders resulting in lipid storage disease, including Tay-Sachs disease and Sandhoff's disease. Now, the model for the molecular structure of β-N-acetyl-D-glucosaminidase A and B is considered in view of the genetic disorders with which they are involved[25].

Mammalian spermatozoa are surrounded by a highly specialized continuous plasma membrane which consists of several glycoproteins. During sperm capacitation, sperm plasma membrane was known to undergo ultrastructural changes as the spermatozoon passes through the female genital tract [26]. The vesiculation of the plasma and outer acrosomal membrane during that process may be caused by the function of uterus hydrolytic enzymes. But, the possible biological function of uterus β-N-acetyl-D-glucosaminidase during that process has not been studied. Although our work does not provide a quantitative evidence that glycoproteins of sperm plasma membrane are the substrates for β-N-acetyl-D-glucosaminidase, a recent paper by Nicolson and Yanagimachi[27] suggest that the sperm plasma membrane is indeed a glycoprotein containing N-acetyl-D-glucosamine-like residues. Therefore, the effective acrosomal reaction in the sperm must involve the use of β-N-acetyl-D-glucosaminidase. This paper considered for the first time the involvement of uterus β-N-acetyl-D-glucosaminidase in sperm capacitation process. The activity of these enzymes on the glycosidic bond of plasma glycoproteins may activate the sperm to penetrate and fertilize the egg. Although no evidence was supplied from our results for the involvement of rat uterus β-N-acetyl-D-glucosaminidase in sperm capacitation, the enzyme obtained in this paper could be considered for testing the effect on the process in the future.

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Synthesis and Properties of Thermotropic Compounds with Two Terminal Mesogenic Units and a Central Spacer (II). Homologous Series of α,ω-Bis(4-*p*-substituted phenoxycarbonyl)phenoxyalkanes

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Two series of thermotropic compounds were prepared and their thermal and liquid crystal properties were examined by differential scanning calorimetry and on the hot-stage of a cross-polarizing microscope. The first series of the compounds has two terminal mesogenic units based on unsubstituted and substituted *p*-(phenoxycarbonyl) phenyl ethers bracketing a central decamethylene spacer, and the second has 4-(*p*-phenylphenoxycarbonyl) phenyl ether moiety as the two terminal mesogenic units and central polymethylene spacers of varying lengths. A thermodynamic analysis of the phase transitions was made and explained in relation to structures and thermotropic behavior of the compounds.

Introduction

Recently we reported on the thermotropic behavior of several different series of polyesters which contained mesogenic units of dyad or triad aromatic esters interconnected through flexible spacers such as polymethylene or polyoxyethylene or disiloxyl groups.[1–5] While those investigations were being conducted, it became of interest to us to compare the structure-property relationships of the polymers with those of low molecular liquid crystal compounds containing two terminal mesogenic units on either side of a central flexible spacer.

Although numerous studies on the structure–liquid crystal property relationships of a wide variety of compounds have been reported[6] since Reinitzer's first observation was made on