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GLYCOSIDASES WITH UNUSUAL SPECIFICITIES

IV. A HYDROLASE THAT SPLITS *p*-NITROPHENYL-2-DEOXY-2-GLYCYLAMIDO-β-D-GLUCOPYRANOSIDE: PROPERTIES OF THE PURIFIED ENZYME

N. V. MOLODTSOV and M. G. VAFINA

Pacific Institute of Bioorganic Chemistry, F.E. Science Centre, Academy of Sciences of the U.S.S.R., Vladivostok-22 (U.S.S.R.)

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SUMMARY

A hydrolase that splits *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside was isolated from a *Chaetopterus variopedatus* extract; the resultant enzyme seems to be an alkaline β-hexosaminidase with broad specificity and may be called β-*N*-glycylglucosaminidase. It was purified 150 times by gel filtration on Sephadex G-200 and DEAE-Sephadex A-50.

The properties of the purified enzyme were investigated.

The Michaelis constant and the pH optimum were determined for *p*-nitrophenyl-2-acetamido- and -2-glycylamido-2-deoxy-β-D-glucopyranosides and -2-deoxy-2-acetamido-β-D-galactopyranoside.

The enzyme is inhibited by *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside at concentrations over 0.4 mM.

INTRODUCTION

Previously [1, 2] we reported that an unusual glycosidase (EC 3.2.1–) found in marine invertebrates and mushrooms splits *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside. The distribution of this glycosidase, which may be called β-*N*-glycylglucosaminidase, was studied.

This paper deals with the isolation of the above unusual glycosidase from an extract of the marine helminth *Chaetopterus variopedatus* and the study of some properties of the purified enzyme.

MATERIALS AND METHODS

Substrates

p-Nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside and -galactopyranoside were synthesized in accordance with Zurabyan et al. [3], while *p*-nitrophenyl-

2-deoxy-2-glycylamido-β-D-glucopyranoside acetate was obtained from *p*-nitrophenyl-2-deoxy-2-*N*-phthalylglycylamido-β-D-glucopyranoside [4] by hydrazinolysis.

Analytical methods

An 0.2 M phosphate buffer (0.1 ml), pH 7.0, containing 1 M NaCl and an enzyme solution (0.1 ml), was added to an 0.1% substrate solution (0.1 ml). The mixture was incubated at 50 °C, the reaction being terminated with the addition of 1 M Na_2CO_3 (2 ml); the resultant *p*-nitrophenol was determined spectrophotometrically at 440 nm. The optimum pH of the enzyme activity and other characteristics were determined at 400 nm. The protein concentration in the solution was determined in accordance with Lowry et al. [5] by using a bovine serum albumin as standard. The enzyme kinetic properties at substrate splitting were measured at pH 6.5 on a "Spekord" spectrophotometer, the *p*-nitrophenol content being recorded automatically.

Isolation and purification of β-N-glycylglucosaminidase

The *C. variopedatus* specimens (Posiet Bay, Sea of Japan) were ground with clean sand, and the mixture was extracted using mild agitation with a 5-fold amount of an 0.05 M phosphate buffer at pH 7.0. The residue was removed by centrifugation at 5500 rev./min (4000 × *g*). The extract obtained was treated with $(NH_4)_2SO_4$ till 80% saturation and centrifuged at 5500 rev./min for 45 min. The residue was dissolved in water, centrifuged at 15 000 rev./min (14 000 × *g*) for 20 min, and chromatographed on a Sephadex G-200 column with elution by an 0.05 M phosphate buffer, pH 7.0. The fractions which actively split *p*-nitrophenyl-*N*-glycylglucosaminide were combined, and the overall fraction of several separations was chromatographed on DEAE-Sephadex A-50, equilibrated with an 0.05 M phosphate buffer, pH 7.0. The active fractions, being eluted from the same buffer with increasing NaCl concentrations, were combined and rechromatographed on DEAE-Sephadex A-50. Fractions of the purified enzyme were lyophilized with $(NH_4)_2SO_4$ (5 mg/ml).

RESULTS AND DISCUSSION

Following sulphate sedimentation of the *C. variopedatus* extract to 80% saturation, virtually the entire β-*N*-glycylglucosaminidase activity was suppressed (see Table I). With chromatography on a Sephadex G-200 column, equilibrated with a 0.05 M phosphate buffer, pH 7.0, the β-*N*-glycylglucosaminidase showed one peak directly after the void volume, which indicated the high molecular weight of the enzyme. In this case, mainly low-molecular proteins and a very insignificant portion of high-molecular proteins separate from the mixture. Following this purification stage, the enzymic activity increased three times, the output being virtually quantitative. The combined active fractions of several separations on a Sephadex G-200 were further chromatographed on DEAE-Sephadex A-50, equilibrated with an 0.05 M phosphate buffer, pH 7.0. Gradient elution of NaCl in the same buffer resulted in two activity peaks in the eluate, with 0.05 and 0.1 M NaCl, respectively. One may assume that these are two isoenzymes, since they have the same molecular weight and do not separate on Sephadex G-200; moreover, the relationship of their activities with respect to *p*-nitrophenyl-2-glycylamido- and -2-deoxy-2-acetamido-β-D-gluco-

TABLE I

PURIFICATION OF *N*-GLYCYLGLUCOSAMINIDASE FROM *CHAETOPTERUS VARIOPEDATUS*

G, splitting of *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside. A, splitting of *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside. Activity unit: number of *p*-nitrophenol μmoles split from substrate during 1 h at 50 °C in 0.06 M phosphate buffer, pH 6.5, with 0.3 M NaCl.

Purification stage	Protein (mg)	Overall activity		Specific activity		Yield per activity		Purification degree		Relationship G/A
		G	A	G	A	G	A	G	A	
1. $(NH_4)_2SO_4$ fraction	226	79	372	0.35	1.79	100	100	1	1	0.2
2. Chromatography on Sephadex G-200	83	83	360	1	6	100	96	2.9	3.36	0.16
3. Chromatography on DEAE-Sephadex A-50	184	200	1110	1	6					
Fraction I	7.25	78	151	11	21	39	13.6	32	11.8	0.5
Fraction II	8.2	51	95	6.25	11.6	25	8.5	18	6.5	0.5
4. Rechromatography of Fraction I	12	104	410	8.5	34					
	0.96	55.5	163	52.5	163	21.4	5	148	91	0.33
Rechromatography of Fraction II	22.8	130	780	5.65	34					
	3	90	300	30	100	17	3.3	86	56	0.3

pyranosides in both peaks is also the same. The main purification of the accompanying β-*N*-acetylglucosaminidase was observed at this stage. The purification degree reaches 30 and 20 as compared to sulphate sedimentation where the yield for the first (0.05 M NaCl) and second (0.1 M NaCl) peak is 39% and 25%, respectively. After rechromatography of both peaks on DEAE-Sephadex A-50, a slight separation of the protein admixtures takes place, and the total degree of purification reaches 150 and 85 for the first and second peak, respectively. The overall activity of β-*N*-glycylglucosaminidase is about 38.5%, but the activity relationship for the above-mentioned substrates somewhat changes and attains 0.33.

Thus, if one calculates the purification degree with respect to the initial extract, the overall value becomes 765 and 1350 for the second and first peak, respectively.

Subsequently, the entire work was carried out on β-*N*-glycylglucosaminidase, which was eluted from DEAE-Sephadex A-50 by 0.05 M NaCl in an 0.05 M phosphate buffer, pH 7.0, i.e. with the first peak.

Optimum pH

Changes in the enzymic activity with different pH values were examined using *p*-nitrophenyl-2-glycylamido- and -2-deoxy-2-acetamido-β-D-glucopyranosides as substrates in a phosphate buffer solution and in the same buffer in the presence of NaCl (Fig. 1). For both substrates, the optimum activity was close to pH 7.0; in the presence of NaCl, a shift takes place to the acid region by 0.5–1.0 pH unit (Fig. 1). A small arm in the 4–5 pH range for *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside indicates a slight admixture of β-*N*-acetylglucosaminidase (EC 3.2.1.30).

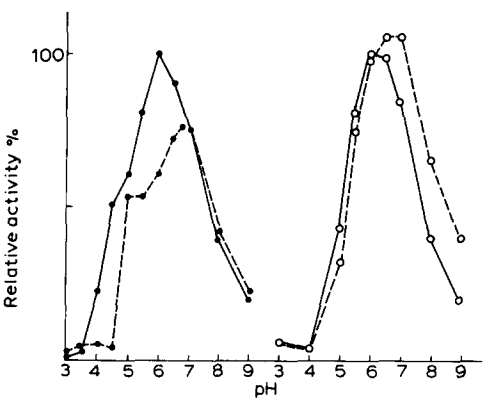

Fig. 1. Effect of pH on enzyme activity. ———, phosphate buffer pH 3–9 with NaCl; – – –, less NaCl; —●—, *N*-acetylglucosaminidase activity; —○—, *N*-glycylglucosaminidase activity.

Temperature stability and activity changes with temperature

The splitting rate of *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside was examined at temperatures from 30 to 85 °C for 15 min of incubation in a phosphate buffer, pH 6.5, with NaCl (Fig. 2). In this case, an increase in the substrate splitting rate to occurred until 70 °C, where it was above 300% of the standard conditions chosen (50 °C, 15 min, pH 6.5 with NaCl). Subsequently, the splitting rate sharply decreased. This was ostensibly due to a thermal denaturation of the enzyme; yet, even at 85 °C, 50% of the activity remained at 50 °C. It should be noted that at

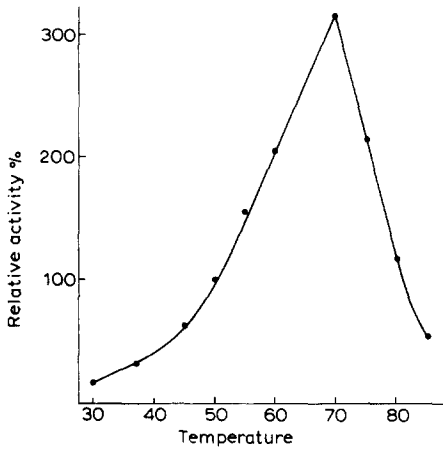

Fig. 2. Temperature effect on enzyme activity. 0.1 ml of enzyme solution, 0.1 ml of 0.2 M phosphate buffer, pH 6.5, with 1 M NaCl and 0.1 ml of 0.1% *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside at were incubated 30–85 °C for 15 min with the subsequent addition of 1 ml of 1 M Na_2CO_3; the amount of *p*-nitrophenol was determined at 400 nm.

37 °C the enzymic activity is about 33% of the standard conditions chosen. The enzyme also showed remarkable stability when maintained in a buffer solution, pH 6.5, with NaCl: with a 50-h incubation at 50 °C, no noticeable fall in activity was observed.

Michaelis constant

The dependence of the substrate splitting rate on (the) enzyme concentration has a linear form. For *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside, the enzyme proved to be inhibited at concentrations greater than 0.4 mM (Fig. 3). For

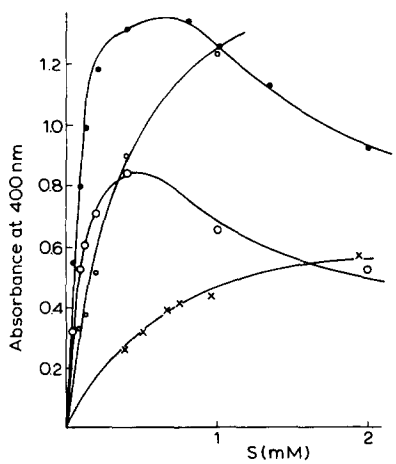

Fig. 3. Effect of the substrate concentration on the splitting rate of *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside (—×—, for an enzyme concentration of 32 μg/ml), *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-galactopyranoside (—o—, for an enzyme concentration of 32 μg/ml) and *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside (—○—, for an enzyme concentration of 10.7 μg/ml and —●—, for an enzyme concentration of 21.4 μg/ml).

TABLE II

MICHAELIS CONSTANTS (K_M) AND MAXIMUM SPLITTING RATES (V) FOR THE HYDROLYSIS OF CERTAIN SUBSTRATES WITH *N*-GLYCYLGLUCOSAMINIDASE

Substrate	K_M (M)	V (μmoles/min) for enzyme concentration of 1 mg/ml
p-Nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside	$1 \cdot 10^{-3}$	0.31
p-Nitrophenyl-2-deoxy-2-acetamido-β-D-galactopyranoside	$0.5 \cdot 10^{-3}$	0.62
p-Nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside	$0.13 \cdot 10^{-3}$	0.94–1.13

p-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside and -2-deoxy-2-acetamido-β-D-galactopyranoside, the curves have classical forms. The calculated values of K_M are cited in Table II.

Influence of inhibitors

The influence of various substances was studied by preincubating the dialyzed enzyme solution at 20 °C during 15 min with the compound examined in a phosphate buffer, pH 6.5, or without it. This particularly concerns the influence of metal ions, whose phosphates are in most cases slightly soluble. Hg^{2+} and Ag^{+} showed the strongest inhibiting effects (Table III).

Molecular weight

The molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200. The graph in Fig. 4 shows it to have been equal to approx. 125 000.

Numerous works in the literature are concerned with the isolation of β-*N*-acetylglucosaminidase from different sources and the estimation of its substrate specificity. The authors [6–11] give special attention to the study of the character of the enzyme–substrate interaction, resulting in the enzyme–substrate complex. In this connection, one should note the works devoted to the influence of the aglycone portion of the substrate and the oligosaccharide activity [6–11], as well as the modification of the glycone portion with the amino group at C_2 [12] and various *O*-substituted hexosaminides, particularly methylated glucosaminides [13]. One could forecast that, depending on the enzyme source, the substrate specificity would somewhat differ. This is moreover true, since a very numerous families of vitally important biopolymers are known to exist, including glycolipids, blood group substances, etc. To conceive the structure and functions of these mixed biopolymers, it appears useful to study both natural compounds and their respective modelling compounds with respect to different glycosidases, particularly β-*N*-acetylglucosaminidases isolated from varied sources. In our view, compounds that simulate glycoside bonds of *N*-aminoacyl derivatives of amino sugars appear to be of particular interest. Such hypothetical bonds are very probable in glycoproteins, although there is no strict evidence for their presence and/or absence in mixed biopolymers. By using *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside as a substrate, we discovered an unusual natural

TABLE III

EFFECT OF CERTAIN COMPOUNDS ON *N*-GLYCYLGLUCOSAMINIDASE ACTIVITY

The enzyme solution (0.1 ml) was incubated for 15 min at 20 °C with a phosphate buffer (0.1 ml), pH 6.5, with NaCl and a solution of the compound studied (0.1 ml); then a 0.1 % substrate solution (0.1 ml) was added, and the mixture was incubated for 30 min at 50 °C. The reaction was terminated by adding 1 M Na_2CO_3 (1 ml); the liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm. G, *p*-nitrophenyl-2-deoxy-2-glycylamido-β-D-glucopyranoside substrate; A_1, *p*-nitrophenyl-2-deoxy-2-acetamido-β-D-glucopyranoside substrate; A_2, galactopyranoside.

Compound	Relative activity (%)						
	M	G		A_1		A_2	
None	—	100		100		100	
N-Acetyl-D-glucosamine	10^{-2}	95		90		70	
	10^{-1}	50		60		40	
N-Acetyl-D-galactosamine	10^{-2}	55		85		90	
	10^{-1}	30		70		70	
N-Benzoyl-D-glucosamine	10^{-1}	100		95		90	
N-Acetylglycyl-D-glucosamine	10^{-2}	90		90		70	
	10^{-1}	70		85		45	
Sodium acetate	10^{-2}	100		115		100	
	10^{-1}	95		95		100	
Acetamide	10^{-1}	80		90		95	
$HgCl_2$	$2 \cdot 10^{-4}$	60	15*	90	30*	100	25*
$AgNO_3$	$5 \cdot 10^{-3}$	45*		90*		55*	
	10^{-2}	20*		15*		5*	
$CuSO_4$	10^{-2}	100		100		100	
	10^{-1}	0		86		—	
p-Nitrophenol	$1.5 \cdot 10^{-4}$	90		—		—	
	$4 \cdot 10^{-4}$	65		—		—	
Aminoethanethiol	10^{-2}	100		100		80	
	10^{-1}	90		90		40	
EDTA	10^{-1}	85		100		80	

* Preincubation less buffer.

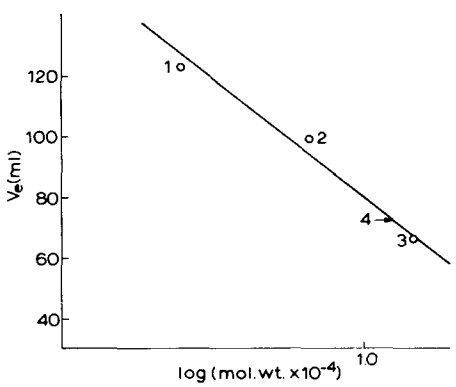

Fig. 4. Determination of enzyme molecular weight by gel filtration on Sephadex G-200: 1, chymotrypsinogen; 2, bovine serum albumin; 3, aldolase; and 4, point of enzyme yield.

glycosidase that degrades the β-glycoside bonds of *N*-aminoacylglucosamine. This may be suggestive of the existence of glycoproteins with aminoacyl bonds from amino sugars with polypeptides in living organisms. Conclusive evidence would be the discovery of the natural substrate of the enzyme described. Besides this, a more detailed study of the substrate specificity of the β-*N*-glycylglucosaminidase is found to be of paramount interest.

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