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PURIFICATION AND PROPERTIES OF RAT GASTROCNEMIUS MUSCLE N-ACETYL-ß-D-GLUCOSAMINIDASE A AND B.

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ABSTRACT

N-acetyl-ß-D-Glucosaminidase was purified by affinity and ion-exchange chromatography. Two major, A and B, and three minor intermediate forms were isolated and characterized. NAG-A and NAG-B were purified 440 and 1200 fold with final yields of 16 and 23 percent respectively. Each activity was represented by a single protein band. After 70 min preincubation at 55°C a loss of 70% activity of NAG-A and 30% activity of NAG-B respectively was observed. Divalent metal ions had no significant effect on either enzyme activity. N-acetyl-D-glucosamine was determined to be a competitive inhibitor for both activities. The method of purification reported here will be of significance in providing larger quantities for the better understanding of both Tay-Sach's and Sandhoff's diseases.

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INTRODUCTION

N-acetyl-ß-D-glucosaminidase (2-acetamido-2-deoxy-ß-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) catalyses the hydrolysis of terminal ß-linked N-acetyl-glucosamine residues of glycoproteins and glycolipids of tissues.[1] The enzyme is widely distributed and has been reported to exist in two major molecular forms, A and B along with 1 to 3 intermediate forms in various tissues including human spleen,[2] human liver,[3] human kidney,[4] and rat brain[5]. Interest in this enzyme was greatly stimulated by the demonstration of a specific deficiency of component A in tissues from patients with Tay-Sachs disease[6] while in Sandhoff's disease the activity of both the A and B components is deficient.[7] Considerable differences in the enzyme have also been reported in normal and atrophic mammalian gastrocnemius muscle.[8]

In view of the involvement of these two isoenzymes and their interrelationship in several lipid-storage diseases, it seems of importance to isolate them in adequate amounts in a pure state so as to enable their chemical characterisation.

In the present paper we describe the purification and properties of glucosaminidase A and B from rat gastrocnemius muscle by ion-exchange chromatography followed by affinity chromatography on a concanavalin A-sepharose (Con-A) column.

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MATERIAL AND METHODS:

Animals: Adult male Wistar rats were used in all experiments.

Chemicals: 4-Methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside, Acrylamide, Tris (2-amino-2 (hydroxymethyl) 1,3-propanediol, Bis (N, N) methylenediacrylamide) were obtained from Koch-Light Laboratories, Colbrook, England. SDS-molecular weight markers, Concanavalin A-sepharose 4B, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, were purchased from Sigma Chemical

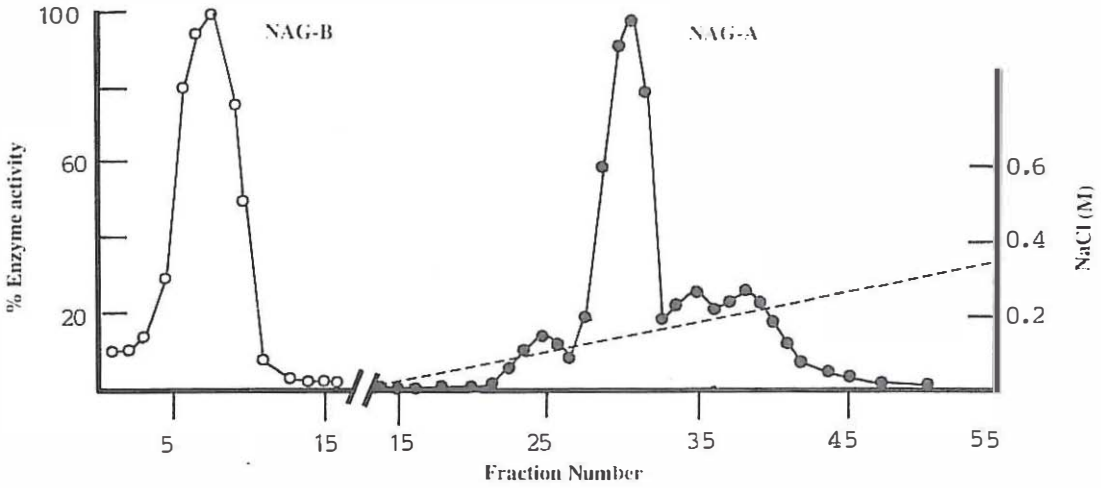

Fig.1. Elution pattern from DEAE-cellulose column.

Co., U.S.A. Cation exchanger CM-52, anion exchanger DE-52 were purchased from Whatman Biochemicals Ltd., Kent, England. All other chemicals employed in this study were of suitable analytical grade.

Preparation of Sample: All operations were carried out at 4 °C unless otherwise mentioned. The animals were killed by cervical dislocation. Gastrocnemius muscles were removed and homogenised in 0.01M potassium phosphate buffer, pH 6.0, to give a 16% (w/v) homogenate. The homogenate was centrifuged at 800 g at 4 °C for 15 min and the pellet was discarded. The supernatant was centrifuged at 70,000 g for 30 min and the pellet obtained was retained while the supernatant was discarded. The pellet was resuspended in 0.01 M-potassium phosphate buffer, pH 6.0, containing 0.1 M NaCl and 1% (w/v) sodium taurocholate. The suspension was sonicated for 90 s and following stabilisation at 4 °C for 2-3h was centrifuged at 70,000 g for 45 min. The pellet was discarded and the supernatant was dialysed for 24 h with three changes against 6 litres of original buffer and then centrifuged at 70,000 g for 45 min. The supernatant (Sl) was retained for chromatography. The (Sl) contained 80% of the total homogenate activity.

Affinity Chromatography:

A column (1×15cm) was packed with Concanavalin A-Sepharose and equilibrated at 4 °C with 6 column volumes of 0.1 M sodium acetate buffer, pH 6.0, containing 0.001 M $MgCl_2$, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$ and 0.5 M NaCl. The protein sample (Sl) was applied onto the Con A-Sepharose column. Following saturation, the column was washed with 100 ml of the equilibrating buffer. N-acetyl-ß-D-glucosaminidase was found to be retained by the Con-A sepharose column which was then eluted with 200 ml of 0.5 M solution of methyl ß-D-glucopyranoside in 0.01 M potassium phosphate buffer, pH 6.2, containing 0.5 M NaCl. The flow rate was maintained at 50 ml/h.

Table I. Purification of N-acetyl-ß-D-glucosaminidase

Purification (Steps)	Enzyme Activity (nmol/min)		Protein Activity (mg)		Specific activity (nmol/mg port/min)	Yield (%)	Purification (fold)
	in 50 µl	in tol.	in 50 µl	in tol.			
Total homogenate	0.230	386	0.7100	1193	0.32		
Supernatant after Treatment with Na taurocholate and sonication	1.580	490	0.075	23	21.12		
Pooled fractions From Con.-A col.	1.450	384	0.025	6.5	58.0	99	
NAG-A CM-52	0.112	60	0.0008	0.4	140.0	16	440
NAG-B DEAE-52	0.380	90	0.0010	0.26	380.0	23	1200

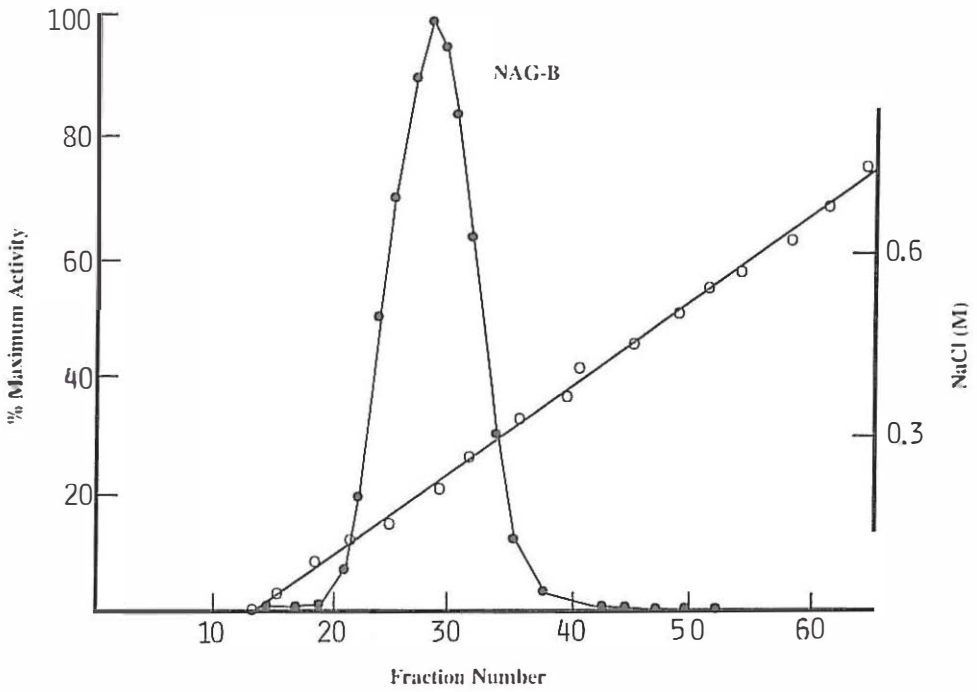

Fig. 2. CM-cellulose elution profile of N-acetyl-ß-D- glucosaminidase B form (NAG-B).

Enzymatically active samples were pooled and concentrated by ultrafiltration with a PM-10 membrane and dialysed for 24h with three changes against 4 litres of original buffer. The dialysate was then centrifuged at 70,000 g for 30 min. The supernatant (S2) was retained while any pellet formed was discarded.

Ion-Exchange Chromatography:

The retained supernatant (S2) was passed through a DEAE-cellulose DE-52 column (1.2 × 22 cm) previously equilibrated with 0.01 M-potassium phosphate buffer, pH 6.2, containing 0.02 M NaCl. The flow rate was maintained at 40 ml/h. Under these conditions one of two major isoenzymes, NAG-B, did not bind to the DEAE-cellulose and the eluate was kept. The column was washed with 200 ml of equilibrating buffer and bound, a second major isoenzyme, NAG-A was eluted with a linear gradient (600 ml) of 0.03-0.5 M NaCl in the same buffer. The elution pattern of NAG-A and NAG-B is shown in Fig. 1. The fractions containing NAG-A activity were pooled and concentrated by ultrafiltration with a PM-10 membrane. The concentrated sample was dialysed for 24 h with three changes against 6 litres of original buffer. The dialysate was centrifuged at 70,000 g for 30 min and the supernatant was kept for characterisation of NAG-A. Three additional (intermediate) activities (I_1,I_2 & I_3) were eluted with major NAG-A activity.

Unbound NAG-B was passed through a CM-cellulose CM-52 column (1.5× 20 cm) equilibrated

Table II. Activity of five glycosidases in purified NAG-A and NAG-B preparations of N-acetyl-ß-D-glucosaminidase from gastrocnemius muscle of rat.

Glycosidase	Substrate	% of NAG-A and NAG-B activity	
		NAG-A	NAG-B
ß-D-Glucosidase pH 4.0	4MU-ß-D-glucopyranoside	0.08	80.0
ß-D-Glucosidase pH 5.3	4MU-ß-D-glucopyranoside	0.06	60.0
α-L-Fucosidase	4MU-ß-L-fucopyranoside	0.12	0.13
α-L-Arabinosidase	4MU-ß-L-arabinoside	0.07	0.06
α-D-Mannosidase pH 4.4	4MU-ß-D-mannopyranoside	0.07	0.09
α-D-Mannosidase pH 6.4	4MU-ß-D-mannopyranoside	0.17	0.21
ß-D-xylosidase	4MU-ß-D-xylopyranoside	0.04	0.03

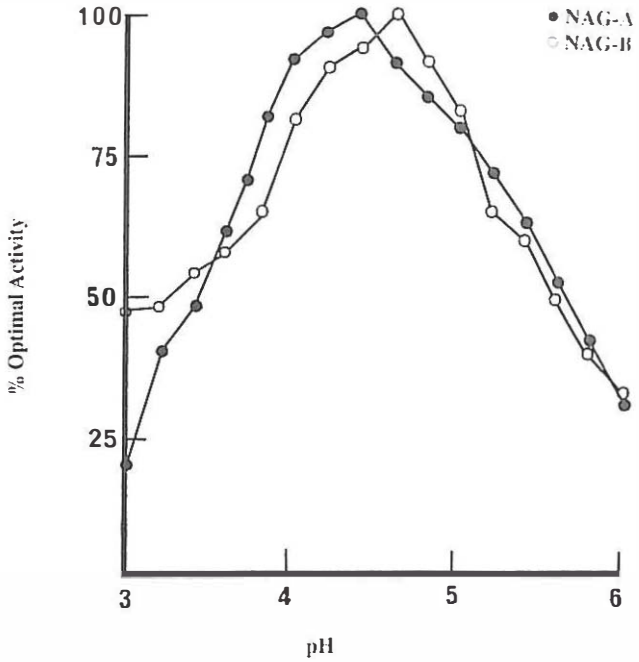

Fig. 3. The effect of pH on N-acetyl-ß-D-glucosaminidase. Assays were performed as described in "Materials and Methods".

with 0.01 M-potassium phosphate buffer, pH 5.0, containing 0.01 M-NaCl. The flow rate was maintained at 50 ml/h. The column was washed with 100 ml of the equilibrating buffer and the bound NAG-B was eluted with a linear gradient (800 ml) of 0.03-0.9 M-NaCl in equilibrating buffer. The elution pattern of CM-cellulose of NAG-B is shown in Fig.2. The fractions (10 ml) containing NAG-B activity were pooled and concentrated by ultrafiltration with PM-10 membrane. The concentrated sample was dialysed for 24 h with three changes against 6 litres of 0.01 M-potassium phosphate buffer (pH 5.0) and the dialysate was centrifuged at 70,000 g for 30 min. The supernatant was kept for characterisation of NAG-B.

Enzyme assay: Glucosaminidase activity was determined by the method previously described by us.[9]

Protein estimation: All samples were subjected to overnight digestion in alkaline (0.1M NaOH) solution. The modified method of Lowry as described by Shatkin[10] was employed for the estimation of non-collagenous preoteins. A protein standard solution of bovine serum albumin (0.5 mg/ml) was used.

Effect

pH Stability of purified enzyme forms was studied by assaying the activity under standard condiitons at various pH values (3.0-6.0) using 0.3 M citrate phosphate buffer.

Aliquots of the purified enzyme forms were preincubated at 55°C for various lengths of time (10-70 min) and assayed under the conditions described above. In metal ion study equal volumes of purified sample of enzyme forms and metal ion solutions (1 mM and 5 mM) were mixed and assayed under the standard conditions.

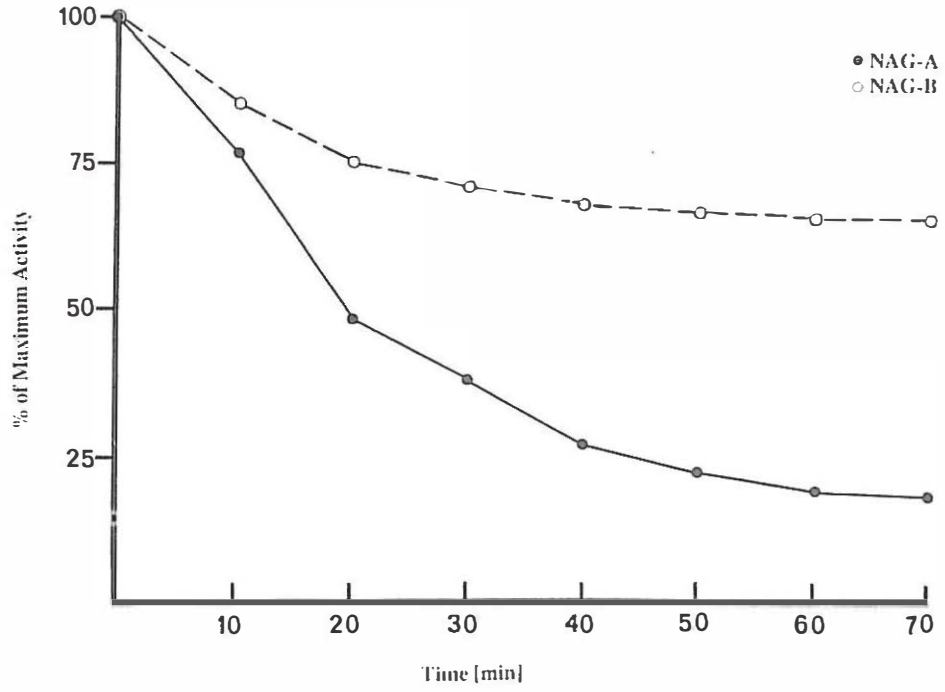

Fig. 4. The effect of temperature at 55°C on N-acetyl-ß-D-glucosaminidase.

Table III. Effect of metal ions on NAG-A and NAG-B

Ion	Con. (mM)	% of activity without metal ions NAG-A	NAG-B
Ca^{2+}	1	98±4.1	102±2.8
	5	97±3.4	107±4.3
Cu^{2+}	1	90±3.1	95±3.2
	5	80±2.9	86±4.7
Cd^{2+}	1	95±3.6	99±4.1
	5	90±4.7	95±3.9
Co^{2+}	1	96±5.1	120±2.9
	5	87±3.4	130±3.1
Zn^{2+}	1	96±1.8	102±4.2
	5	79±3.4	107±3.6
Ba^{2+}	1	85±2.7	105±3.1
	5	78±4.1	115±3.4

Electrophoresis:

Polyacrylamide gel electrophoresis of the purified enzyme forms was performed on rod gel of 8% (w/v) polyacrylamide as described by Davis[11] at pH 8.5 in Tris-glycine buffer. About 30-80 μg of protein was applied onto the gels, which were run at 5mA/tube for 6-10 h. After the electrophoresis each gel was cut into two longitudinal halves. One half was transversely sliced into 5mm pieces and each piece was homogenised in 0.2mM citrate-phosphate buffer containing. 1.25mM

4- methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside and assayed as described.[9] The other half was stained for protein by coomassie brilliant blue R. The enzyme activity was also observed under the U.V. light after the gel was incubated with enzyme substrate.

Sodium Dodecyl Sulphate (SDS) polyacrlyamide gel electrophoresis for the determination of molecular weights of purified enzymes was carried according to the method of Weber and Osborn[12] using SDS-marker proteins.

Kinetic constants and inhibition studies:

Apparant Michaelis constant (Km) and maximal velocities (Vmax) were determined for purified enzyme forms (A and B) using 4-methylumbelliferyl-2-aectamido-2-deoxy-D-glucopyranoside as substrate. Enzyme samples were incubated at 37 °C for 20 min. in quadruplicate using 100 μl of varying concentrations of substrate in 100μl of citrate-phosphate buffer at the optimum pH of the enzymes. Kinetic parameters were calulated using ICL 190 S computer and a programme entitled POLY written in fortran.

Inhibition constants (Ki) were determined by mixing the purified enzyme preparations of A and B forms with varying concentrations (5, 10 and 15 mM) of N-acetyl-D-glucosamine and assayed under standard conditions.

RESULTS

Affinity Chromatography

When supernatant (S_1) was subjected to Con-A sepharose N-acetyl-ß-d-glucosaminidase was found to be retained by the affinity resin. After the elution with ∝-D-glucopyranoside (0.5 M) it was observed that recovery of enzyme activity from Con-A sepharose column was more than 90% of the total activity applied.

DEAE-Cellulose Chromatography

The elution pattern of rat glucosaminidase (Fig.1) isoenzymes (NAG-A and NAG-B) from DEAE-cellulose column shows that NAG-B was not bound to the resin and was eluted with buffer in fractions 4-11. Whereas the NAG-A was eluted with a linear gradient of NaCl, the additional minor intermediate forms (I_1, I_2 and I_3) were eluted with NAG-A activity at the concentration of 0.12, 0.21, and 0.24 M NaCl respectively. NAG-A was eluted at a concentration of 0.16M NaCl. At a concentration of NaCl greater than 0.3M no further enzymatic activity was eluted.

CM-Cellulose Chromatography

The unobserved NAG-B from DEAE-cellulose column was passed through CM-cellulose column and elution pattern is shown in Fig 2. The NAG-B was eluted at about 0.21 M NaCl. The various stages of purification of both NAG-A and NAG-B from gastrocnemius muscles are summarised in Table I. NAG-A was purified 430-fold with a final specific activity of 137.5 n mol/mg protein/min. NAG-B was purified 1200-fold with a final specific activity of 386 n mol/mg/protein/min. The total yields of NAG-A and NAG-B were 16% and 23% respectively. The ability of glucosaminidase A & B to hydrolyse both N-acetylglucosaminides and N-acetylgalactosaminides was determined to 8.1 and 7.4 respectively. The purified proportions of NAG-A and NAG-B were found to have neligible amounts of other glycosidase activities (Table II).

Effect of pH, Temperature and Metal Ions:

The activity of purified NAG-A and NAG-B over the range 3.0 to 6.0 in citrate phosphate buffer was determined. The NAG-A and NAG-B exhibited optimum activity at pH 4.4 to 4.6 respectively (Fig.3). All three intermediate forms I_1, I_2, I_3 showed their maximum activity at pH 4.4. NAG-B had significantly higher activity at pH 3.0 than did the A form of enzyme. At pH 3.0, NAG-B retained approximately 50% of the activity observed at pH 4.6, while NAG-A retained only 20% of its activity at pH 4.4. Assay of the crude extract containing the unseparated A and B form of the enzyme revealed an intermediate level of activity (30-40%) at pH 3.0 relative to the activity at pH 4.5.

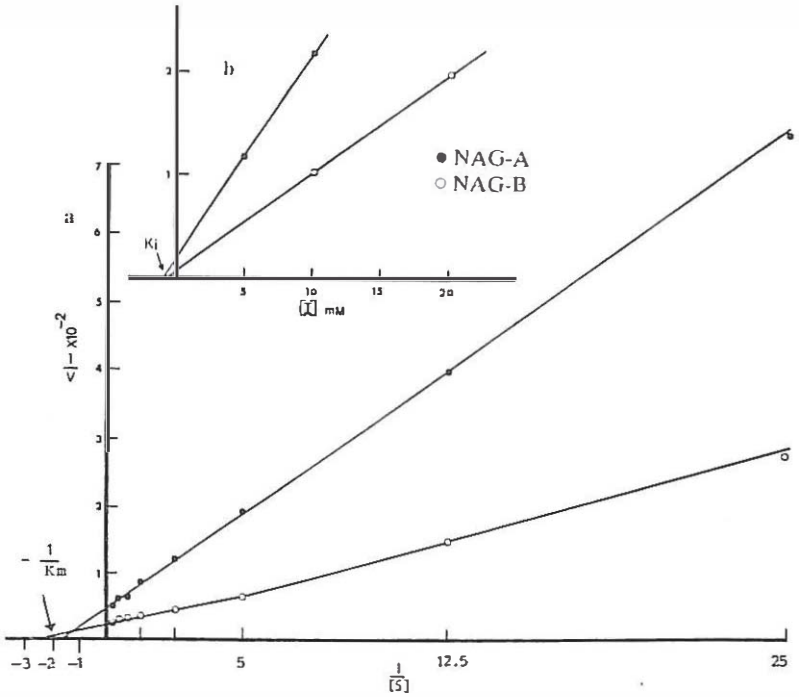

Fig.5. (A) Linewear-Burk plots of the activity of N-acetyl-ß-D- glucosaminidase.
(B) Determination of inhibition constants (Ki) for N-acetyl-ß-D- ghcosaminidase.

This level of intermediate activity for the crude extract was consistant with the proportion of the A and B forms of the enzyme.

Purified NAG-A lost 70% of the initial activity after preincubation for 70 min at 55 °C, whereas NAG-B lost only 20% of its initial activity after 70 min at 55 °C (Fig.4).

Treatment of NAG-A and NAG-B with 1mM and 5mM solutions of various metal chlorides for 30 min at 37 °C had no significant effect on either activity (Table III).

Kinetic and Inhibition Studies:

Apparent Michaelis constants (Km) for purified NAG-A and NAG-B from rat gastrocnemius muscles were determined. Double reciprocal plots based on linear regression analysis were found to be linear for NAG-A and NAG-B and isoforms (Fig.5.A). Km and Vmax values determined by using computer programme are presented in Table IV.[17] Km for NAG-A and NAG-B were 0.526 and 0.480 respectively. The corresponding Vmax values 217.4 and 575.8 nmol/mg/protein/min were obtained respectively.

N-acetyl-D-glucosamine was found to be a competitive inhibitor of NAG-A and NAG-B isoforms from rat gastrocnemius muscle under the conditions described in methods. The slope values were calculated using computer programme and plotted against the concentration of N-acetyl-D-glucosamine (Fig. 5, B). Ki value for NAG-A and NAG-B were 0.85 mM and 0.75 mM respectively (Table IV).

Table IV. Kinetic parameters of N-acetyl-ß-D-glucosaminidase purified from rat gastrocnemius muscle.

Isoenzyme	Final substrate Concentration (mM)	Km (mM)	Vmax nmol/mg protein/min	Ki (mM)
NAG-A	0.02-3.2	0.526 ±0.047	217.4 ±2.747	0.851 ±0.031
NAG-B	0.02-3.2	0.484 ±0.005	575.8 ±3.089	0.752 ±0.028

Electrophoresis and Purity of Enzyme:

Polyacrylamide disc electrophoresis of purified NAG-A and NAG-B revealed that each is presented by a single protein band containing the respective enzyme activity when stained with 4-methylumbelliferyl-ß-D-N-acetyl glucosaminide. The enzymic distribution of NAG-A and NAG-B is shown in Fig 6.

On SDS-polyacrylamide rod gel electrophoresis NAG-A exhibited the presence of one major protein band at a molecular weight of approximately 40,000 along with a faint protein band at a molecular weight of approximately 16,000. NAG-B exhibited a single band of molecular weight of about 53,000.

DISCUSSION

Both Tay-Sach's and Sandhoff's diseases result in

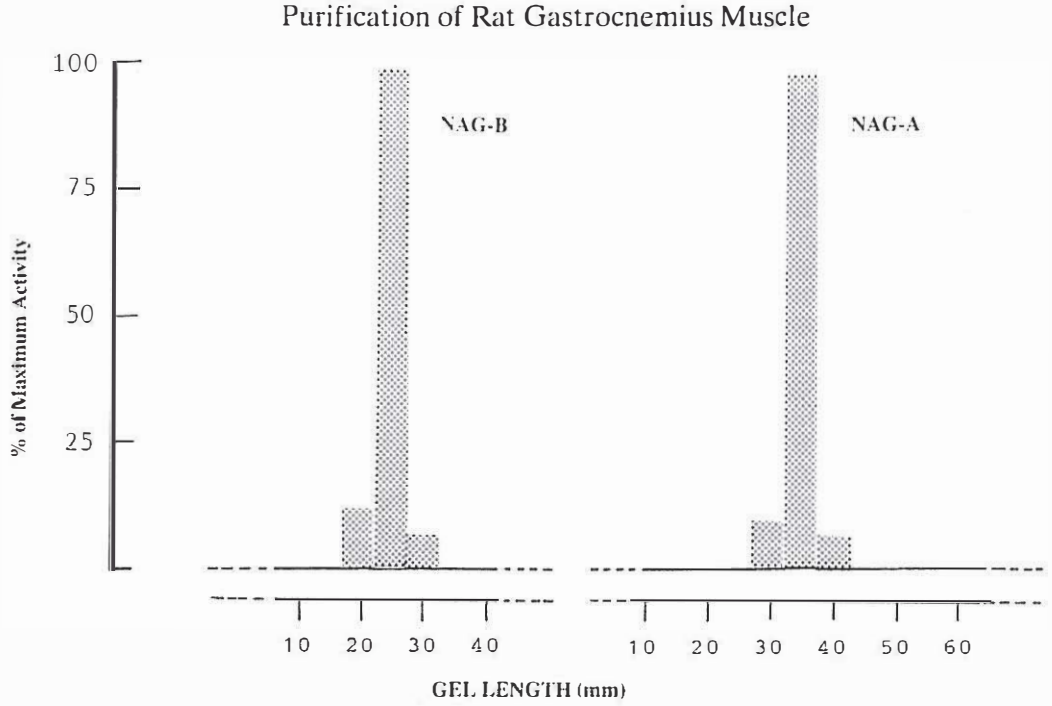

Fig.6. Distribution of the activity of N-acetyl-ß-D-glucosaminidase isoenzymes A and B purified from rat gastrocnemius muscle.

abnormal metabolism of GM_2 ganglioside. Both the disorders are characterized by progressive mental and motor deterioration. In these diseases deficiency of NAG-A or A and B together has been reported.[3,6,7] The potential of successful enzyme-replacement theraphy in lysosomal storage diseases has been greatly enhanced. Concurrently, methods are being developed and improved for the purification of homogeneous enzymes.

N-acetyl-ß-D-glucosaminidase has been purified from several mammalian visceral tissues but it has not been previously purified from rat skeletal muscle.

The method of purification of NAG-A and NAG-B reported here will be of significance in providing larger quantities as well as their detailed characterization.

We have purified NAG-A and NAG-B from rat-gastrocnemius muscle 440 and 1200 fold respectively (Table I). The purified enzymes were found to be homogeneous proteins.

Characterization of NAG-A and NAG-B of N-acetyl-ß-D-glucosaminidase indicate that NAG-B form was more stable to heat and more active at lower pH than was the NAG-A form. A similar finding was reported by Brattain, et al.[2] Treatment with metal ions had no significant effect on activity of either NAG-A and NAG-B. A similar finding was reported by Main, et al.[11] However, Izumi and Suzuki[5] observed an inhibition (28%) of isoenzyme B in the presence of 5mM Cu^{2+}. The results in the present study indicate that neither NAG-A or NAG-B require any of these metal ions for their activity or stability. The apparent Km values obtained in the present study are similar to those reported for partially purified isoforms of N-acetyl-ß-D-glucosaminidase for rat brain.[5] For both NAG-A and NAG-B the inhibition by N-acetyl-D-glucosamine was purely competitive since the ki value obtained showed no significant difference. On SDS-electrophoresis NAG-A dissociated into two protein bands corresponding to 40,000 and 16,000 molecular weights. Wiktorowicz[13] reported dissociation of NAG-A isolated from human kidney into three protein components with molecular weights of 18,000, 35,000 and 55,000. NAG-B exhibited only a single band on SDS electrophoresis. On freezing for 30 days at 20 °C, NAG-A showed NAG-B like electrophoretic mobility. The significance of this change is not clear. Presumably, it represents an alteration of the enzyme to a more heat-stable conformation. The ability of glucosaminidase A and B to hydrolyse both N-acetyl-glucosaminides and N-acetyl-galactosaminides has been reported by several workers.[2,11] From the present study it appears that the galactosaminidase activity of either NAG-A or NAG-B is only 13-14% of the glucosaminidase activity. The highest activity recorded for other glycosidases is that of neutral α-D-mannosidase (pH 6.4) (Table II).

Three additional minor activities (I_1, I_2 and I_3) were eluted with the major NAG-A activity. Robinson and Stirling[2] isolated two isoforms, A and B of N-acetyl-ß-D-glucosaminidase from human spleen which were initially assumed to correspond to the NAG-A and NAG-B isolated in the present study. I_1, I_2 and I_3 bear

no relationship to other minor forms of the enzyme reported in the literature. All three intermediate forms exhibited optimum activity at pH 4.4 and possibly represent fragmented forms of NAG-A. However, their low concentration prevented further investigation.

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