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HEXOSAMINIDASE ACTIVITY OF CULTURED HUMAN SKIN FIBROBLASTS

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SUMMARY

Human skin fibroblasts were grown in tissue culture and both hexosaminidase A and hexosaminidase B were separated on a DEAE-cellulose column. The initial extract and the two separated components were utilized for establishing the K_m and V values for the four substrates, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide (4-MU-GluNAc), 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminide (4-MU-GalNAc), *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (pNP-GluNAc) and *p*-nitrophenyl-β-D-*N*-acetylgalactosaminide (pNP-GalNAc). The data obtained for the K_m values on the GluNAc compounds were essentially identical. The 4-MU-GalNAc yielded similar values for all three enzyme sources; however, differences were found with pNP-GalNAc as substrate. Several commercial batches of serum albumin were found to contain appreciable hexosaminidase activity.

INTRODUCTION

An enzyme which catalyzes the hydrolysis of the β-hexosaminide bond has been purified from a variety of biological sources. In almost every instance derivatives of either β-D-*N*-acetylglucosamine or β-D-*N*-acetylgalactosamine were employed as substrates, and the rate of hydrolysis with the former usually exceeds that of the latter[1–4]. Three distinct hexosaminidases, however, have been reported to be present in rat and calf brain tissue[5]. One of these is nonspecific as to the nature of the amino sugar used as substrate. In addition, a specific β-galactosaminidase and a specific β-glucosaminidase have been purified from this source.

The presence of two predominant, electrophoretically distinct β-hexosaminidases was reported in spleen tissue which have been operationally designated as hexosaminidase A and B (ref. 3). Hexosaminidase A can be converted by neuraminidase

Abbreviations: 4-MU-GluNAc, 4-methylumbelliferyl-β-D-*N*-acetylglucosaminide; 4-MU-GalNAc, 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminide; pNP-GluNAc, *p*-nitrophenyl-β-D-*N*-acetylglucosaminide; pNP-GalNAc, *p*-nitrophenyl-β-D-*N*-acetylgalactosaminide; 4-MU, 4-methylumbelliferone.

digestion to a form that is electrophoretically indistinguishable from hexosaminidase B. The activity of hexosaminidase A is more thermolabile than hexosaminidase B, an observation which has been employed for the differential quantitation of these two catalytic proteins in a variety of tissue fluids and extracts[6-8].

The substrates most commonly employed for assaying hexosaminidase activities are either the 4-methylumbelliferyl or *p*-nitrophenyl derivatives of β-D-*N*-acetylglucosamine or β-D-*N*-acetylgalactosamine. This study presents data on the K_m values and V values for these four substrates with crude extracts, partially purified hexosaminidase A and hexosaminidase B of normal human fibroblasts grown in tissue culture.

MATERIALS AND METHODS

4-Methylumbelliferyl-β-D-*N*-acetylglucosaminide (4-MU-GluNAc), 4-methylumbelliferyl-β-D-*N*-acetylgalactosaminide (4-MU-GalNAc), *p*-nitrophenyl-β-D-*N*-acetylglucosaminide (pNP-GluNAc) and *p*-nitrophenyl-β-D-*N*-acetylgalactosaminide (pNP-GalNAc) were obtained either from Koch–Light Limited (Colnbrook, England) or Pierce Chemical Company (Rockford, Ill.). Human skin fibroblasts from normal individuals were grown in tissue culture as previously described[9] and kindly supplied by Dr Aubrey Milunsky.

The cell pellet was suspended in 1 ml of water and sonicated for two 30-s bursts with a Bronson W175 sonifier, this represents the initial fibroblast extract and contained 7 mg protein. Hexosaminidase A and hexosaminidase B were obtained from this preparation by fractionation on a 3 g DEAE-cellulose column (DE-52, Whatman)[10]. The packing material was prepared in 0.01 M phosphate buffer (pH 6.0) and the sample applied. The column was eluted with 70 ml of 0.04 M NaCl in the same buffer to obtain hexosaminidase B and 75 ml of 0.2 M NaCl in the same buffer to yield hexosaminidase A. Individual 1-ml samples were collected at +4 °C. Aliquots were subjected to electrophoresis with cellulose acetate, and the strips were incubated with 4-MU-GluNAc to detect the individual bands as described[11].

The incubation tubes contained 500 nmoles substrate, 10 μmoles citrate–phosphate buffer; 25 μmoles NaCl, 1 mg bovine serum albumin, 3–10 μg enzyme protein in a total volume of 0.2 ml and were shaken at 37 °C for 1 h. Each tube received 0.7 ml 2.75% trichloroacetic acid, 0.45 ml 0.5 M KOH and 0.7 ml glycine buffer (pH 10.2). The liberated 4-methylumbelliferone (4-MU) was measured fluorimetrically in an Amino-Bowman spectrofluorimeter with the excitation wavelength at 366 nm and the emission wavelength at 466 nm. The *p*-nitrophenol liberated was measured in a Beckman spectrophotometer at 420 nm. Each assay was carried out in duplicate.

K_m and V values were obtained by the double reciprocal plot procedure of Lineweaver and Burk[12].

RESULTS

General studies

The pH activity curve indicated an optimum value at 4.0 with citrate–phosphate buffer as shown in Fig. 1. There is a marked stimulation of hexosaminidase

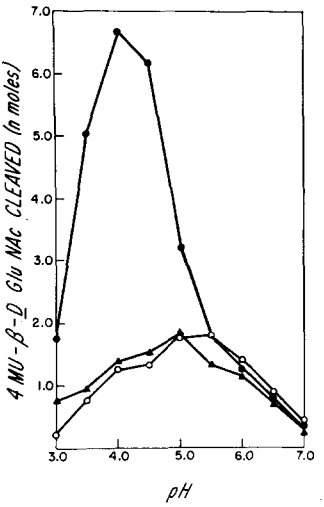

Fig. 1. Effect of pH on the rate of hydrolysis of 4-MU-GluNAc by crude fibroblast extract. △—△, citrate–phosphate buffer; ●—●, citrate–phosphate buffer *plus* serum albumin; ○—○, acetate buffer *plus* serum albumin.

activity by serum albumin as previously reported[7] and the inhibitory effect of acetate buffer is evident. Several commercial preparations of serum albumin were assayed directly for hexosaminidase activity and found to contain from 0 to 31 nmoles 4-MU liberated per mg protein. Only those preparations without demonstrable enzyme activity were employed in subsequent studies. The reaction was linear with time and enzyme concentration.

Kinetic studies

The effect of varying 4-MU-GluNAc, 4-MU-GalNAc, pNP-GluNAc and pNP-GalNAc concentration on their hydrolysis by the crude extract, hexosaminidase A and hexosaminidase B was examined. The crude extract showed two distinct bands on electrophoresis; the one which migrated the furthest was assumed to correspond to hexosaminidase A and the slowest assumed to be hexosaminidase B. Electrophoresis of the isolated column fractions revealed that a single enzyme component was present in the two separated enzyme fractions. The K_m and V values were calculated for all three enzyme sources for each of the four substrates, and these data are presented in Table I. It is apparent that the K_m values obtained for 4-MU-GluNAc and pNP-GluNAc are identical with all three enzyme sources. Similarly the K_m values for 4-MU-GalNAc are essentially the same with all three enzyme sources. The K_m values obtained for the hydrolysis of pNP-GalNAc of both hexosaminidase A and B are greater than that of the initial extract. When both fractions are mixed and present together in similar amounts in the incubation tubes, a value almost identical to that of the starting material is obtained.

The purification achieved, based on V values, varied from 10.13 for hexosaminidase B with pNP-GalNAc as substrate to 4.8 for hexosaminidase B with 4-MU-GalNAc as substrate. The ratio of hexosaminidase A and B for the utilization of the four individual substrates is presented in Table II. It appears that a reciprocal re-

TABLE I

K_m AND V VALUES FOR HUMAN SKIN FIBROBLAST EXTRACTS, HEXOSAMINIDASES A AND B

	pNP glycosides				*4-MU glycosides*			
	GalNAc		*GluNAc*		*GalNAc*		*GluNAc*	
	K_m[a]	V[b]	K_m[a]	V[b]	K_m[a]	V[b]	K_m[a]	V[b]
Extract	1.79	0.617	8.30	4.76	1.19	0.76	8.31	4.39
Hexosaminidase A	2.08	4.53	8.29	33	1.16	5.26	8.33	43.8
Hexosaminidase B	3.12	6.25	8.33	40	1.10	3.71	8.3	32
Hexosaminidase A and B	1.76	5.04	—	—	—	—	—	—

[a] $10^4 \times$ concentration (M).
[b] μmoles cleaved/h per mg protein.

TABLE II

RATIO OF V OF HEXOSAMINIDASE A/HEXOSAMINIDASE B FOR THE ARTIFICIAL SUBSTRATES

Substrate	*Hexosaminidase A* / *Hexosaminidase B*
4-MU-GluNAc	1.36
4-MU-GalNAc	1.42
pNP-GluNAc	0.82
pNP-GalNAc	0.72

lationship exists; thus hexosaminidase A, as compared to hexosaminidase B, hydrolyzes the 4-MU derivatives nearly twice as effectively as the pNP derivatives. With all three enzyme sources, the *N*-acetylglucosamine derivative is cleaved preferentially to the *N*-acetylgalactosamine.

DISCUSSION

The differential assay of hexosaminidase A and B in tissues has been employed for the detection of affected individuals and heterozygote carriers for Tay–Sachs' disease[7,8]. Biochemically, the disease is characterized by the accumulation of G_{M_2} ganglioside, its corresponding asialo derivatives and a decrease or lack of hexosaminidase A in tissues and body fluids[13]. The terminal carbohydrate unit in these sphingoglycolipids is an *N*-acetylgalactosamine residue. Due to the absence of the A component of hexosaminidase, it has been speculated that G_{M_2} is one of its naturally occurring substrates. Experimental evidence has been provided with purified enzyme preparations to support this hypothesis[14]. In general, for diagnostic purposes, the ratios of hexosaminidase A and B in a tissue sample is based either upon differential heat stability or electrophoretic separations. The ability to hydrolyze either 4-MU-β-D-GluNAc or pNP-β-D-GluNAc by preparations is employed to quantitate hexosaminidase activity in these clinical diagnoses. Most studies on hexosaminidase have utilized either the 4-MU or the pNP-β-hexosaminide in kinetic studies, and no apparent differences have been reported.

The present series of experiments suggests that the K_m values for both 4-MU

and pNP-GluNAc of the initial fibroblast extract, hexosaminidase A and hexosaminidase B, are essentially identical at $8.3 \cdot 10^{-4}$ M. This value is close to that reported for other tissues[3,14]. Similarly the K_m values for 4-MU-GalNAc of all three enzyme sources appear to be the same at $1.1 \cdot 10^{-4}$ M. The only kinetic differences appear when pNP-GalNAc is employed as substrate. The K_m values for hexosaminidase A and hexosaminidase B are higher than that of the initial extract. Surprisingly, when the preparations of hexosaminidase A and B are mixed together, the K_m value observed corresponds to that obtained with the original fibroblast extract. This suggests that a cooperativity may exist between hexosaminidase A and B, resulting in an altered affinity for different substrates.

The increased enzymatic activity found when albumin is present in the incubation mixture presumably is due to its established effect on the prevention of protein denaturation[15]. It is obvious that care must be taken in the choice of albumin samples employed for such purposes due to the presence of hexosaminidase activity as shown in Table I. It should be noted that individual batches from the same supplier are not consistent, such as I and V.

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