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ISOLATION OF β-*N*-ACETYL-D-HEXOSAMINIDASES FROM LUPIN SEED

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Key Word Index—*Lupinus luteus*, Leguminosae, lupin seed, hexosaminidase, concanavalin A

Abstract—A rapid procedure for the isolation of β-*N*-acetyl-D-hexosaminidase from lupin seed meal is described This involves affinity chromatography of a seed extract on concanavalin A, followed by chromatography on DEAE-Sepharose The purified enzyme was obtained in three forms, hexosaminidases A, B and B_1, capable of hydrolysing both *p*-nitrophenyl β-2-acetamido-2-deoxy-D-glucopyranoside and *p*-nitrophenyl β-2-acetamido-2-deoxy-D-galactopyranoside Enzyme A was relatively less active towards the galactosaminide substrate, than were the B forms of the enzyme

INTRODUCTION

β-*N*-Acetyl-D-glucosaminidase (EC 3 2 1 30) activity has been detected in a wide variety of biological materials [1] and in most cases occurs in association with β-*N*-acetyl-D-galactosaminidase (EC 3 2.1 53) activity [2] β-*N*-Acetyl-D-hexosaminidase (EC 3 2 1 52) from Jack bean meal was isolated in electrophoretically homogeneous and crystalline form and it was concluded from pH and thermal inactivation and kinetic analysis that the one enzyme hydrolyses β-2-acetamido-2-deoxy-D-glucopyranoside and β-2-acetamido-2-deoxy-D-galactopyranoside substrates at the same site [3] β-*N*-Acetylhexosaminidase has been isolated from lupin seed meal and three electrophoretically distinct forms have been demonstrated One form of the enzyme appears to be more active for *p*-nitrophenol β-2-acetamido-2-deoxy-D-galactopyranoside

RESULTS AND DISCUSSION

Glycosidase activity of crude lupin extracts

Crude extracts of lupin seed meal and lupin nodules were assayed for glycosidase activity using the eight substrates listed in Table 1 Activity with all eight substrates was demonstrated with the seed extract, *p*-nitrophenyl-α-D-mannopyranoside and *p*-nitrophenyl-β-D-galactopyranoside being the most actively hydrolysed The nodule extract showed no activity with *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-mannopyranoside and *p*-nitrophenyl-α-L-fucoside, but similar levels of activity compared with the seed extract for the other five substrates

Purification

β-*N*-Acetyl-hexosaminidase was isolated from the crude extracts by affinity retention on a concanavalin A (conA)-AffiGel column, followed by elution with methyl-α-D-mannopyranoside (MM) When tested with the eight substrates shown in Table 1, the MM eluate showed activity only with *p*-nitrophenyl β-2-acetamido-2-deoxy-D-glucopyranoside and *p*-nitrophenyl β-2-acetamido-2-deoxy-D-galactopyranoside The glycosidases capable of hydrolysing substrates one to six (Table 1) are therefore either not glycoproteins or do not contain α-D-glucopyranosyl, α-D-mannopyranosyl, or sterically related residues required to bind to conA

When the lyophilized MM seed meal eluate from the conA-AffiGel column was subjected to SDS-PAGE, three to four main bands and several minor bands of proteins were detected The SDS gel pattern for the nodule protein MM eluted from the conA column showed two main bands The nodule preparation was not investigated further

Further purification of the enzyme from lupin seed was achieved by DEAE-Sepharose chromatography with a NaCl gradient, resulting in the elution of three separate bands of activity (Fig 1) The peaks of activity were designated B, B_1 and A in order of elution of increasing salt concentration The specific activities of these enzymes are given in Table 2 The specific glucosaminidase ac-

Table 1 Glycosidase activities of lupin extracts

Substrate (*p*-Nitrophenyl-)	Sp activity (μmol/mg protein/hr)	
	Seed extract	Nodule extract
α-D-glucopyranoside	0 10	0
β-D-glucopyranoside	0 11	0 18
α-D-mannopyranoside	0 73	0 62
β-D-mannopyranoside	0 11	0
α-L-lucoside	0 10	0
β-D-galactopyranoside	0 33	0 28
β-2-acetamido-2-deoxy-D-glucopyranoside	0 16	0 70
β-2-acetamido-2-deoxy-D-galactopyranoside	0 11	0 28

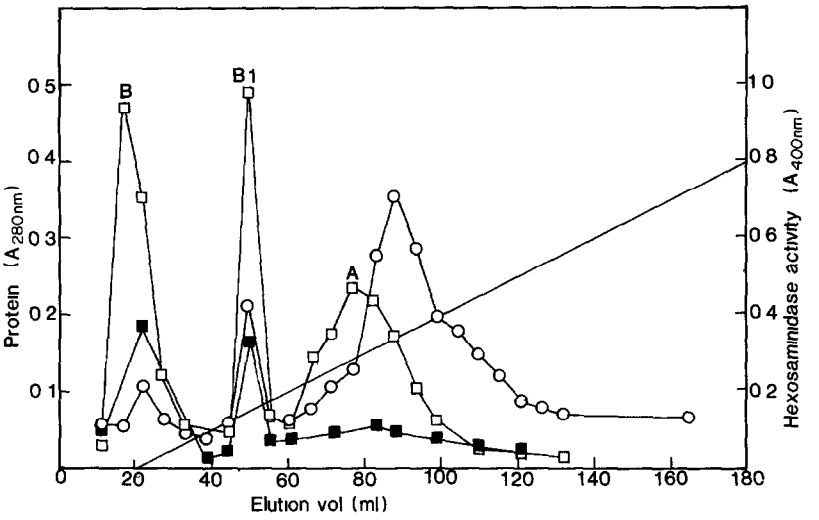

Fig 1 DEAE-Sepharose chromatography of conA eluate The NaCl gradient (0–0 8 M NaCl) is shown □–□, glucosaminidase activity, ■–■, galactosaminidase activity, ○–○, protein A280

Table 2 Hexosaminidase activities of enzymes A, B and B_1

	Sp activity (μmol substrate hydrolysed/min/mg protein)		Activity ratio
	Glucosaminidase	Galactosaminidase	
Enzyme B	21 4	11 3	1 9
Enzyme B_1	15 2	5 0	3 0
Enzyme A	11 9	2 5	4 8

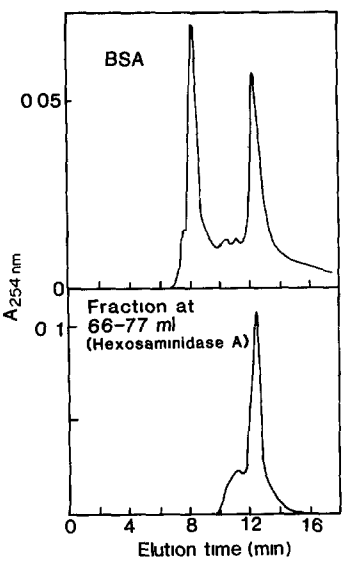

Fig 2 HPLC of fractions from DEAE-Sepharose chromatography and of bovine serum albumin (BSA) Injection was made at zero time, using *ca* 10 μg protein in each case The peak of hexosaminidase A at 12 5 min eluted just after the monomer of BSA (MW 67 000)

tivities of the crude extract and the extract eluted from the conA-AffiGel column were 0 16 and 4 7 μmol/min/mg of protein respectively Similar results were reported for placental β-*N*-acetyl-hexosaminidase [8]

Following dialysis and lyophilization of selected DEAE-Sepharose fractions, hexosaminidase A, eluted from 66–77 ml, was resolved into two peaks of protein by HPLC (Fig 2) As can be seen in Fig 2, hexosaminidase A from the front of the peak of activity (Fig 1) was almost pure, in agreement with results obtained by gel electrophoresis

General properties of β-N-acetyl hexosaminidase from lupin seed

When the peak fractions from the DEAE-Sepharose column were subjected to gel electrophoresis, single bands were obtained on Tris–glycine gels SDS gels yielded major bands corresponding to MWs of 66 000 for enzyme A and 40 000 for both enzymes B and B_1, by comparison with Pharmacia low MW standards The MW of enzyme A was also determined by sedimentation equilibrium Three series of $\ln\Delta y$ values (Δy is the fringe displacement) were plotted against r^2 (r is the radial co-ordinate) to give straight line plots with slopes of $1\ 57 \pm 0\ 05$ The estimated value of partial specific volume was taken to be 0 73 and thus the apparent MW was calculated to be $66\,000 \pm 2000$ It therefore appears that this enzyme consists of a monomer of 66 000 daltons, confirmed by HPLC (Fig 2)

The SDS gel pattern for the nodule protein eluted with MM from the conA column also gave a major band estimated to be of MW 66 000 This indicates that the nodule hexosaminidase is the same as hexosaminidase A from the seed

Hexosaminidases B and B_1 exhibited greater activity towards *p*-nitrophenyl β-2-acetamido-2-deoxy-D-galactopyranoside than did A (Table 2) The ratio of the rate of hydrolysis of the two hexosaminidase substrates varies from two for enzyme B to five for enzyme A (Table 1)

Some glycosidases have been shown to contain carbo-

hydrate and to have haemagglutinating properties [9–11] When analysed for carbohydrate with phenol–sulphuric acid reagent, enzyme A was shown to contain 6 8 mg carbohydrate per 100 mg of protein, equivalent to a mol ratio, hexose to protein, of 18 and B_1 to contain 12 5 mg carbohydrate per 100 mg of protein Protein was determined by the Folin method

Both enzymes were tested for haemagglutination activity using a 2% suspension in PBS-1 of washed heparinised rabbit red cells A concanavalin A solution in PBS was used as a positive control for comparison, and no haemagglutination was observed

CONCLUSION

Lupin β-N-acetylhexosaminidases have properties similar to those generally reported for β-N-acetylhexosaminidases from various sources, in that they exist in more than one active form, have the ability to bind to conA [12, 13] and the capacity to hydrolyse both glucosaminide and galactosaminide substrates It is of interest that the lupin enzyme shows differential activity towards the above two substrates Lupin seed appears to be a convenient source for the rapid preparation of β-N-acetylhexosaminidases for further studies

EXPERIMENTAL

Plant material (a) *Seed meal extract* Meal was prepared from seeds of *Lupinus luteus* L by grinding in a Janke and Kankel water-cooled mill The meal was rinsed briefly in phosphate buffered saline (PBS-1 0 15 M NaCl, 10 mM NaP_i, pH 7 2) to remove coloured material and resuspended in PBS-2 (PBS-1 plus 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$) (1 g meal/5 ml buffer) Extraction was continued for 2 hr at room temp with stirring After centrifugation the supernatant (11 mg protein/ml) was applied to a concanavalin A-AffiGel column All procedures following the initial extraction were performed at 4° (b) *Nodule extract* A crude nodule extract was prepared by macerating frozen lupin nodules in 0 45 M sucrose, 50 mM KP_i buffer (pH 7 2) containing 2% soluble PVP (1 g nodules/1 ml buffer) After filtration through two layers of cheesecloth and centrifugation at 11 000 rpm for 20 min at 4°, the supernatant was dialysed twice versus PBS-1 and concd four times using Amicon diaflo filtration with a PM10 membrane

The supernatant (18 7 mg protein/ml) was passed through the conA AffiGel column

Glycosidase assay p-Nitrophenyl substrate (2 mM, 0 5 ml) in 0 05 M sodium citrate buffer, pH 4 8, and 50 μl enzyme soln were incubated for 30–60 min at 25° The reaction was stopped by addition of 3 ml 0 2 M sodium borate buffer, pH 9 8, and the amount of p-nitrophenol liberated was calculated from the absorbance of the soln at 400 nm [4] by using an ε value of $1\,45 \times 10^4$ for the p-nitrophenolate ion

Concanavalin A chromatography Concanavalin A (conA) was prepared from Jack bean meal (Sigma) using an AffiGel-ovalbumin column as described by Biorad (Catalogue G, 1981, page 48) and then coupled with 15 ml AffiGel-15

The conA column was washed with PBS2 until the $A_{280\,nm}$ was less than 0 02 Elution was carried out with 0 5 M methyl-α-D-mannopyranoside in PBS-2, after which the protein-containing fractions were dialysed twice versus distilled H_2O and lyophilized

DEAE-Sepharose chromatography The active material from the conA column was purified on columns of DEAE-Sepharose (1 6 cm × 24 cm) equilibrated in 0.05 M Tris–HCl, pH 7, and eluted using the following gradient 100 ml 0 8 M NaCl in Tris into 100 ml Tris, using 15 mg of protein The main peak of activity eluted with the salt gradient is enzyme A B and B_1 are used to denote the enzymes eluted earlier in Fig 1

The fractions showing maximum activity in the peaks at 22, 50 and 77 ml were concd, dialysed against distilled H_2O and lyophilized The proteins were then characterized by gel electrophoresis as described below

Gel electrophoresis Disc electrophoresis was performed as described by Davis [5] using Tris–glycine buffer, pH 8 3, and SDS-PAGE to characterize protein fractions MW standards (10 000–90 000) were used to establish the molecular size of the fractions Gels were stained with Coomassie blue R as described by Davis [5] for SDS gels and with 0 4% Coomassie blue G in 3 5% perchloric acid for the Tris–glycine gels [6]

Ultracentrifugation The MW of enzyme A was established by sedimentation equilibrium [14], with use of interference optics in a Spinco Model E centrifuge The enzyme was dissolved in 0 05 M Tris buffer, pH 7 0 (0 2 mg/ml), and dialysed versus the same buffer overnight at 4° Ultracentrifugation was performed at 30 000 rpm for 24 hr

Total carbohydrate This was determined by the phenol–H_2SO_4 method [15] using lactose (500 mg/ml) as standard

Protein was determined by the Lowry method [7] using crystalline BSA as standard

HPLC was performed using a Waters model 6000A pump, model U6K injector with a Waters I250 protein column equilibrated in 0 1 M acetate buffer (pH 6 8) The flow rate was 1 0 ml/min and column pressure was 800 psi

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