CHARACTERIZATION AND LOCALIZATION OF FOUR FORMS OF *N*-ACETYL-β-D-HEXOSAMINIDASE FROM FENUGREEK *(TRIGONELLA FOENUM GRAECUM)* GERMINATED SEEDS

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1. Introduction

The enzyme *N*-acetyl-β-D-glucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamido-deoxy glucohydrolase, EC 3.2.1.30) appears in various tissues in several isozymic forms. Three isozymes namely hexosaminidases A and B [1–4] and hexosaminidase C [5–7] have been described in human tissues. Multiple forms of *N*-acetyl-β-D-hexosaminidase have been isolated from other mammalian tissues [8–12], from hen oviduct [13] and from *Dictyostelium discoideum* [14,15].

In this paper, we report the first isolation of 4 forms of *N*-acetyl-β-D-hexosaminidase from plants and we demonstrate that these 'isoenzymes' originate from different parts of the germinated seeds.

2. Materials and methods

2.1. *Germination*

Fenugreek seeds (Cooperative Pharmaceutique Française, Melun) were treated during 10 min at room temperature with a 10% solution of sulfuric acid, then with cold running water till neutrality and finally with cold absolute ethanol during 30 min and put to swell for 12 h in distilled water. The swollen seeds were set in the dark at room temperature on several layers of wet filter paper and covered with one sheet of wet filter paper. The germination was stopped after 48 h.

2.2. *Purification*

The intact germinated seeds or the previously removed endosperm, cotyledons and embryonic axis of the germinated seeds were suspended, ground into 2 litres 0.9% sodium chloride solution, filtered on gauze and centrifuged at 4000 rev/min during 1 h. The supernatant of each extract was adjusted to pH 4.6 with 1 M citric acid and solid ammonium sulfate was added until 0.8 saturation. The precipitate was collected by centrifugation (15 000 rev/min during 1 h), dissolved in 10 mM sodium phosphate buffer pH 6.7 and dialysed against the same buffer during 3 days. The solution was concentrated by ultrafiltration on diafiber Amicon H1DX50 and applied to DEAE-cellulose (type DE 22) column (2.5 × 50 cm) equilibrated with 10 mM sodium phosphate buffer pH 6.7. The column was washed with 1.4 litre of the equilibrating buffer and then eluted with stepwise increases in sodium phosphate concentration in the equilibrating buffer, 10 ml fractions were collected at a flow rate of 34 ml/h and the *N*-acetyl-β-D-hexosaminidase activity of each fraction determined. These active fractions were concentrated by ultrafiltration, dialysed against 10 mM sodium phosphate buffer pH 6.7 and submitted to a rechromatography on DEAE-cellulose column (2.5 × 50 cm) under the same conditions.

Each peak previously dialysed against 10 mM sodium phosphate buffer pH 6.15 was subjected to affinity chromatography on a column (2 × 20 cm) of Sepharose 6-B-(ε-aminocaproyl)-*N*-acetyl-β-D-glucosaminylamine prepared according to the procedure of Lotan et al. [16]. The column was washed with 10 mM sodium phosphate buffer pH 6.15, then eluted with 0.2 M sodium phosphate buffer pH 6.8.

8 ml fractions were collected at a flow rate of 16 ml/h. The *N*-acetyl-β-D-hexosaminidase activity eluted in a single peak was dialysed and concentrated in ultra-thimble UH 100 (Schleicher and Schüll).

2.3. *Enzyme assays*

N-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities were assayed using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (pNPGLcNAc) and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (pNPGalNAc) respectively. Both substrates were products of Koch-Light Laboratories Ltd (Colnbrook Bucks; G.B.).

A 900 μl sample of 5 mM *p*-nitrophenyl-glycoside in McIlvaine buffer pH 5.0 was incubated at 37°C with 100 μl of enzyme solution for 10 min. The reaction was stopped by the addition of 500 μl of 1 M sodium carbonate and the liberated *p*-nitrophenol was estimated at 400 nm using a Zeiss PMQ II spectrophotometer MT 4 cells.

2.4. *Analytical methods*

Protein was estimated by the method of Lowry et al. [17] or by the fluorescamine method of Udenfried et al. [18] using crystalline bovine serum albumine (Sigma) as standard. The effect of inhibitors on enzyme activity were examinated by incubating the enzyme at its optimum pH with aldonolactone for 20 min at 37°C before the hydrolysis of *p*-nitrophenyl-glycosides. K_is were determined by the method of Dixon [19].

Michaelis constant (K_M) and maximum velocity (V_{max}) of each peak of *N*-acetyl-β-D-hexosaminidase activity were estimated from the reciprocal Lineweaver–Burk plots [20]. Possibility of one single site for the two activities was investigated according to Dixon and Webb [21].

Analytical gel electrophoresis was performed on 5% cross linked polyacrylamide gels at pH 9.5, by the method of Davis [22]. The gels were stained by the method of Chrambach et al. [23] using Coomassie Brilliant Blue and by the method of Hayashi [24] using naphtol-AS-BI-*N*-acetyl-β-D-glucosaminide or galactosaminide (Koch Light Ltd, Colnbrook, Bucks, UK).

3. Results and discussion

From fenugreek germinated seeds 4 forms of *N*-acetyl-β-D-hexosaminidase have been separated on DEAE-cellulose column (fig.1). Purification of these 4 forms was achieved by rechromatography on DEAE-cellulose column under the same conditions and by affinity chromatography on a Sepharose 6-B-(ε-aminocaproyl)-*N*-acetyl-β-D-glucosaminylamine

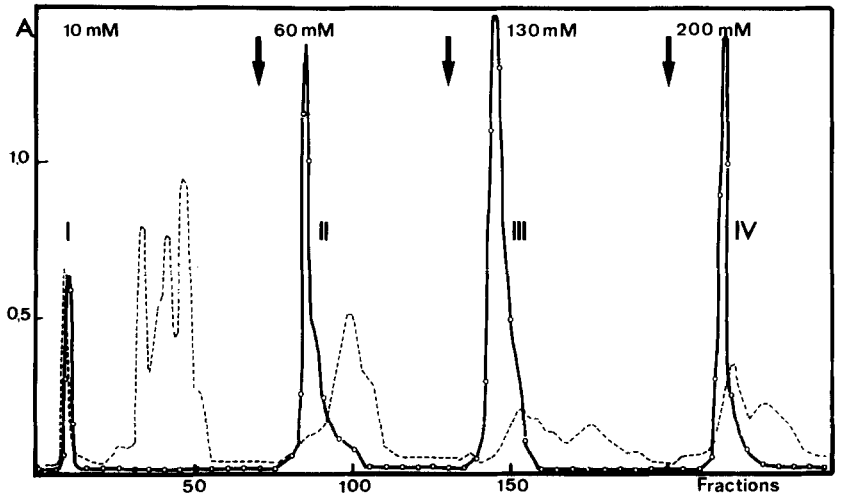

Fig.1. Elution pattern of *N*-acetyl-β-D-glucosaminidase from germinated fenugreek seeds on DEAE-cellulose column (2.5 × 50 cm). The column is washed with 10 mM sodium phosphate buffer pH 6.7 and then eluted with stepwise increases in sodium phosphate concentrations. (- - - -) Absorbance at 280 nm. (-0-0-0-) *N*-acetyl-β-D-glucosaminidase activity.

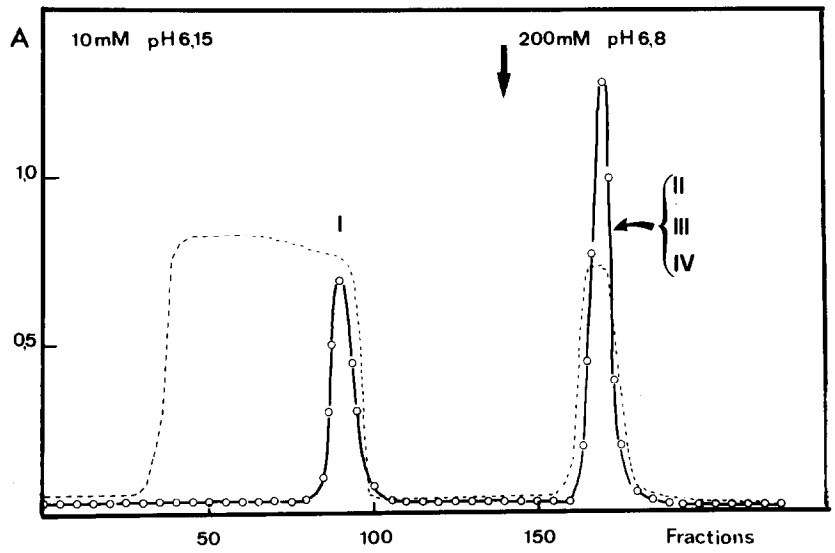

Fig.2. Elution pattern of *N*-acetyl-β-D-glucosaminidase from the Sepharose 6-B-(ε-aminocaproyl) *N*-acetyl-β-D-glucosaminylamine column. The column is washed with 10 mM sodium phosphate buffer pH 6.15 and then eluted with 0.2 M sodium phosphate buffer pH 6.8. (- - - -) Absorbance at 280 nm. (-0-0-0-) *N*-acetyl-β-D-glucosaminidase activity.

column (fig.2). Form I was eluted with the equilibrating buffer, the other forms (II, III and IV) were eluted by increasing the ionic strength and the pH of the buffer. It can be noticed, that, from Jack bean meal and wheat germ, Rafestin et al. [25] seem to have isolated only two forms of hexosaminidases.

In table 1 are reported the activities of the 4 forms of *N*-acetyl-β-D-hexosaminidase in the cotyledon, the endosperm and the embryo extracts. It appears that *N*-acetyl-β-D-hexosaminidase form I is found only in the cotyledons, form II in endosperm and cotyledons and forms III and IV are localized especially in embryo. So, it appears that, like in animals, plant 'isoenzymes' are specifically or essentially synthetized by well-defined tissues.

Specific acitivities and purification factor of the 4 forms of *N*-acetyl-β-D-hexosaminidase are given in table 2. It can be seen that the Sepharose-ligand appears to be very efficient for the publication of the 4 forms, since a purification factor of 20 880 was obtained after affinity chromatography for the form III of *N*-acetyl-β-D-hexosaminidase. Enzymatic studies demonstrated that any α- or β-glucosidase, α- or β-galactosidase, α- or β-mannosidase and *N*-acetyl-α-D-hexosaminidase activities can be detected in the 4 peaks after affinity chromatography on the Sepharose-ligand column. After gel electrophoresis each of the 4 forms of *N*-acetyl-β-D-hexosaminidase give a single band which was stained by the Coomassie Brillant Blue reagent or by the enzymatic reagents.

All forms which have been isolated possess both *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities. For each form, the ratio of these 2 activities remained relatively constant throughout the purification procedure (table 2).

Table 1
Localization of the *N*-acetyl-β-D-hexosaminidase activities in the different parts of germinated fenugreek seeds

Isozymic forms	Endosperm	Cotyledons	Embryo
I	0	18.8	0
II	95.0	79.0	0
III	0.17	0.7	7.9
IV	4.5	1.1	92.0

Results were expressed as percent of the activity of enzyme found in each extract

Table 2
Purification of four forms of *N*-acetyl-β-D-hexosaminidase from germinated fenugreek seeds

Preparation	*N*-acetyl-β-D-hexosaminidase forms	Protein (mg)	Specific[a] activity	Purification rate	Activity ratio $\frac{N\text{-acetyl-}\beta\text{-D-glucosaminidase}}{N\text{-acetyl-}\beta\text{-D-galactosaminidase}}$
Ammonium sulfate crude extract		3049	0.035	1	2.5
DEAE-cellulose chromatography	I	273	1.35	40	1.30
	II	18	0.85	24.2	2.20
	III	205	14.70	420	6.00
	IV	77	6.70	191	17.00
DEAE-cellulose rechromatography	I	38	1.57	44.8	1.27
	II	2.80	6.80	194	2.30
	III	8.10	20.10	574	6.20
	IV	21	12.70	362	17.00
Affinity chromatography	I	2	6.48	185	1.25
	II	0.13	23.40	670	2.35
	III	0.14	731.00	20 880	6.50
	IV	0.80	166.00	4740	18.00

[a]Specific activity is defined as the quantity (μg) of *p*-nitrophenol released per min and per mg of protein.

Table 3
Kinetic values of *N*-acetyl-β-D-hexosaminidases from germinated fenugreek seeds

Enzymatic form	pNPGlcNAc as substrate		pNPGalNAc as substrate		pNPGlcNAc + pNPGalNAc as substrate α = 1.03		
	K_M^A (mM) (1)	V_{max}^A (μg·min·mg)	K_M^B (mM)	V_{max}^B (μg·min·mg)	$V_{max}^A + V_{max}^B$	V_{max}^{A+B} (2) observed	V_{max}^{A+B} (3) theoretical
Form I	0.04	40	0.15	33	73	35.7	37.8
Form II	0.62	20	0.55	12	32	16.6	16
Form III	0.08	2700	0.09	410	3110	1515	1580
Form IV	0.37	434	0.04	23	457	64.5	63.5

(1) K_M^A, V_{max}^A, K_M^B, V_{max}^B are average value from 6 to 8 experiments.
(2) Average value from 3 experiments.
(3) Values obtained from the formula $\frac{K_m^A}{\alpha K_M^B} = \frac{V_{max}^A - V_{max}^{A+B}}{V_{max}^{A+B} - V_{max}^B}$.

However, the ratio of the 2 enzymatic activities appeared to be quite different for the 4 forms of *N*-acetyl-β-D-hexosaminidase from germinated fenugreek seeds. The purified enzyme exhibit different kinetic parameters with artificial *p*-nitrophenyl-substrates (table 3).

Substrates competition studies realized in the presence of pNPGlcNAc and pNPGalNAc in a ratio of 1.03 and competitive inhibition studies realized in the presence of *N*-acetyl-β-D-glucosamino (1→5) lactone and *N*-acetyl-β-D-galactosamino (1→5) lactone gave the results which are described in tables 3 and 4. These results suggest that in the 4 forms of *N*-acetyl-β-D-hexosaminidase isolated from germinated fenugreek seeds the *N*-acetyl-β-D-glucosaminidase and *N*-acetyl-β-D-galactosaminidase activities are located on the same enzyme molecule and a single site wore the two different activities. Similar results have been obtain on the whole *N*-acetyl-β-D-hexosaminidases of Jack bean meal [26] and beef spleen [9].

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Table 4
Inhibition by lactones of *N*-acetyl-β-D-hexosaminidase from germinated fenugreek seeds

Enzymatic form	*N*-acetyl-glucosamino-(1→5) lactone as inhibitor		*N*-acetyl-galactosamino-(1→5) lactone as inhibitor	
	pNPGlcNAc as substrate	pNPGalNAc as substrate	pNPGlcNAc as substrate	pNPGalNAc as substrate
	K_i (mM) (1)	K_i (mM)	K_i (mM)	K_i (mM)
Form I	0.068	0.071	0.010	0.012
Form II	0.119	0.134	0.011	0.011
Form III	0.08	0.08	0.010	0.010
Form IV	0.12	0.14	0.011	0.012

(1) Average value from 3 experiments.

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