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ESTERASE ACTIVITIES OF β-*N*-ACETYL-D-GLUCOSAMINIDASE PREPARATIONS

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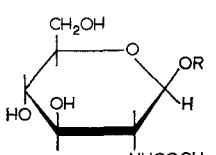
SUMMARY

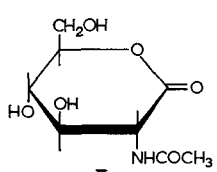
Evidence indicating that the enzyme β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) can catalyse the hydrolysis of esters has been presented. This is probably the first demonstration of catalytic action of a glycosidase upon structures other than glycosides. The specificity of the esteratic activities has been examined, and the possible significance of the results has been discussed.

INTRODUCTION

Pig epididymis is a particularly rich source of β-*N*-acetyl-D-glucosaminidase (EC 3.2.1.30) activity[1]. Although the significance of this high activity is unknown, extracts of the tissue constitute a convenient source of the enzyme for purification[2]. Certain properties of the purified enzyme have been reported[2,3], and evidence has recently been presented[4] suggesting that the enzyme catalyses the hydrolysis of 2-acetamido-2-deoxy-β-D-glucopyranose derivatives (I) by a mechanism similar to that advanced for egg-white lysozyme[5,6].



I



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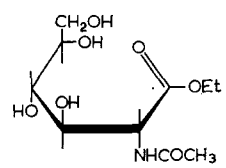

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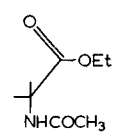

IV

In the course of work[4] undertaken to rationalise the high affinity of the epididymal enzyme for 2-acetamido-2-deoxy-D-glucono-(1,5)-lactone (II), binding studies using a series of simple structural analogues of the lactone (*viz.*, acetic acid, acetamide, *N*-methyl acetamide, 2-acetamidoacetic acid, ethyl 2-aminoacetate, ethyl 2-acetamidoacetate, and ethyl 2-propionamidoacetate) indicated that these materials compete with 2-acetamido-2-deoxy-β-D-glucopyranoside substrates for the highly specific 'acetamido' receptor site of the enzyme[3]. Since the latter site must lie close to that at which the catalysed reaction takes place and since there are strong similarities

of structure and of polarity between 2-acetamido-2-deoxy-β-D-glucopyranosides and competitive inhibitors such as the lactone (II) and ethyl 2-acetamidoacetate (IV), the question arises as to whether detectable catalytic action of the enzyme might be demonstrated against compounds other than the known pyranoside substrates.

The present communication describes work carried out to investigate this possibility.

MATERIALS AND METHODS

The β-*N*-acetyl-D-glucosaminidase preparations were, Stages 2, 6b and 7b of the procedure of FINDLAY AND LEVVY[2], an almond emulsin sample from British Drug Houses, and an extract of limpet visceral hump[7].

Ethyl, phenyl and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosides were prepared according to LEABACK AND WALKER[8]. Crystalline 2-acetamido-2-deoxy-D-glucono-(1,5)-lactone, which had been prepared according to British Patent 1138 367, was a gift from the late Mr. N. M. Cross. Ethyl 2-acetamido-2-deoxy-D-gluconate (m.p. 151–153°, $[\alpha]_D^{21}$ + 22.5°; *c* 1 in water) was prepared by a method analogous to the corresponding 2-benzamido compound[9].

Ethyl 2-acetamidoacetate was prepared by the addition of acetic anhydride (67 ml) over 15 min to a cooled solution of glycine ethyl ester hydrochloride (88 g) and $NaHCO_3$ (120 g) in water (II) followed by evaporation to dryness under reduced pressure after 16 h, and four extractions of the residue with ethyl acetate (200 ml). The solution was dried ($CaCl_2$) and evaporated to a syrup which crystallised. The residue was re-distilled twice at about 150° and 20 mm Mg; and recrystallized from ethyl acetate–light petroleum (b.p. 60–80°) to give 26 g ethyl 2-acetamidoacetate, m.p. 47–48°, with absorption maxima at 1750 and 1660 cm^{-1} (ref. 10, m.p. 48°). Ethyl 2-propionamidoacetate (m.p. 50–52°) was prepared similarly and showed absorption maxima at 1750 and 1660 cm^{-1}.

Unless otherwise stated, enzyme assays were carried out in 0.05 M sodium citrate buffer (pH 4.3) in the presence of 0.01% bovine serum albumin (Armour Products). After 30 min at 30° with the substrates specified the reactions were terminated and ethanol of *p*-nitrophenol were estimated by the methods described earlier[11].

RESULTS AND DISCUSSION

Moderate activities of a partially purified pig epididymal β-*N*-acetyl-D-glucosaminidase preparation (Stage 6b) were incubated with either 5 mM ethyl 2-acetamido-2-deoxy-β-D-glucopyreuoside (I, C_2H_5), with 5 mM ethyl 2-acetamido-2-doexy-D-gluconate (III) or with 200 mM ethyl 2-acetamidoacetate (IV). The results in Table I (Expt. 1) show that under the conditions specified, comparable quantities of ethanol were liberated enzymically from the ethyl esters and glycoside, whereas there was no detectable enzyme activity towards 200 mM ethyl 2-propionamidoacetate, 200 mM ethyl 2-aminoacetate, or 200 mM ethyl acetate using similar conditions. Thus, the acetamido specificity of the esterase activity parallels that of the β-*N*-acetyl-D-glucosaminidase activity of the preparation and that of the inhibition of the glycosidase by acylamido derivatives of ethyl acetate[3,4].

Results in Table I (Expts. 1, 2 and 3) show that the relative amounts of esterase

TABLE I

ESTERASE ACTIVITIES OF β-*N*-ACETYL-D-GLUCOSAMINIDASE PREPARATIONS

Enzyme preparations (sufficient to catalyse the hydrolysis of about 2 μmoles of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in 30 min at 30° under standard conditions) were incubated with the compounds indicated; after 30 min at 30°, a pyrophosphate–glycine–semicarbazide solution[11] (3 ml) was added to the reaction mixture (0.4 ml) and the liberated ethanol assayed by the yeast alcohol dehydrogenase–NAD^+ system as described by BOROOAH *et al.*[11].

Expt. No.	*Enzyme preparation*	*Substrate*	*Substrate concn. (mM)*	*Inhibitor added*	*Ethanol liberated enzymically (μmoles)*	*Ratio of activities* b/a	c/a
1	Epididymal enzyme (Stage 6b)	(a) I, R = C_2H_5	5	—	0.09	—	—
		(b) IV	200	—	0.05	0.56	—
		(c) III	5	—	0.035	—	0.39
		(d) Ethyl 2-propionamidoacetate	200	—	<0.011	—	—
		(e) Ethyl glycinate	200	—	<0.011	—	—
		(f) Ethyl acetate	200	—	<0.011	—	—
2	Epididymal enzyme (Stage 2)	(a) I, R = C_2H_5	5	—	0.08	0.56	—
		(b) IV	200	—	0.045		
3	Epididymal enzyme (Stage 7b)	(a) I, R = C_2H_5	5	—	0.08	0.50	—
		(b) IV	200	—	0.04		
4	Epididymal enzyme (Stage 6b) after heat inactivation (66%)	(a) I, R = C_2H_5	5	—	0.07	0.43	—
		(b) IV	200	—	0.03		—
5	Epididymal enzyme (Stage 6b) but 88% heat inactivated	(a) I, R = C_2H_5	5	—	0.06	0.50	—
		(b) IV	200	—	0.03		
6	Epididymal enzyme (Stage 6b)	IV	200	a. None	0.08	—	—
				b. I, R = C_6H_5 (2.5 mM)	0.03	0.38	—
				c. I, R = C_6H_5 (5 mM)	<0.01	—	0.12
7	Epididymal enzyme (Stage 6b)	IV	200	a. None	0.08	—	—
				b. Lactone II (1.1 μM)	0.03	0.38	—
				c. Lactone II (11 μM)	<0.01	—	0.12
8	Epididymal enzyme (Stage 6b) but eluted from Sephadex G-200	(a) I, R = C_2H_5	5	—	0.075	0.53	—
		(b) IV	200	—	0.04		
9	Crude limpet extract	(a) I, R = C_2H_5	5	—	0.095	0.42	—
		(b) IV	200	—	0.04		
10	Almond emulsin	(a) I, R = C_2H_5	5	—	0.10	—	—
		(b) IV	200	—	0.04	0.40	—

and glycosidase activities remain (within the limits of the analytical technique) unchanged at various stages in the purification of the glycosidase; this was also true (Expts. 4 and 5) of residual activities after heat treatment of the enzyme preparation and of the enzyme after elution from Sephadex G-200 using a procedure similar to that described by CAYGILL *et al.*[12] (Expt. 8).

The esteratic activities were strongly inhibited by the products of the reaction and the precision of the assays was further limited by high blank readings (blanks due to acidic and basic hydrolysis of the esters under standard assay conditions, were usually equivalent to about 1 μmole of ethanol), by inhibitory products of nonenzymic substrate hydrolysis and by interference of the ester IV with the estimation of ethanol by yeast alcohol dehydrogenase. These limitations made determinations of the kinetic parameters of the esterase activities impracticable: for very small amounts of ethanol liberated (up to about 0.1 μmole), esterase activities towards ethyl 2-acetamidoacetate (IV) were maximally active at about pH 3.5, were approximately linear with enzyme concentration and with time up to about 30 min.

The esterase activity was inhibited progressively by increasing concentrations of the phenyl glycoside (I, R = C_6H_5) and by very low concentrations of the lactone II (Table I, Expts. 6 and 7, respectively). The lactone II is a powerful, competitive inhibitor with high specificity towards β-*N*-acetyl-D-hexosaminidase activities. Thus, the identity of the enzyme responsible for the esterase and glycosidase activities is indicated by studies on the specificity of the enzyme for the acetamido group, by the relative abundance of the activities in various enzyme preparations, and by the inhibition of the esterase activity by structures known to combine specifically at the active site of the glycosidase.

Preliminary experiments with crude β-*N*-acetyl-D-glucosaminidase preparations from almond emulsin and from a mollusc show similar proportions of esterase activity (Table II, Expts. 9 and 10, respectively) and suggest that the phenomenon is general. It is noteworthy that in contrast to certain 'un-natural' substrates for other enzymes (*e.g.* *p*-nitrohenyl acetate, and *p*-nitrophenyl chitobiose for chymotrypsin and lyso-

TABLE II

STABILITY OF THE 1,5-LACTONE AND β-*N*-ACETYL-D-GLUCOSAMINIDASE ACTIVITIES

(*a*) Stability of β-*N*-acetyl-D-glucosaminidase: Enzyme (Prep. 6b) was incubated at 30° for the time stated in the presence of 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin and then assayed for enzyme activity under standard conditions. (*b*) Stability of lactone II: An absolutely fresh solution (4 μM) of the crystalline lactone II was incubated at 30° for the time stated in the presence of 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin before the addition of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (5 mM) and enzyme (Prep. 6b) and assayed under standard conditions. (*c*) Inhibition by 2-acetamido-2-deoxy-D-gluconate: 200 μM lactone II was left for 16 h at 5° with 2.02 M, pH 7.0 sodium phosphate buffer, diluted to about 4 μM and incubated at 30° for the time stated with 0.05 M sodium citrate buffer pH 4.3 and assayed under standard conditions. (*d*) Stability of lactone II in presence of enzyme: 40 μM lactone II was incubated for the time stated with β-*N*-acetyl-D-glucosaminidase (Prep. 6b), 0.05 M sodium citrate buffer pH 4.3 and 0.01% albumin, then diluted 20-fold and assayed under standard conditions.

Time (min)	*(a)*		*(b)*		*(c)*		*(d)*	
	p-Nitrophenol liberated (μmoles)	*% Activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*	*p-Nitrophenol liberated (μmoles)*	*% Uninhibited activity*
0	0.178	100	0.132	20	0.180	100	0.135	21
15	0.178	100	0.154	23	0.177	98	0.155	23
30	0.178	100	0.166	25	0.176	98	0.173	26
45	0.176	99	0.220	33	0.175	97	0.215	35
60	0.178	100	0.250	39	0.176	98	0.250	39

zyme, respectively), the initial rates for the enzymic hydrolysis of the esters III and IV were of the same order as that for the ethyl glycoside (I).

Since the lactone (II) is the most powerful competitive inhibitor of β-*N*-acetyl-D-glucosaminidase known[2,3], decreased inhibitory power should accompany any conversion of the 1,5-lactone to the corresponding aldonic acid or to the 1,4-lactone. Any catalytic activity that β-*N*-acetyl-D-glucosaminidase might show towards the 1,5-lactone should, therefore, manifest itself as decreasing inhibitory power of the solution. Results of experiments designed to demonstrate such an effect are represented in Table II. It can be seen that at pH 4.3 and 30°, the enzyme is stable for at least 60 min, that the de-lactonised lactone recovers its inhibitory power very slowly, that fresh solutions of the lactone show a rapid fall of inhibitory power over the first 30 min but that the time-course of the process up to 60 min is not affected by the presence of the enzyme.

It was concluded that no enzyme-catalysed transformation of the lactone could be detected under conditions similar to those where hydrolytic activities towards the esters III and IV have already been demonstrated.

While there is no evidence available concerning the mechanism of the enzyme-catalysed hydrolysis of the ethyl compounds I, III and IV, it is tempting to suggest that these compounds might be hydrolysed by a mechanism (enzymic protonation of the ethoxy oxygen, *etc.*) analogous to that advanced[4] for the enzymic hydrolysis of the aryl glycosides (I), and that the lactone is not similarly hydrolysed owing to what must be a very different orientation of the lactone (ester) oxygen at the active site of the enzyme.

The present work includes what are probably the first demonstrations of catalytic activities of a glycosidase upon ester bonds. This work was greatly aided by the presence of a highly specific site for the absorption of acetamido derivatives adjacent to the catalytic centre of the enzyme. Not all enzymes would have sites with such high specificity close to the catalytic centres, and it seems possible that some of the many esterases of low substrate specificity and of unknown biological function may have unsuspected activity towards structures apparently very dissimilar (and biologically more significant) than the ester substrates commonly employed in the assay procedures.

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