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ON THE ACTIVE SITE OF β-HEXOSAMINIDASE FROM LATEX OF *FICUS GLABRATA*

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Key Word Index—*Ficus glabrata*; Moraceae; latex; β-hexosaminidase; enzyme purification; kinetics; regulation.

Abstract—*N*-Acetyl-β-D-hexosaminidase (EC 3.2.1.52), active against both *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, is present in latex of *Ficus glabrata*. The final specific activity was increased 150-fold from crude extract after ammonium sulphate fractionation and affinity chromatography on Concanavalin A–Sepharose. The activity ratio β-*N*-acetylglucosaminidase–β-*N*-acetylgalactosaminidase remained constant. Substrate competition, competitive inhibition studies and Arrhenius plots confirm that, in the hexosaminidase, only one kind of active site is responsible for both activities. Acetate and acetamide are more effective competitive inhibitors than iodoacetamide, *N*-acetylglucosamine and *N*-acetylgalactosamine more than glucosamine and galactosamine, and α-methylmannoside more than mannose, suggesting that the active site binds the *N*-acetyl moiety of the substrate and a hydrophobic interaction of the methyl group is involved. The difference between the strength of the inhibition by mannosamine with respect to glucosamine and galactosamine, that do not inhibit, seems to be due to the position at C-2 of the amino group in the pyranose ring.

INTRODUCTION

β-*N*-Acetylhexosaminidase, Hex (EC 3.2.1.52), is a lysosomal enzyme involved in the catabolism of glycoproteins, glycolipids and glycosaminoglycans. In addition to this physiological significance, an enzyme that splits *N*-acetylhexosamine linkages may be useful as analytical tool to study the structures of some *N*-acetylhexosamine containing glycoconjugates. The Hex is present in multiple molecular forms in mammalian tissues, extracellular fluids and plants [1–5]. In human tissues there are two major Hex isoenzymes, A and B [6]; while human serum contains one or more intermediate forms I_1 and I_2 [7]. All these isoenzymes have been reported to be similar with respect to pH optima, K_m values and behaviour towards inhibitors, but to differ in thermostability [8] and electrophoretic mobility [9]. The interest in this enzyme was increased by the findings of Okada and O'Brien [9], who reported a specific deficiency of the A component in tissues and serum of patients with Tay-Sachs disease and those by Sandhoff [10] who described a deficiency of both A and B components in a different form of the same disease. Although extensive studies have been carried out on the properties of the mammalian Hex [11], there is still much to know about the metabolic role, physiological significance and regulation of these enzymes in different tissues, since there are few reports that compare the properties of this enzyme with Hex in other animals and plants. Consistent differences between the intracellular and extracellular enzymes from a number of species would indicate that these differences have a functional significance. Moreover the similarities in the behaviour of the enzyme from various sources indicate a common feature which is essential in the enzyme catalysis. The enzymes have, in general, been reported as catalysing the release of *N*-acetylglucosamine and *N*-acetylgalactosamine from β-glucoside linkages at comparable rates, but the two activities have not been separated. However, many of the studies have measured only the glucosaminidase activity but, where both activities have been compared kinetically, it appears that a common active site for the two types of glycoside is present [12–15].

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RESULTS AND DISCUSSION

Enzyme purification

All procedures were carried out at 4° unless otherwise indicated.

Step 1: *preparation of the crude extract.* The latex was collected from oblique nicks on the trunk of *Ficus glabrata* and was diluted with an equal volume of water to give a homogeneous liquid, which was centrifuged at 3300 *g* for 10 min and the supernatant treated four times with petrol and once with carbon disulphide.

Step 2: *ammonium sulphate fractionation.* Ammonium sulphate was added, with stirring, to the aqueous phase obtained from step 1, to give 30% saturation. The resulting precipitate was removed by centrifugation at 12 000 *g* for 20 min and discarded. The supernatant was adjusted to 60% saturation with solid ammonium sulphate added with stirring and the suspension was centrifuged at 20 000 *g* for 20 min; the supernatant was discarded. The precipitate was dissolved in and dialysed against water.

Step 3: *affinity chromatography on Con-A Sepharose.* The solution from step 2 was then dialysed against

12.5 mM succinate buffer, pH 6, containing 0.5 mM magnesium chloride, manganese chloride, calcium chloride and 0.02% sodium azide. The solution was concentrated by ultrafiltration (Amicon PM-10 filter, nominal cut-off 10 000 MW). The concentrated solution was pumped onto a Concanavalin A–Sepharose 4B column (2.5 × 30 cm) equilibrated with the above buffer. The column was washed with 0.01 M sodium phosphate buffer, pH 7 and 0.5 M sodium chloride, then with 0.01 M sodium phosphate buffer alone. The enzyme was eluted with a linear gradient of α-methylmannoside (0.05–0.5 M) in 0.01 M sodium phosphate buffer, pH 7. (This step was carried out at room temperature.) The partial purification procedure is summarized in Table 1.

The Hex adsorbed on Concanavalin A–Sepharose and eluted by α-methylmannoside confirms that from a plant source also the enzyme was a glycoprotein and that this affinity chromatography method is very efficient for the purification of Hex. This step alone increases activity *ca* 100-fold with a recovery of 70%. The enzyme preparation possesses both β-*N*-acetylglucosaminidase (β-Glc*N*Acase) and β-*N*-acetylgalactosaminidase (β-Gal*N*Acase) activities; the ratio of the two activities remained relatively constant throughout the purification procedure. These results suggest that, in the latex of *Ficus glabrata*, the β-Glu*N*Acase and the β-Gal*N*Acase are located on the same enzyme molecule. Similar results have been obtained on Hex from jack bean meal [12] and germinated fenugreek seeds [14] and also from animal sources [11]. Therefore, in an attempt to determine whether a single site carries the two different activities, kinetic studies have been worked out.

The pH optima for the two substrates, p*N*PGlu*N*Ac and p*N*PGal*N*Ac, were 4 and 4.5, respectively, for this Hex. The Hex from latex hydrolyses both the p*N*PGlu*N*Ac and p*N*PGal*N*Ac at different rates and the V_{max} values are, respectively, 188 ± 50 and 100 ± 20 nmol/min · mg protein. In addition, the apparent K_m for β-Glc*N*Acase (2.8 ± 0.6 mM) was greater than that for β-Gal*N*Acase (0.7 ± 0.2 mM), using *p*-nitrophenyl-*N*-acetyl-D-glycosides as substrates. These values are the means ± standard deviations of five experiments and are similar to those of other β-*N*-acetylhexosaminidases [12, 13, 16].

The kinetics of competition for the enzyme were studied with mixed substrates to determine whether the enzyme has one or two active sites for hydrolysis of β-*N*-acetylglucosaminide and β-*N*-acetylgalactosaminide. A maximum velocity of 109 nmol of *p*-nitrophenol/min · mg was calculated for competition between substrates according to the theoretical rate expression (see Table 2). The experimental velocity is 113 nmol *p*-nitrophenol/min · mg.

This finding means that both substrates compete for a single active site. A corollary of this theoretical rate law for a single active site with multiple substrates is that the total rate of the reaction will be less than the sum of the individual rates of reaction measured separately [17]. The total rate of the reaction in our case is 113 nmol *p*-nitrophenol/min · mg which is less than the sum of the rate of the individual reactions measured separately: 155 + 95 = 250 nmol *p*-nitrophenol/min · mg.

Additionally, if an enzyme catalyses the hydrolysis of two substrates at the same active site, a competitive inhibitor must have the same K_i when it is assayed with either substrate [17]. The K_i values of the tested competitive inhibitors are also practically identical for both activities (see Table 3). Moreover, Table 3 shows that acetate and acetamide are more effective inhibitors than iodoacetamide, *N*-acetylglucosamine and *N*-acetylgalactosamine more than glucosamine and galactosamine, and α-methylmannoside and mannosamine more than mannose. These results suggest that the active site of latex Hex binds the equatorial acetamido group at C-2 of the pyranose ring of the substrate molecule, according to Neuberger and Pitt-Rivers [18], who were the first to suggest a specific 'acetamido receptor site'. It seems that hydrolysis of 2-acetamidoglucopyranoside by Hex involves the hydrophobic interaction of the methyl group of the 2-acetamido group by binding of the active site of the enzyme, as proposed by Yamamoto [19]. However, the difference between the strength of inhibition by mannosamine and the other amino sugars, which do not inhibit, depends on the position of the amino group at C-2 of the pyranoside ring. It seems, therefore, that at pH 4–4.5, the positively charged axial amino group in mannosamine may attract a negatively charged group in the active site of the enzyme to form a non-productive complex, or may otherwise hinder access of the enzyme to the substrate, as suggested by Mian *et al.* [20].

Table 4 show that, among the various metal ions tested, Ag^+, Hg^{2+} and Fe^{3+} were the most potent inhibitors with the same effectiveness against both substrates.

The Arrhenius plots for hydrolysis of p*N*PGlu*N*Ac and p*N*PGal*N*Ac (range 4–40°) show an apparent activation energy of 13.2 ± 0.5 and 12.8 ± 0.4 kcal/mol, respectively. When an enzyme acts on several substrates the same activation energy is often obtained. The activation energy, therefore, seems to be a characteristic of the enzyme more than of the substrate [21].

There is experimental evidence for the presence of β-*N*-acetylhexosaminidase activity in a larger number of tissues, but we have not found any reference for the presence of this enzyme in the extracellular fluid of plants like latex of *Ficus glabrata*.

Although the non-identity of two different enzymes can

Table 1. Partial purification of β-*N*-acetylhexosaminidase from latex of *Ficus glabrata*

Procedures	Total units	Sp. act. (units/mg)	Recovery* (%)	Activity ratio (β-Glc*N*Acase–β-Gal*N*Acase)
Crude extract	1760	1.1	100	1.82
Ammonium sulphate precipitate	1235	1.4	70	1.91
Concanavalin A–Sepharose chromatography	880	155	50	1.93

*Percentage recovery was calculated relative to the crude extract.

Table 2. Mixed substrate analysis for p*N*PGlu*N*Ac (*S'*) and p*N*PGal*N*Ac (*S''*)

S'	v_1	S''	v_2	v_1+v_2	$S'+S''$ v_{obs}	v_{calc}
4.8	155	1.4	95	250	113	109

S' and *S''* concentrations are in mM. The experimental values are expressed as nmol/min·mg protein and are the average of three experiments. The calculated values were obtained from the formula:

$$v = \frac{K_m'' V_{max}' S' + K_m' V_{max}'' S''}{K_m' K_m'' + K_m'' S' + K_m' S''}$$

Table 3. Competitive inhibitors of β-*N*-acetylhexosaminidase from latex of *Ficus glabrata*

Inhibitor (mM)		K_i(M) p*N*PGlu*N*Ac	p*N*PGal*N*Ac
Acetate	(30)	3×10^{-2}	5×10^{-2}
Acetamide	(30)	2×10^{-2}	3×10^{-2}
Iodoacetamide	(77)	$>10^{-1}$	$>10^{-1}$
N-Acetylgalactosamine	(15)	5×10^{-3}	3×10^{-3}
N-Acetylglucosamine	(15)	1.0×10^{-2}	0.8×10^{-2}
Glucosamine	(77)	$>10^{-1}$	1.5×10^{-1}
Galactosamine	(77)	1×10^{-1}	$>10^{-1}$
Mannosamine	(30)	1×10^{-2}	2.5×10^{-2}
Mannose	(77)	$>10^{-1}$	$>10^{-1}$
α-Methyl-D-mannoside	(30)	2.5×10^{-2}	1.5×10^{-2}

The data are the average of five measurements. The final concentration of inhibitors is indicated in parentheses.

Table 4. Effect of metal ions on activity of β-*N*-acetylhexosaminidase from latex of *Ficus glabrata*

Inhibitor	Inhibitor concentration (mM)	% relative activity p*N*PGlu*N*Ac	p*N*PGal*N*Ac
$AgNO_3$	0.077	26	25
$HgCl_3$	0.077	25	22
$Fe_2(SO_4)_3$	7.7	20	18
$CaCl_2$	7.7	85	78
$ZnSO_4$	7.7	64	58
$BaCl_2$	7.7	88	80
$MgSO_4$	7.7	80	78
$CuSO_4$	7.7	48	52

be proved more easily than their identity, the evidence presented in this study indicates that the latex β-Glc*N*Acase and β-Gal*N*Acase activities are catalysed by the same enzyme at the same site. The apparent lack of stereospecificity by this enzyme for the 4-hydroxyl group in the sugar moiety agrees with previous reports from plant sources [12], confirming that it may be of biological significance.

EXPERIMENTAL

Latex. The latex was obtained from a *Ficus glabrata* tree (Moraceae) from Merida, Venezuela.

Enzyme assay. Enzymatic activity was assayed essentially as described in ref. [22]: 0.1 ml of 5.2 mM p*N*PGlc*N*Ac or 1.7 mM p*N*PGal*N*Ac soln in 50 mM Na citrate–NaPi buffer at the optimum pH for each enzyme was incubated for 15–30 min at 37° with 30 μl of adequately diluted enzyme preparation. The reaction was stopped by adding 0.1 ml 2 M NH_4OH and by diluting to 1 ml with H_2O. The released *p*-nitrophenol was quantitatively estimated in a Beckman DB-GT spectrophotometer at 400 nm; the molar ratio adsorption coefficient for the *p*-nitrophenol ion was 18.1×10^3/M·cm. One enzyme unit was defined as the amount of the enzyme that converts 1 nmol of substrate/min into *p*-nitrophenol at 37°.

Protein determination. Protein was determined by the method of ref. [23] with BSA as standard. Sp. act. was expressed as units/mg protein.

Kinetic measurements. In the kinetic expts, substrate concn ranged from 0.57 to 3.47 mM for p*N*PGlu*N*Ac and from 0.167 to 1 mM for p*N*PGal*N*Ac. The Michaelis constant and maximal velocity were determined from the Lineweaver–Burk plots [24]. In inhibition studies, aliquots of enzyme soln were mixed with different inhibitor concns and incubated at 37° for 5 min before addition of the substrate. Inhibition constants were evaluated according to ref. [25]. Substrate competition analysis was interpreted by using the formula derived from a model with one enzyme catalysing two reactions simultaneously [17]. The pH optima were ascertained in Na citrate–NaPi buffer, pH 3–7, at 37°. All the kinetic determinations were made at optimum pH values.

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