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β-*N*-ACETYLHEXOSAMINIDASE FROM SOYBEAN

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Key Word Index—*Glycine max*; Leguminosae; glycosidases; *N*-acetylglucosaminidase; *N*-acetylgalactosaminidase; *N*-acetylhexosaminidase; kinetics; inhibition; purification.

Abstract—The β-*N*-acetylhexosaminidase from soybean (*Glycine max*) seeds was purified more than 100-fold. The ratio of β-*N*-acetylglucosaminidase activity to *N*-acetylgalactosaminidase activity remained unchanged throughout the whole purification procedure.

Inhibition by glucosamine, galactosamine and their acetyl derivatives was of the competitive type. Irrespective of the activity tested, the same inhibition constants resulted. This indicates that both activities are due to the same enzyme molecule.

The enzyme binds to concanavalin A, but no interaction with the lectin from the same plant could be found.

INTRODUCTION

Plant seeds contain several glycosidase activities. There is evidence that in Jack bean (*Canavalia ensiformis*) [1], mung bean (*Vigna radiata*) [2], and castor bean (*Ricinus communis*) [3], the GlcNAc'ase and GalNAc'ase activities reside on the same enzymes which in consequence are designated as *N*-acetylhexosaminidases.

Several leguminous lectins interact with GalNAc and its glycosides. Among these is the soybean (*Glycine max*) lectin (SBA) which binds to both GalNAc and galactose, strongly preferring the former [4].

Thus, though it is doubtful whether plants contain GalNAc [5], they do contain enzymes that can process GalNAc glycosides, and lectins that specifically interact with this sugar.

Presumably, SBA and the soybean *N*-acetylhexosaminidase occur together in the protein bodies of the seeds since this is the case in mung bean [6] and pea (Gers-Barlag and Rüdiger, unpublished). The common occurrence of two proteins that specifically interact with a sugar that presumably is absent from plants prompted us to study the soybean *N*-acetylhexosaminidase. In particular, we were interested to see if the GlcNAc'ase and GalNAc'ase activities could be separated from each other, and if the *N*-acetylhexosaminidase interacted with the lectin from the same plant as do other glycosidases [7–9].

Enzymes: β-*N*-Acetylglucosaminidase, β-*N*-acetyl-D-glucosaminide *N*-acetylglucosaminohydrolase (EC 3.2.1.30); β-*N*-acetylgalactosaminidase, β-*N*-acetyl-D-galactosaminide *N*-acetylgalactosaminohydrolase (EC 3.2.1.53).

Abbreviations: ConA, concanavalin A, the lectin from *Canavalia ensiformis*; SBA, soybean agglutinin, the lectin from *Glycine max*; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; GlcNAc'ase, β-*N*-acetylglucosaminidase; GalNAc'ase, β-*N*-acetyl-galactosaminidase.

RESULTS AND DISCUSSION

Purification

The enzyme was purified by a series of chromatographic procedures as shown in Table 1. At each step, the α-galactosidase, β-galactosidase, α-mannosidase, GlcNAc'ase and GalNAc'ase activities were assayed. The former three separated from the *N*-acetylhexosaminidases in the first steps and were completely absent after affinity chromatography on immobilized ConA. In contrast, at each step the β-GlcNAc'ase and β-GalNAc'ase activities occurred together.

The enzyme bound to phenyl-Sepharose at a high ammonium sulphate concentration and was desorbed at a medium ionic strength. Another leguminous glycosidase, the α-mannosidase from Jack bean, is much more hydrophobic than the soybean *N*-acetylhexosaminidase since it is only desorbed under drastic conditions, i.e. with salt-free water [10]. After concentration by the use of DEAE-cellulose, the soybean enzyme was further purified on ConA-Sepharose at pH 5. Similar to the Jack bean α-mannosidase [8], the soybean *N*-acetylhexosaminidase was not desorbed by sugars that are specific for ConA but by salts. Some contaminant proteins ran through, others were desorbed from the lectin column by glucose or α-methylmannoside (Fig. 1a). A final purification was achieved by FPLC on the high resolution gel filtration medium Superose 6. At this stage, a M_r of 133 000 ± 3000 was determined by gel filtration.

As seen from Table 1, the GlcNAc'ase/GalNAc'ase activity ratios remain constant (1.3 to 1.4) throughout all purification steps. Somewhat higher ratios have been described for the enzymes from Jack bean [1] and castor bean [2].

Kinetics

The pH optima of the GlcNAc'ase and GalNAc'ase activities were 5.0 and 4.6 with half-widths of 2.7 and

Table 1. Purification of the GlcNAc'ase/GalNAc'ase from 3.5 g of soybean seeds

	Protein	Sp. act. (mIU/mg)	Yield (%)	Purif. factor
Extract	343	10.6/7.8	100/100	1.00/1.00
Precipitation pH 6	140	20.8/15.7	78/82	1.96/2.00
Chromatography:				
1. Phenyl-Sepharose	34.1	63.1/45.9	59/58	5.94/5.86
2. DEAE-Cell.	28.2	69.7/48.7	54/51	6.57/6.20
3. ConA-Sepharose	8.9	114/93.4	32/30	12.6/11.9
4. Superose	0.74	1200/881	24/24	113/113

Figures on the left refer to the GlcNAc'ase, on the right to GalNAc'ase activities.

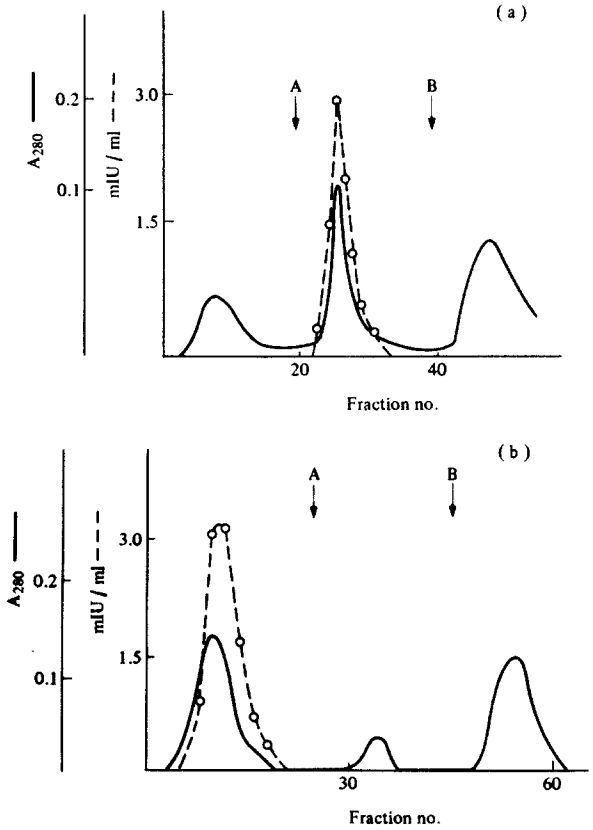

Fig. 1. Purification of *N*-acetylhexosaminidase from soybean on ConA-Sepharose. 3.2 mg/10 ml of prepurified material (Table 1) was applied to columns (2.5 × 8 cm) of ConA-Sepharose. After washing, the columns were developed with 0.5 M NaCl (arrow A) and 0.2 M glucose (arrow B). Fractions of 4 ml/4 min were collected. Buffers were 0.05 M sodium acetate, pH 5.0 (a) and 0.05 M Tris–HCl, pH 8.0 (b).

2.4 pH units, respectively. Different pH optima for both substrates have also been found by others [1, 3]. For convenience, all measurements reported in Table 1 were performed at pH 5.0. For the determination of K_M and V_{max} values, however, the respective optimal pH values were chosen. Measurements were performed with an enzyme preparation after affinity chromatography on ConA-Sepharose (Table 1) at three enzyme (5.40–10.8 μg/ml) and four substrate (0.22–1.75 mM) concentrations. The data were evaluated by a non-linear regression method [11]. As seen from Table 2, both activities exhibited essentially the same K_M and V_{max} values. With the Jack bean and mung bean enzymes, Li and Li [1] and Ohtakara and Tran-Thi [2] found that K_M and V_{max} values were lower for the GalNAc derivatives than for those of GlcNAc.

Inhibition

We found that the enzyme is subject to inhibition by both products (GlcNAc and GalNAc) and by the corresponding free amino sugars. Preliminary evaluation by the method of Dixon [12] showed that in all cases competitive patterns result (not shown). The final evaluation was made by non-linear regression [11].

It is to be expected that a particular inhibitor will exhibit the same K_I value irrespective of the kind of substrate, if both substrates are processed at the same catalytic site. Table 3 gives the inhibition constants.

Table 2. K_M and V_{max} values (2.7 μg enzyme preparation) of the GlcNAc'ase/GalNAc'ase from soybean seeds

	K_M (mM)	V_{max} (mIU)
GlcNAc'ase	0.48 ± 0.04	3.28 ± 0.09
GalNAc'ase	0.47 ± 0.06	2.86 ± 0.12

Table 3. Inhibition of the GlcNAc'ase/GalNAc'ase from soybean seeds [K_I values (mM) are given]

Inhibitor	GlcNAc'ase (mM)	GalNAc'ase (mM)
GalNAc	8.2 ± 0.6	7.1 ± 0.5
GlcNAc	39.7 ± 5.3	40.4 ± 4.3
Galactosamine	17.6 ± 1.3	18.2 ± 1.5
Glucosamine	5.6 ± 0.2	6.2 ± 0.6

Apparently, the substrate type does not influence the K_I value. This indicates that the same active site hydrolyses both GlcNAc and GalNAc glycosides.

Remarkably, inhibition of both activities by GalNAc is more effective than by GlcNAc. A reverse order was found for the free amino sugars. Sandhoff and Wässle [13], who studied human liver *N*-acetylhexosaminidases found a similar inhibition pattern by these four monosaccharides. Glucose and galactose are very poor inhibitors: at 100 mM the residual activities are 90 and 85%, respectively.

As found for the Jack bean [1] and mung bean enzymes [3], the soybean *N*-acetylhexosaminidase requires intact thiol groups since 0.5 mM $AgNO_3$ resulted in complete and 10 mM $ZnCl_2$ in 80% inhibition. Acetate has been reported to inhibit some *N*-acetylhexosaminidases [1–3]. We could find no differences in activity using 0.05 M citrate or acetate buffers at pH 5. Therefore most measurements were performed in acetate buffer.

Interaction with lectins

Recently, we found that glycosidases from several leguminous seeds interact as well with lectins from the same plants as from other sources [7–9]. This is also true for the soybean α-mannosidase and α-galactosidase which bind to ConA by their carbohydrate moieties and to the soybean lectin by ionic forces [14]. Figure 1a shows that the soybean N-acetylhexosaminidase binds to immobilized ConA at pH 5. Binding is abolished at high salt concentrations whereas lectin-specific sugars are without effect. In contrast, no binding occurs at pH 8 (Fig. 1b).

In a similar approach with immobilized SBA, we were unable to find an interaction of the GlcNAc'ase/GalNAc'ase neither at pH 5 nor at pH 8 (results not shown).

EXPERIMENTAL

Materials. Soybeans were bought from Lima, B-9830 Sint-Martens-Latem, (lot PV 103/85), *p*-nitrophenylglycosides from Sigma Chemie, phenyl-Sepharose, Sepharose 6B and Sephadex G-50 from Deutsche Pharmacia, and DEAE-cellulose (DE 52) from Whatman. ConA was isolated from Jack beans (Sigma Chemie) by the use of Sephadex G-50 (15), SBA by affinity chromatography according to ref. [16]. Both lectins were immobilized to Sepharose 6B as described in ref. [17]. Protein densities, determined according to ref. [18], were 10 and 9 mg per ml gel, respectively.

Purification. Dry soybeans were shelled and finely ground in a coffee mill. The powder was defatted with CH_2Cl_2 and dried under a hood. 3.5 g of the defatted meal was suspended in 35 ml 0.05 M Tris–acetate buffer, pH 8.0, and stirred for 2 hr at 4°. The suspension was percolated and the filtrate clarified by centrifugation for 15 min at 12000 *g*. The crude extract was adjusted to pH 6.0 by slow addition of 5 M HOAc, stirred for 30 min, centrifuged and readjusted to pH 8.0 by dialysis against the extraction buffer.

The soln was brought to 0.6 M $(NH_4)_2SO_4$ by addition of the solid salt and applied to a column (3.5 × 20 cm) of phenyl-Sepharose equilibrated with 0.6 M $(NH_4)_2SO_4$ in 0.05 M Tris–acetate buffer, pH 8.0. After washing the column with 2 vol of the equilibration buffer, the proteins were eluted with 2 vols of a gradient [0.6 to 0.0 M $(NH_4)_2SO_4$] at a speed of 54 ml/hr. Both enzyme activities centred around 0.23 M $(NH_4)_2SO_4$. For concentrating, the active fractions were pooled, dialysed against 0.05 M Tris–acetate buffer, pH 8.0, and applied to a column (3.5 × 20 cm) of DEAE-cellulose in the same buffer. After the column had been washed with two vols of the starting buffer, the enzyme was desorbed as a sharp peak with 0.5 M NaCl in the buffer.

This material was dialysed against 0.05 M NaOAc buffer, pH 5 (1 mM $MgCl_2$ and $CaCl_2$), clarified by centrifugation and applied to a column of ConA-Sepharose as described in the legend of Fig. 1. No difference was found if at all steps citrate was used instead of acetate.

As a final step, we used gel filtration on a column (1 × 30 cm) of Superose 6 in 0.05 M Tris–HCl, 0.1 M NaCl. For determining the M_r Sephadex G-100 was used. β-Amylase (200000), yeast alcohol dehydrogenase (150000), ovalbumin (45000) and cytochrome *c* (12400) served as references.

Enzyme determinations. Glycosidase activities were assayed by hydrolysis of *p*-nitrophenylglycosides as described in refs [1], [8]. pH optima were determined in 0.1 M McIlvain [19] or citrate buffers.

Interaction with SBA. 24 mIU/10 ml of a seed extract (2 mg) was applied to a column (0.4 × 1.5 cm) of SBA-Sapharose in 0.05 M NaOAc buffer, pH 5.0, and in 0.05 M Tris–HCl, pH 8.0. After washing with the starting buffers, desorption was attempted with 1 M NaCl in the respective buffer.

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