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PURIFICATION OF ACID β-GALACTOSIDASE AND ACID NEURAMINIDASE FROM BOVINE TESTIS: EVIDENCE FOR AN ENZYME COMPLEX

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The isolation of an acid neuraminidase from bovine testis is described. Under all experimental conditions this neuraminidase copurifies with acid β-galactosidase, but not with other lysosomal hydrolases. Immunotitration with an antiserum raised against purified human placental β-galactosidase results in the coprecipitation of both enzyme activities. Our data indicate that acid neuraminidase and β-galactosidase are present as an enzyme complex. The possible physiological relevance is discussed.

INTRODUCTION

Neuraminidases catalyze the hydrolysis of sialic acid residues α-ketosidically bound to oligosaccharides, glycoproteins, glycopeptides and glycolipids. They are widely distributed in eukaryotic and prokaryotic cells (1).

The neuraminidases of prokaryotic cells are very well studied (1,2), but relatively little information is available on eukaryotic neuraminidases (3-6).

In the last few years a number of different genetic defects of lysosomal acid neuraminidase have been found in man (for reviews see 7,8). Especially interesting are patients who have a combined deficiency of two lysosomal enzymes, neuraminidase and β-galactosidase (9,10). To understand the possible relationship between neuraminidase and β-galactosidase, it is necessary to have more information about the structure and properties of the different molecular forms of mammalian neuraminidases. Such information will also help to elucidate the molecular nature of genetic diseases associated with a neuraminidase deficiency.

Despite the recent availability of sensitive assay procedures with methylumbelliferyl substrate, the lability and strongly membrane bound character of the enzyme hampered studies on mammalian neuraminidases (3,4) (for review, see 6).

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In the present report we describe the copurification of a bovine testis acid neuraminidase with lysosomal β-galactosidase throughout the isolation procedure developed for the latter enzyme. Evidence is presented that β-galactosidase and neuraminidase molecules exist as an enzyme complex.

MATERIALS AND METHODS

Purification procedure

All purification steps were carried out at 4°C, unless otherwise noted. The procedure used is a modification of the isolation method developed for bovine testis β-galactosidase as described earlier (11).

Bovine testes were obtained at the local slaughterhouse and kept frozen at -70°C until use. Testes devoid of epididymes (0.5 kg) were homogenized in 2 vol. distilled water in a Waring blendor for 3 min. The homogenate was brought to pH 4.0 by dropwise addition of 2N HCl. After 30 min stirring the acid homogenate was centrifuged at 25000 x g for 30 min. The clear supernatant was adjusted to pH 6.2 with 2N NaOH. This supernatant was applied to a Concanavalin A-Sepharose column (60 ml bed volume) (Pharmacia) that was previously equilibrated with 50 mM sodium phosphate buffer pH 6.2. After loading, the column was washed overnight with equilibration buffer containing 100 mM sodium chloride. The column was brought to room temperature and washed with equilibration buffer containing 1M sodium chloride and eluted with the same buffer with 1M α-methylglucoside added to it. The eluate was concentrated on an Amicon Hollow Fiber Concentrator and dialysed overnight against 10 vol. of 20 mM sodium acetate buffer pH 5.2 containing 100 mM sodium chloride. This preparation was applied to a 2 ml p-aminophenyl-thiogalactoside-CH-Sepharose column (prepared as described earlier (12)), equilibrated with the same buffer as used for dialysis. The column was then washed extensively with 10 times the bed volume of equilibration buffer and subsequently with the same volume of equilibration buffer containing 1M sodium chloride. β-Galactosidase and neuraminidase were eluted by adding 100 mM γ-galactonolacton to the last buffer. The eluate was concentrated and dialysed in an Amicon Ultrafiltration cell with a PM-10 filter. Dialysis was against 25 mM sodium acetate buffer containing 100 mM sodium chloride. The final preparation was frozen and stored at -70°C.

Assay of protein and enzyme activity

Protein was measured by the method of Lowry (13).

The activity of neuraminidase and the other enzymes was measured with 4-methylumbelliferyl substrates, according to procedures described earlier (8,10). One unit of activity is defined as the amount of enzyme forming 1 μmol of 4-methylumbelliferone per minute at 37°C.

SDS-Polyacrylamide gel electrophoresis

Electrophoresis was carried out on a 12% polyacrylamide slab gel as described by Laemmli (14), with a modified acrylbisacryl ratio of 30 : 0.315.

Gel filtration

A 1 x 95 cm column of Sephadex G-200 (Pharmacia) was equilibrated at 4°C with 25 mM sodium acetate buffer pH 5.2 containing 100 mM sodium chloride. 2 mg of the purified enzyme preparation was mixed with glycerol (final conc. 10%) and applied to the column. The column was run overnight and fractions of 1 ml were collected. All fractions were assayed for both neuraminidase and β-galactosidase.

Immunotitration

10 µl Concanavalin A-Sepharose eluate containing 6.03 mU β-galactosidase and 0.066 mU neuraminidase was added to 10 µl 1M sodium phosphate buffer pH 6.0 containing 1M sodium chloride and 5% bovine serum albumin. An increasing amount of an IgG preparation (5-100 µl) (prepared from an antiserum raised against purified human placental β-galactosidase (15) using Protein A-Sepharose) was added to this mixture. 20 mM sodium phosphate buffer pH 6.0 containing 100 mM sodium chloride was added to a final volume of 120 µl. After 3 hours incubation at 4°C 20 µl of a 1 : 1 protein A-Sepharose suspension in 20 mM sodium phosphate buffer pH 6.0 containing 100 mM sodium chloride was added. After 2 hours all tubes were centrifuged at 10,000 x g for 1 minute. Supernatants were assayed for both neuraminidase and β-galactosidase.

RESULTS AND DISCUSSION

In our initial attempts to purify active acid neuraminidase from a mammalian source, we observed that bovine testis contains large amounts of acid neuraminidase. We also found a considerable neuraminidase activity in the 25000 x g supernatant after acid precipitation, which enabled us to purify an active mammalian neuraminidase. This neuraminidase has a pH optimum around 4.5 and a K_m value of 3 mM using the 4-methylumbelliferyl-N-acetylneuraminic acid substrate.

In the course of studies on the molecular properties of β-galactosidase we found that neuraminidase copurified during purification of bovine testicular β-galactosidase, after two subsequent affinity chromatography steps. The purification and recovery of neuraminidase and β-galactosidase at different stages of a representative purification is summarized in table 1.

Chromatography on Concanavalin A-Sepharose yields a preparation in which neuraminidase and β-galactosidase are purified 25- and 30-fold respectively.

After p-aminophenylthiogalactoside-CH-Sepharose affinity chromatography, which seemed to be specific for β-galactosidase purification, not only a 512-fold purification of β-galactosidase was achieved, but also a 316-fold purification of neuraminidase. Neuraminidase had a somewhat lower overall recovery (17%) than β-galactosidase (28%). However, no neuraminidase activity was present in the unretained material and no residual neuraminidase could be detected on the column after elution. The lower recovery of neuraminidase is therefore probably due to a more pronounced denaturation during the last affinity chromatography step. The activity of neuraminidase is measured with a substrate concentration below the K_m, since higher substrate concentrations result in high blancs, which disturb accurate measurements. The specific

Table 1

Copurification of Neuraminidase (Neur) and β-Galactosidase (Gal).
Results are for 500 g wet weight testes.

Step	Volume	Protein	Activity		Specific activity		Activity	Purification		Yield	
			Neur.	Gal.	Neur.	Gal.	Neur/Gal	Neur.	Gal.	Neur.	Gal.
	ml	mg	mU[a]		mU/mg		$x10^{-2}$	-fold		%	
25000 xg Supernat. pH4	730	5542	2008	99604	0.36	18.0	2.0	1	1	100	100
Con A Sepharose	5.0	87.5	796	47033	9.10	537.5	1.7	25	30	40	47
PAPS-gal-Sepharose[b]	2.5	3.05	347	28083	113.8	9208	1.2	316	512	17	28

[a] nmol 4-methylumbelliferone released/min.

[b] PAPS-gal-Sepharose: p-aminophenylthiogalactoside-CH-Sepharose.

Table 2

Lysosomal hydrolase activities in the purified bovine testicular neuraminidase preparation

	25000 xg Supernatant mU/mg	Purified mU/mg	Purification factor
Neuraminidase	0.36	113.8	x 316
β-Galactosidase	18.0	9208	x 512
β-Glucuronidase	5.7	13.8	x 2
α-Galactosidase	2.4	17.4	x 7
β-Glucosidase	2.6	13.8	x 5
β-Hexosaminidase	204	562	x 3
α-Glucosidase	1.6	1.1	x 1
α-Mannosidase	1.2	1.5	x 1

activities of neuraminidase are therefore an underestimation of the real values.

Table 2 demonstrates that only β-galactosidase and neuraminidase are copurifying during our procedure: none of the other hydrolases tested were substantially purified. The overall yield of these other acid hydrolases varied from 0.02% to 1%. This indicates that the copurification is specific for neuraminidase and β-galactosidase.

Both neuraminidase and β-galactosidase elute in the void volume of a Sephadex G-200 column (Fig. 1), suggesting that both enzymes are present as a high molecular weight aggregate ($>$ 300 kd).

This observation prompted us to try and separate the two enzymes via a variety of separation techniques, e.g. sucrose gradients, aceton fractionation, cellogel electrophoresis and hydrophobic chromatography. However, under all conditions tested, β-galactosidase and neuraminidase could not be separated, which fits the assumption, that both enzymes are present as a complex.

Additional evidence for the existence of an enzyme complex was derived from immunotitration experiments (Fig. 2). In the presence of antibodies raised against purified human placental β-galactosidase, both neuraminidase and β-galactosidase activities were precipitated. The precipitation curves for both enzyme activities are identical, which indicates the simultaneous removal of

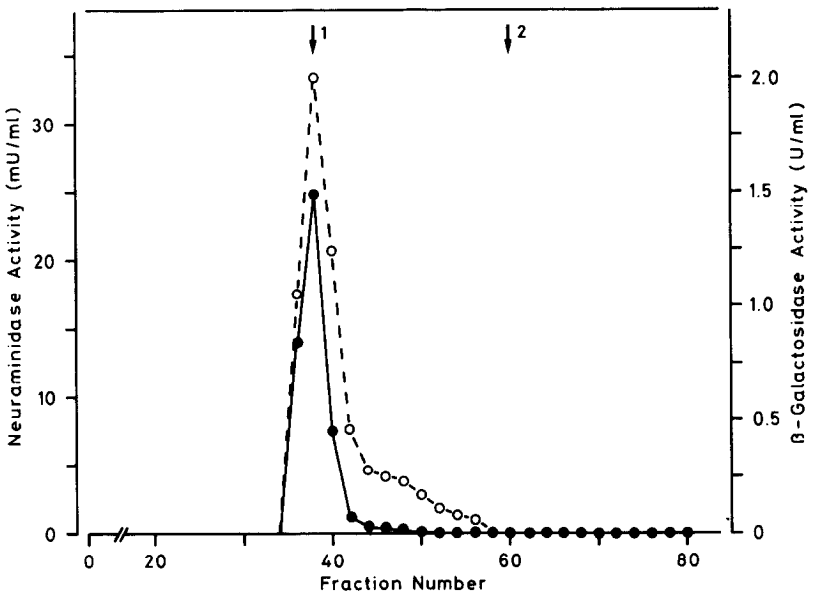

Fig. 1 Elution profile obtained after Sephadex G-200 column chromatography of the purified enzyme preparation.
● Neuraminidase activity
○ β-galactosidase activity
Arrow 1 denotes position of the void volume
Arrow 2 denotes position of bovine serum albumin

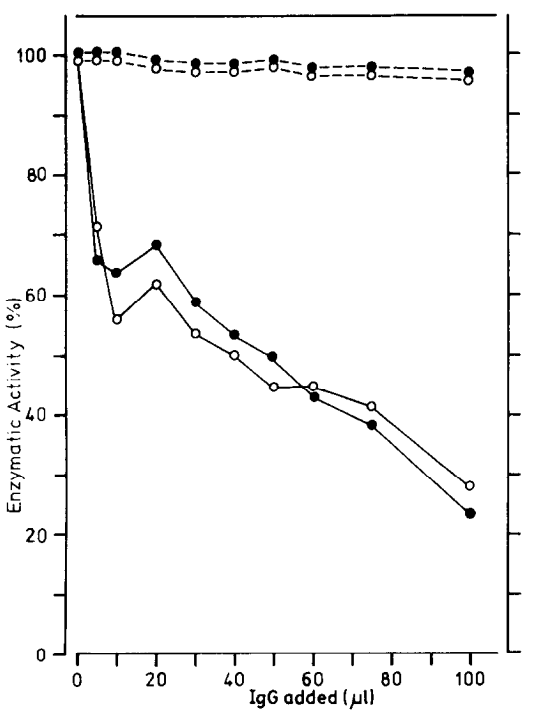

Fig. 2 Immunotitration of bovine testicular neuraminidase and β-galactosidase

●——● Neuraminidase ○——○ β-galactosidase	Titrated with IgG raised against purified human placental β-galactosidase
●--● Neuraminidase ○--○ β-galactosidase	Titrated with IgG derived from pre-immune serum

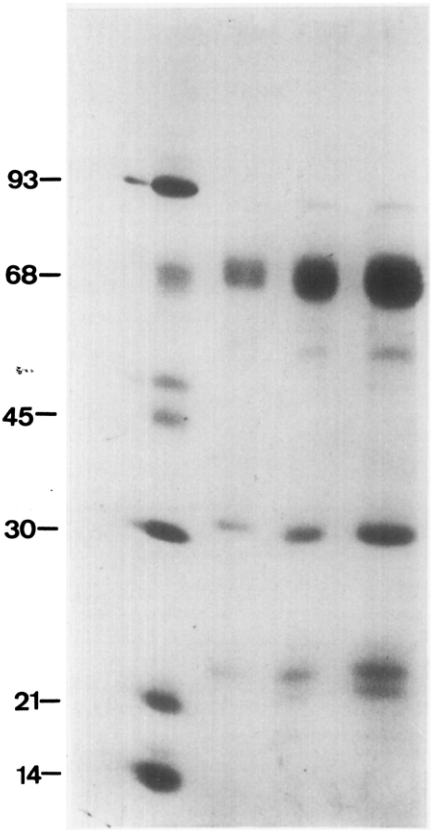

Fig. 3 Polyacrylamide gel electrophoresis of the purified enzyme preparation in the presence of SDS.
From left to right: marker proteins, 10, 20 and 40 µg of enzyme preparation, respectively.

both enzymes and that β-galactosidase and neuraminidase react with the same antibody population.

Polyacrylamide gel electrophoresis of the purified preparation in the presence of SDS shows three major protein bands with molecular weights of 64 kd, 32 kd and 22 kd (Fig. 3). Since bovine testis β-galactosidase monomer has a molecular weight of 64 kd, it is likely that the neuraminidase activity in our purified preparation is associated with the 32 kd and/or 22 kd protein band(s). Distler et al. (16) also reported a 64 kd band for bovine testis β-galactosidase, but did not mention any other components. In human liver, Norden et al. (17), and human fibroblasts, Hoeksema et al. (18) β-galactosidase monomer (64 kd) and high molecular weight aggregates (600-700 kd) were found. Recently our group (d'Azzo et al., 15) using immunoprecipitation, discovered that mature β-galactosidase (64 kd) is formed from a precursor (85 kd) and that in addition a protective glycoprotein (32 kd) is required

to prevent excessive intralysosomal proteolytic degradation. This protective protein and its 54 kd precursor are deficient in fibroblasts from patients with combined β-galactosidase and neuraminidase deficiency.

Preliminary experiments on solubilized neuraminidase from normal human fibroblasts (to be published) also point to the presence of β-galactosidase and neuraminidase in an enzyme complex. We are currently studying the relationship between these two enzymes and the 32 kd protective protein.

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