Purification and characterization of N-glycanase, a concanavalin A binding protein from jackbean (*Canavalia ensiformis*)

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Removal of the N-glycan from the concanavalin A (Con A) glycoprotein precursor is a key step in its conversion into an active lectin. N-Glycanase (EC 3.5.1.52), the enzyme from jackbean catalysing this process, has been purified to homogeneity as judged by native PAGE. One of the purification steps is binding of the enzymic activity to Con A–Sepharose and its elution by methyl α-mannoside. On SDS/PAGE the principal components were found to be 78 kDa, 74 kDa, 54 kDa, 32 kDa and 30 kDa polypeptides. These did not react with Con A on an affinity blot. Cleveland mapping indicated that some of these polypeptides had related primary structures. The enzyme has a broad pH optimum in the region of 5.0.

INTRODUCTION

The carbohydrate-binding proteins known as lectins are found in the seeds of a wide range of plants [1]. Their function within the seed remains unclear, although in roots they may play a role in mediating the symbiotic relationship with nodulating bacteria [2]. One of the best studied lectins is concanavalin A (Con A) found in jackbean (*Canavalia ensiformis*) seeds.

Con A biosynthesis is complex, involving virtually unprecedented processes [3–12]. It is thought that the nascent polypeptide is co-translationally segregated into the endoplasmic reticulum, glycosylated and the signal sequence cleaved to form a glycoprotein precursor which does not possess lectin activity [5,9]. It is then transported via the Golgi into the protein-storage vesicles. Labelling with [^{14}C]glucosamine and pulse–chase experiments suggest that removal of the oligosaccharide, catalysed by N-glycanase, is the first stage of post-translational processing and occurs in a single step [6].

Subsequent processing involves endoproteolytic cleavage C-terminal to several asparagine residues and a ligation or transpeptidation step, resulting in the post-translational creation of a new peptide bond between amino acid residues 118 and 119 [4,5]. The final product in the mature seed consists of two forms. In one form the protomeric unit consists of a single polypeptide chain in which the amino and carboxyl halves from the precursor have exchanged positions. The other form is a protomeric unit consisting of two chains in which the amino and carboxyl halves of the original polypeptide are not covalently linked.

Interestingly, endoproteolytic cleavage and formation of the peptide bond are not required for conversion of the glycoprotein precursor into a protein having carbohydrate-binding activity. The crucial step in this process appears to be deglycosylation of the precursor [13,14]. Here we describe the purification and characterisation of jackbean N-glycanase, the enzyme responsible for deglycosylation of the Con A precursor and its conversion into an active lectin.

EXPERIMENTAL

Materials

Pthalaldehyde was obtained from Kodak Eastman, Sephacryl S200, Con A–Sepharose, FPLC phenyl-Superose and Mono Q columns from Pharmacia, DE-52 cellulose from Whatman Biosystems Ltd., *Staphylococcus aureus* V8 protease and recombinant endo-β-*N*-acetylglucosaminidase (Endo H) from Boehringer Mannheim, Enzymobeads from Bio-Rad, jackbean meal from Sigma Chemical Company and *Flavobacterium meningosepticum* peptide N-glycanase (PNGase F) from Genzyme Biochemicals Ltd.

Protein characterization

SDS/PAGE, electrophoretic transfer to nitrocellulose and silver staining were carried out using standard methods [15–17]. Protein concentration was determined by the dye-binding method [18]. Alkaline electrophoresis on native slab gels used standard Laemmli buffers with SDS absent [15,19]. Native acidic gel electrophoresis was carried out using a discontinuous buffer system (125 mM sodium acetate in the stacking gel, 375 mM sodium acetate in the separating gel, 140 mM acetic acid/0.33 M β-alanine in the reservoir buffer) [20]. Peptide mapping and Con A affinity blotting were carried out as described [21,22]. Matrix-assisted laser desorption MS with time-of-flight detection was carried out using a Kratos Kompact II spectrometer with sinapinic acid as the laser absorbing matrix.

Preparation of ^{125}I-Con A glycoprotein precursor

The ^{125}I-Con A glycoprotein precursor was purified with two modifications of the previously described method [13] which was found to give improved yield and purity. (1) The precursor was released from immature jackbean microsomes by sonication into 10 mM sodium phosphate, pH 7.4/1 M NaCl (4 ml/g of coty-

Abbreviations used: Con A, concanavalin A; Endo H, endo-β-*N*-acetylglucosaminidase; PNGase F, *Flavobacterium meningosepticum* peptide N-glycanase; Bistris-propane, 1,3-bis[tris(hydroxylmethyl)methylamino]propane.

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ledon initially used). (2) After DE-52 chromatography, the precursor-enriched fraction was concentrated to a volume of 2 ml by partial lyophilization and applied to a column (1.4 × 90 cm) of Ultrogel AcA44 previously equilibrated in 10 mM sodium acetate, pH 5.0/150 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$. Iodination was carried out by the Enzymobeads method. The labelled precursor had a specific activity of ~ 3 Ci/g.

N-Glycanase assays

Routinely, N-glycanase was detected in chromatographic fractions as the activity causing an increased mobility of ^{125}I-Con A precursor on SDS/PAGE as a result of loss of the glycan. A digest (total volume 20 μl) contained 2–5 μl of enzyme extract, 150000 c.p.m. of ^{125}I-Con A precursor, 100 mM sodium acetate (pH 4.5) and 1 mg/ml BSA. Digestion was carried out at 30 °C for 30 min–16 h depending on N-glycanase concentration. Digestion was stopped by the addition of 1.1 × standard concentration SDS sample buffer to a final volume of 200 μl and heating to 95 °C for 5 min. SDS/PAGE was carried out on 13 % polyacrylamide gels [15]. Immediately after electrophoresis, gels were dried and autoradiographed. The mixture for assay of activity from native gel slices had the same composition as that described above, except that the soluble enzyme extract was replaced by a small gel slice (5 μl volume) which had been previously equilibrated for 5 min in 100 mM sodium acetate, pH 4.5.

The glycopeptide from ovalbumin was isolated following CNBr cleavage [ovalbumin/CNBr (1:2.4, w/w) in 70 % formic acid for 36 h at room temperature] [23,24] by lyophilization and gel filtration of resolubilized material on Sephadex G25, previously equilibrated in 50 mM triethylamine/acetic acid, pH 5.0. The N-glycanase assay, based on the release of ammonia from the cleaved amino-oligosaccharide, was carried out using the ovalbumin CNBr glycopeptide (100 nmol) as a substrate [25,26].

Purification of N-glycanase

All operations were carried out at 0–4 °C. Jackbean meal (100 g) was stirred for 2 h with 1 litre of 10 mM sodium phosphate, pH 7.4/150 mM NaCl/1 mM EDTA/1 mM PMSF/1 mM iodoacetamide. After centrifugation (17000 ***g***, 1 h), the supernatant (915 ml, 6.8 mg/ml protein) was retained. Ammonium sulphate was added to 55 % saturation (351 g/l) and the pH was adjusted to 7.4. After stirring for 30 min the extract was centrifuged (17000 ***g***, 30 min) and the supernatant was discarded. The protein pellet was redissolved in 70 ml of 10 mM Tris/HCl, pH 7.4/1 M NaCl and dialysed overnight against this buffer (5 litres). After removal of precipitated protein by centrifugation, $CaCl_2$ and $MnCl_2$ were added to the supernatant (114 ml, 27 mg/ml protein) to final concentrations of 1 mM each. The extract was then applied to a column of Sephacryl S-200 (4.6 × 50 cm) previously equilibrated in 10 mM Tris/HCl, pH 7.4/1 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$. N-Glycanase activity was present in the unbound fraction (470 ml, 3 mg/ml protein). The Sephacryl matrix could be regenerated by eluting affinity bound Con A with 200 mM methyl α-mannoside. To the fraction which passed straight through the column, EDTA/Na^+ (pH 8.0) was added to a final concentration of 10 mM, and ammonium sulphate was added to 70 % saturation (pH maintained at 7.4 with ammonia solution). After centrifugation, the precipitated protein was redissolved and dialysed against 2 × 5 litres of 10 mM 1,3-bis[tris(hydroxylmethyl)methylamino]propane (Bistris-propane)/HCl, pH 6.8. Buffer solution was added and, after centrifugation to remove precipitated protein, the extract (180 ml, 6.3 mg/ml protein) was applied to a DE-52 cellulose column (24 × 4.5 cm) previously equilibrated in 10 mM Bistris-propane/HCl, pH 6.8. The column was washed with buffer and eluted with a linear gradient of 0–0.4 M NaCl in the same buffer over 2000 ml. Fractions containing N-glycanase activity were pooled (380 ml, 1.8 mg/ml protein), made up to 506 ml by the addition of 40 mM Tris/HCl, pH 7.4/4 M NaCl/4 mM $CaCl_2$/4 mM $MnCl_2$ and applied to a column of Con A–Sepharose (2.2 × 21 cm) previously equilibrated in 10 mM Tris/HCl, pH 7.4/1 M NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$. The column was washed first with 300 ml of this buffer, then with 10 mM Tris/HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$. It was eluted with 200 mM methyl α-mannoside, 10 mM Tris/HCl, pH 7.4/150 mM NaCl/1 mM $CaCl_2$/1 mM $MnCl_2$.

Fractions in the methyl α-mannoside eluate containing N-glycanase activity were dialysed against 10 mM Tris/HCl, pH 8.0. After filtration (0.22 mm Sartorius Minisart), the dialysate (36 ml, 0.13 mg/ml protein) was applied to a Mono Q column (5/5 series, Pharmacia FPLC system) equilibrated in 10 mM Tris/HCl, pH 8.0. The enzyme was eluted with a gradient of 0–0.4 M NaCl over 30 ml. The pooled active fractions (4.7 ml, 0.085 mg/ml protein) were made to 1.5 M $(NH_4)_2SO_4$/1 mM EDTA and applied to a phenyl-Superose column (5/5 series, Pharmacia FPLC system) previously equilibrated in 10 mM sodium phosphate, pH 7.4/1.5 M $(NH_4)_2SO_4$/1 mM EDTA. After washing the column with this buffer, the enzyme was eluted with a linear gradient to 5 mM sodium phosphate, pH 7.4, over 30 ml. Active fractions were stored at −70 °C.

Glycosidase treatments

Phenyl-Superose-purified N-glycanase was dialysed against 10 mM *N*-ethylmorpholine acetate, pH 8.0, and lyophilized. For digestion with PNGase F, jackbean N-glycanase (1 μg) was dissolved in 20 μl of 50 mM sodium phosphate, pH 8.2/20 mM EDTA/0.1 % (w/v) SDS and heated to 95 °C for 5 min. After cooling, PNGase F [5 μl, 1 unit in 5 % (v/v) Nonidet P40] was added and the mixture was incubated at 37 °C for 16 h. The reaction was stopped by addition of 25 μl of 2 × SDS/PAGE sample buffer and heating to 95 °C for 5 min before analysis by SDS/PAGE. For Endo H treatment, jackbean N-glycanase (1 μg) was dissolved in 8 μl of 50 mM sodium acetate, pH 5.5/-0.02 % (w/v) SDS and heated to 95 °C for 5 min. After cooling, Endo H (2 m-units) was added and the mixture was incubated at 37 °C for 10 h. The reaction was stopped by the addition of 10 μl of 2 × SDS sample buffer and incubation at 95 °C for 5 min before analysis by SDS/PAGE and Con A affinity blotting. As positive controls, similar Endo H treatments were carried out on jackbean α-mannosidase (2.3 μg) and Con A precursor (1 μg).

RESULTS

Purification of N-glycanase

The meal from mature seeds was used as the starting material for purification of N-glycanase, as preliminary analysis of jackbean extracts over the time course of development had showed that the enzyme activity persisted to seed maturity (results not shown). Extraction was followed by ammonium sulphate fractionation to remove the bulk of canavalin, the major storage protein of jackbeans. Affinity chromatography on Sephacryl S-200 was used to remove Con A which bound to the matrix, whereas processing activities did not bind. The next purification step was chromatography at pH 6.8 on DEAE-cellulose (Figure 1A).

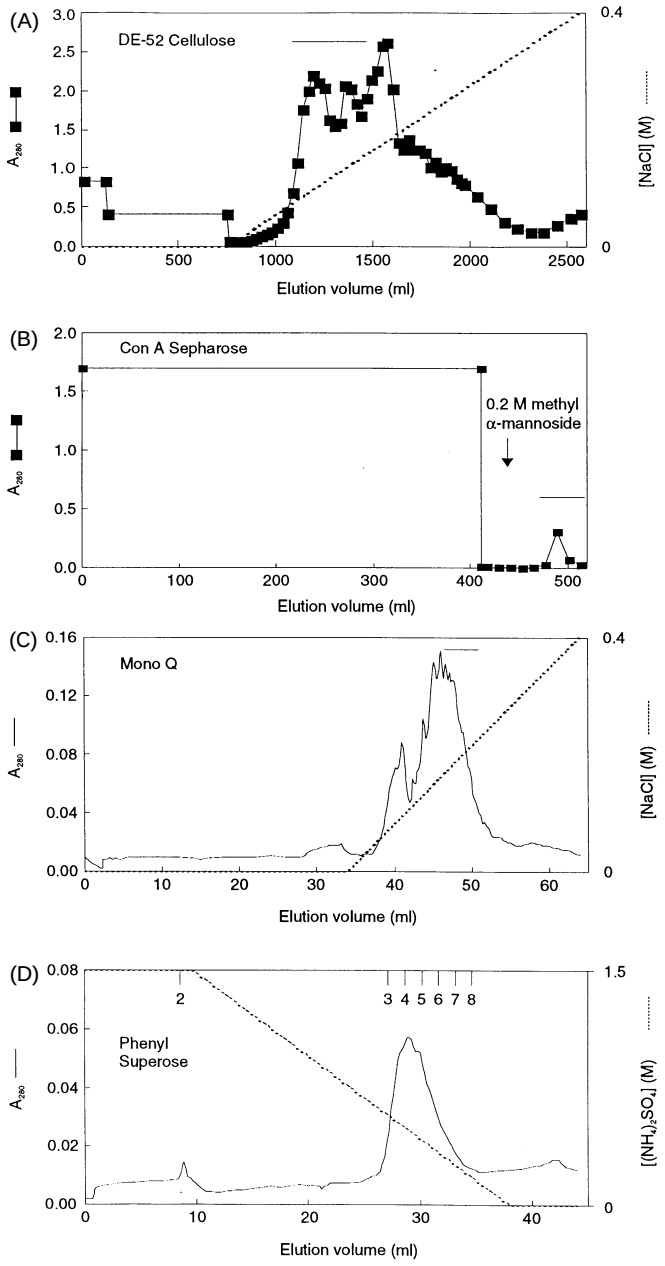

Figure 1 Purification of N-glycanase

(**A**) DE-52-cellulose chromatography; (**B**) Con A–Sepharose chromatography; (**C**) Mono Q FPLC; (**D**) phenyl-Superose FPLC. A horizontal bar indicates fractions containing activity pooled for the next step. In the final step (**D**) the positions of fractions assayed for activity and analysed by SDS/PAGE in (**B**) and (**C**) are indicated.

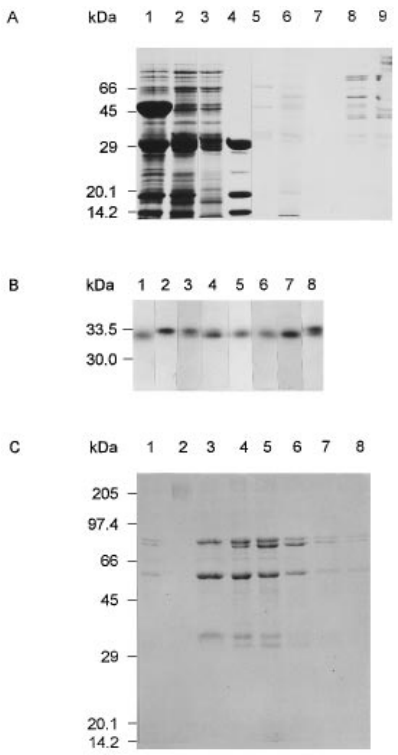

Figure 2 Analysis of N-glycanase purification by SDS/PAGE

(**A**) SDS/PAGE of fractions produced during the purification of N-glycanase (10% gel, stained with Coomassie Blue R250). Lanes: 1, initial jackbean supernatant: 2, 55% ammonium sulphate precipitate; 3, Sephacryl S-200 unbound material (containing N-glycanase); 4, Sephacryl S-200 methyl α-mannoside eluate (affinity purified Con A); 5, DE 52 active fractions; 6, Con A-Sepharose active fractions (α-mannoside eluate); 7, Mono Q active fractions; 8 and 9, phenyl-Superose active fractions. Lanes 1–8, sample buffer containing 5% (v/v) 2-mercaptoethanol; lane 9, no reductant present. (**B**) Assay of N-glycanase activity elution from phenyl-Superose. Autoradiograph of SDS 13%-polyacrylamide gel. ^{125}I-Con A glycoprotein precursor was incubated with 2 μl of extract for 30 min at 30 °C and analysed by SDS/PAGE as described in the Materials and methods section. Lane 1, digestion with extract applied to column; lanes 2–8, digestion with fractions eluted at the points indicated in Figure 1(D). Only the central section of the gel is shown since no other components were observed in the ^{125}I-Con A precursor digests. (**C**) SDS/PAGE analysis of phenyl-Superose-purified N-glycanase (10% gel, stained with Coomassie Blue R250). Lane 1, material applied to column; lanes 2–8, material eluted from the column at positions in the gradient indicated in Figure 1(D).

During Con A–Sepharose chromatography (Figure 1B) the enzyme was consistently separated into both a non-binding fraction and a highly enriched fraction which bound to the matrix. The bound fraction was eluted with methyl α-mannoside. Separation into the two fractions did not appear to be due to column overloading, nor was there a significant amount of Con A in the applied extract which would compete with Con A immobilized on the column. Attempts to purify the non-binding fraction were unsuccessful due to the inability of any chromatographic technique to give sufficient separation of the enzyme activity from contaminating protein in the extract. Since this non-binding fraction was not used, the activity is not indicated in Figure 1(B). At this stage of the purification, N-glycanase activity in the methyl α-mannoside eluate was free of any protease activity acting on the Con A precursor.

The methyl α-mannoside eluate was then further purified by FPLC with Mono Q (Figure 1C) and phenyl-Superose (Figure 1D). SDS/PAGE of fractions containing N-glycanase activity after each step of purification is shown in Figure 2(A). In the final step, elution of enzyme activity correlated closely with elution of a protein peak (Figures 1D and 2B). Chromatography on Superose 12 did not give significant further purification.

Composition of the purified enzyme

SDS/PAGE analysis of the eluate from phenyl-Superose chromatography (Figure 2C) shows that fractions enriched in enzyme activity contained major polypeptides of molecular mass 78, 74, 54, 32 and 30 kDa. In the absence of 2-mercaptoethanol, higher molecular-mass species are observed, suggesting the presence of

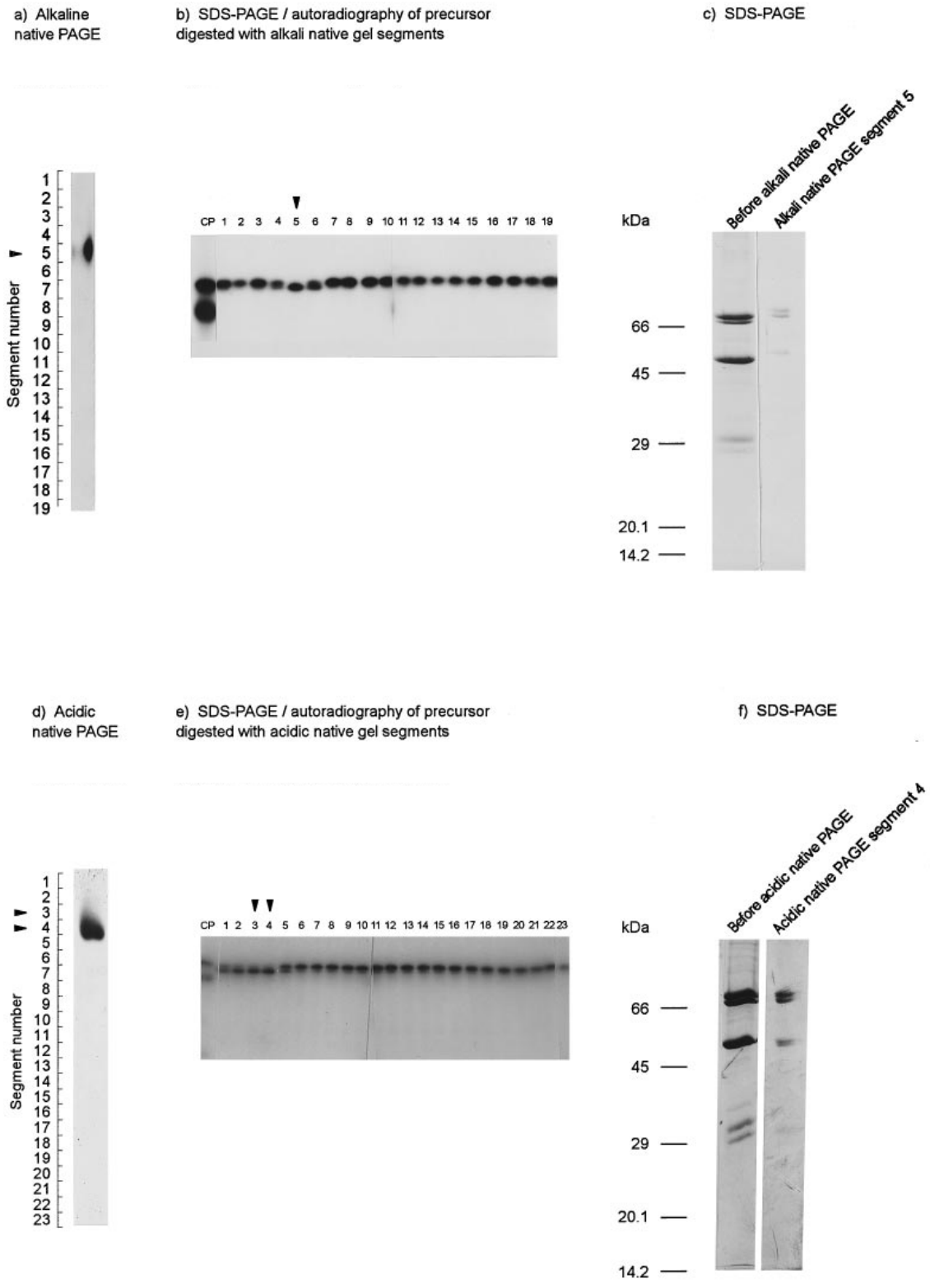

Figure 3 Native gel electrophoresis of purified N-glycanase

(**a**) Native alkaline gel (8% gel, stained with Coomassie Blue R250). The scale on the left indicates the positions of gel slices of a parallel track of N-glycanase used to assay for activity. (**b**) Assay of N-glycanase activity following native alkaline gel electrophoresis: excised gel segments were incubated for 10 min in 100 mM sodium acetate, pH 4.5, and then individually incubated with ^{125}I-Con A precursor (60 min, 25 °C). The Figure shows an autoradiograph of an SDS 13%-polyacrylamide gel showing the products of digestion catalysed by each gel segment. The number above each track refers to the gel segment indicated in the left hand scale of (**a**). CP is a mixture of mature Con A (30 kDa) and Con A precursor (33.5 kDa). Only the central section of the gel is shown since no other components were observed. (**c**) SDS/PAGE of N-glycanase following native alkaline PAGE (SDS 10%-gel stained with Coomassie Blue R250). (**d**) Native acidic gel (8%-gel stained with Coomassie Blue R250. (**e**) Assay of N-glycanase activity following native acid gel electrophoresis. The methods used are as described in (**b**) with lane numbers indicating digestion with gel segments indicated by the scale in (**d**). (**f**) SDS/PAGE of N-glycanase following acid native gel electrophoresis. Gel segments containing maximal activity are highlighted by arrowheads.

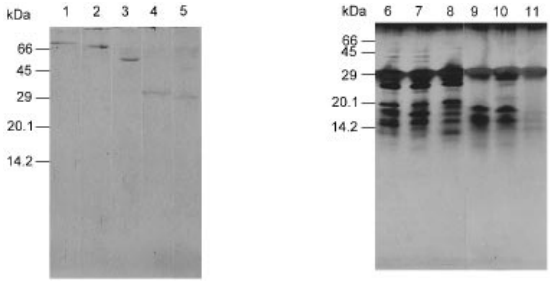

Figure 4 *S. aureus* V8 protease mapping of N-glycanase components

The principal components of phenyl-Superose-purified N-glycanase were separated by SDS/PAGE, stained with Coomassie Blue R250 and the polypeptides were excised in gel slices. These were equilibrated in sample buffer and reseparated on SDS 15%-polyacrylamide gels either in the absence (lanes 1–5) or presence (lanes 6–10) of 200 ng/track of *S. aureus* V8 protease [21]. Lanes 1 and 6, 78 kDa polypeptide; lanes 2 and 7, 74 kDa polypeptide; lanes 3 and 8, 54 kDa polypeptide; lanes 5 and 9, 32 kDa polypeptide; lanes, 6 and 10, 30 kDa polypeptide; lane 12, V8 protease alone. Lanes 1–5, stained with Coomassie Blue R250; lanes 6–11, silver stained.

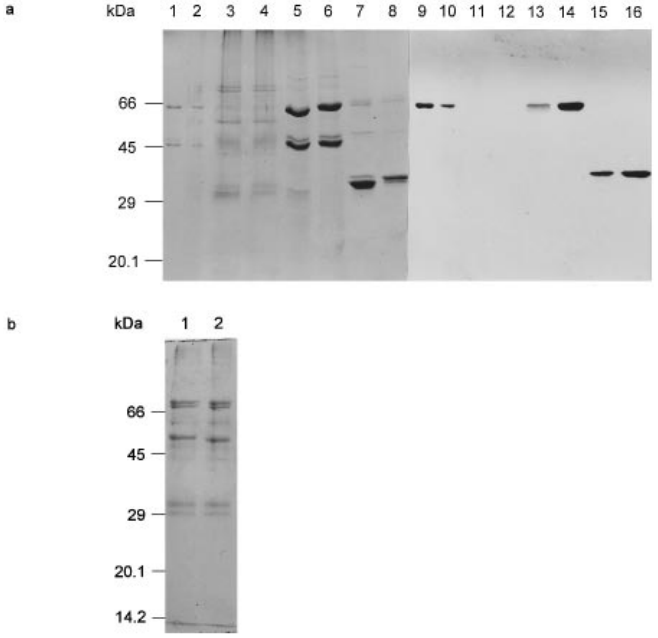

Figure 5 Lack of recognition of N-glycanase polypeptides by Con A and effect of incubation with Endo H and *F. meningosepticum* N-glycanase

(**a**) Samples were separated on an SDS 10%-polyacrylamide gel. Lanes 1 and 9, jackbean α-mannosidase (0.47 μg); lanes 2 and 10, jackbean α-mannosidase (0.23 μg); lanes 3 and 11, jackbean N-glycanase treated with Endo H; lanes 4 and 12, untreated jackbean N-glycanase; lanes 5 and 13, α-mannosidase (3 μg) treated with Endo H; lanes 6 and 14, α-mannosidase (3 μg) untreated; lanes 7 and 15, Con A precursor (2 μg) treated with Endo H; lanes 8 and 16, Con A precursor (2 μg) untreated. Lanes 1–8, Coomassie Blue stained gel; lanes 9–16, Con A affinity blot. (**b**) SDS 10%-polyacrylamide gel stained with Coomassie Blue R250. Lane 1, control digest of jackbean N-glycanase; lane 2, PNGase F digest of jackbean N-glycanase.

disulphide bonds (Figure 2A, lane 9). N-Glycanase polypeptides were not recognized on an immunoblot by an antiserum raised against Con A, indicating that the 30 kDa and 32 kDa polypeptides are not structurally related to Con A (results not shown). On Superose 12 FPLC, the enzyme activity from phenyl-Superose eluted at a position corresponding to a molecular mass of 69 kDa.

On native gel electrophoresis at basic pH, the enzyme activity from phenyl-Superose appeared as a single component (Figure 3a) which co-migrated with enzyme activity assayed directly from native gel segments (Figure 3b). Analysis by SDS/PAGE shows that this region of the native gel has the same polypeptide composition as the enzyme preparation before native PAGE (Figure 3c). Similarly, on an acidic native gel, the enzyme ran essentially as a single component with the same polypeptide composition as the original enzyme preparation, comigrating with the enzyme activity (Figures 3d–3f).

Protease mapping of the polypeptide components of N-glycanase

Mapping of the polypeptide components of phenyl-Superose-purified N-glycanase by partial digestion with *S. aureus* V8 protease suggests that the 78, 74 and 54 kDa polypeptides contain related elements of primary structure and that the 32 and 30 kDa species are also inter-related (Figure 4).

Con A affinity blotting of N-glycanase polypeptides

After SDS/PAGE and transfer onto nitrocellulose, the molecular species corresponding to N-glycanase activity were not recognized by Con A (Figure 5a, lanes 4 and 12), a surprising observation, as the purification of the enzyme includes a step of binding to Con A and elution with methyl α-mannoside. Positive controls included jackbean α-mannosidase (lanes 1, 2, 9 and 10), which possesses a 66 kDa glycosylated polypeptide [8,27], and Con A precursor (lanes 8 and 16).

Susceptibility of jackbean N-glycanase to digestion with glycosidases

The effect of incubating the denatured jackbean N-glycanase preparation with Endo H is shown in Figure 5(a) lane 3, and compared with a control digest without Endo H (lane 4). The slight increase in intensity in the 30 kDa region is due to the Endo H polypeptide itself and is also seen in the positive control digests of jackbean α-mannosidase (lane 5) and Con A precursor (lane 7). Extensive deglycosylation of the positive controls is indicated by loss of recognition by Con A on an affinity blot (lanes 13–16). Jackbean N-glycanase polypeptides therefore appear to be resistant to Endo H digestion.

However, incubation of denatured jackbean N-glycanase with PNGase F appears to result in a reduction of mass of each of the 78, 74 and 54 kDa polypeptides by about 2 kDa, whereas the 32 and 30 kDa polypeptides are unaffected (Figure 5b). The reduction in size is consistent with deglycosylation of these polypeptides. Incubation of denatured jackbean N-glycanase on its own under these conditions had no effect on its polypeptide composition (results not shown).

Characterization of N-glycanase catalytic activity

Although the purified enzyme catalyses a shift in molecular mass of the ^{125}I-Con A precursor that is consistent with removal of the glycan, this method does not provide a precise indication of the nature of the cleaved bond.

However, affinity blotting [22] of the Con A precursor shows that digestion with the purified enzyme results in loss of recognition by Con A (Figure 6A). This indicates, as expected for an N-glycanase activity, that the Con A-reactive oligosaccharide has been removed from the precursor as a result of digestion by the enzyme.

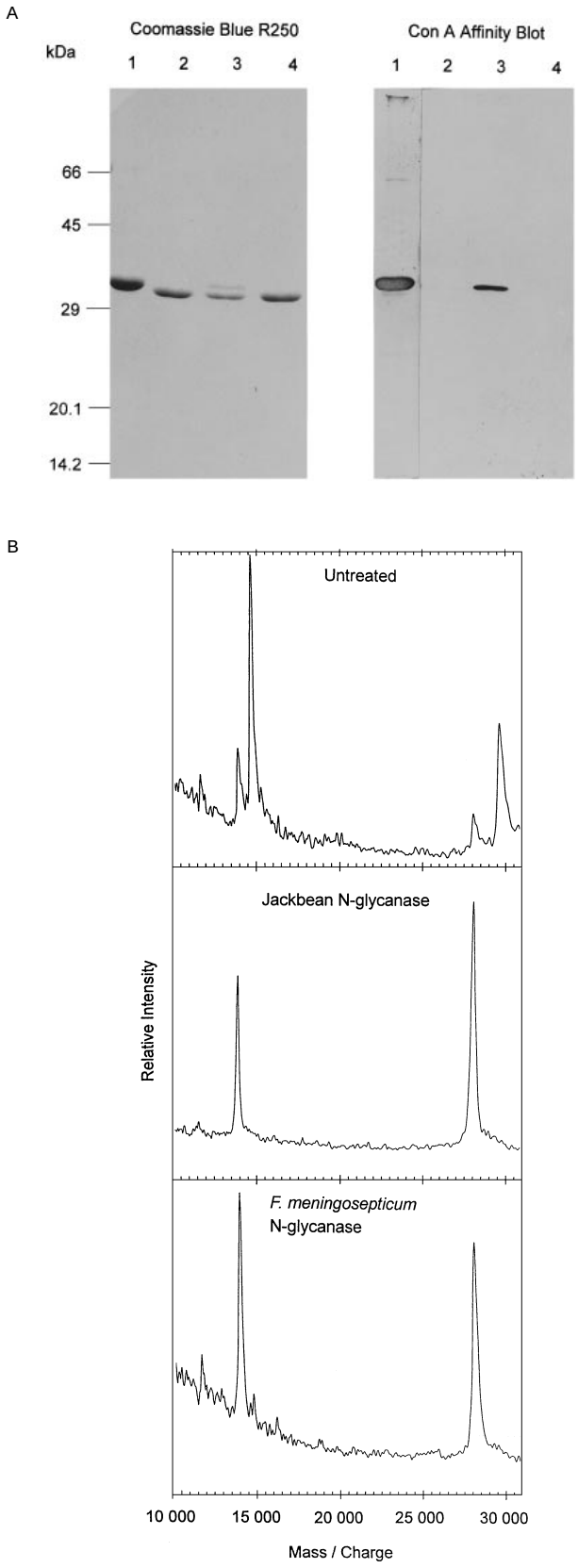

Figure 6 Characterization of Con A precursor before and after digestion with jackbean N-glycanase and PNGase F

(**A**) SDS/PAGE and Con A affinity blotting of Con A precursor following digestion with each glycosidase: lane 1, untreated precursor; lane 2, jackbean N-glycanase-treated precursor; lane 3, Endo H-treated precursor; lane 4, *F. meningosepticum* N-glycanase-treated precursor. (**B**) MS of Con A precursor before and after treatment with N-glycanases. The samples were from the same digests as those analysed in (**A**). Before analysis, trifluoroacetic acid was added to a final concentration of 1% to denature each sample. The maximum deflection on the vertical scale is 8 mV.

Table 1 Ammonia release from N-glycanase digestion of ovalbumin glycopeptide

Mean values obtained from two determinations for each sample are given.

Digest components	Apparent ammonia release (nmol)
Glycopeptide alone	6.5
Jackbean extract alone	7.8
Jackbean extract + glycopeptide	34.9
N-Glycanase alone	0.52
N-Glycanase + glycopeptide	69.0

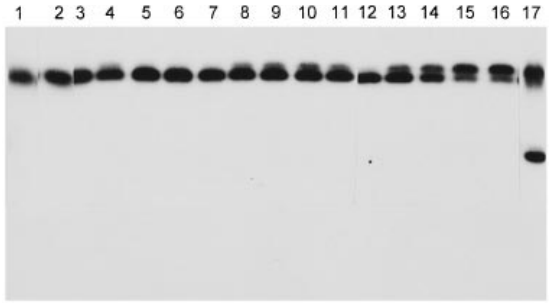

Figure 7 Dependence on pH of jackbean N-glycanase activity

Each digest (total volume 20 μl, 16 h incubation, 25 °C) contained 100 mM buffer, 5 μl of enzyme, 1 mg/ml BSA and ^{125}I-precursor (160000 c.p.m.). Digests were stopped by the addition of 180 μl of 1.11 × normal concentration sample buffer and heated to 95 °C for 5 min. The products of digestion were separated by electrophoresis on SDS 12%-polyacrylamide gels and revealed by autoradiography. Since no other components were seen in the digests, only the region of the gel around the precursor is shown. Lanes: 1, formate/Na^+ pH 3.5; 2, formate/Na^+ pH 4.0; 3, formate/Na^+ pH 4.5; 4, acetate/Na^+ pH 4.0; 5, acetate/Na^+ pH 4.5; 6, acetate/Na^+ pH 5.0; 7, acetate/Na^+ pH 5.5; 8, histidine/HCl pH 5.5; 9, histidine/HCl pH 6.0; 10, histidine/HCl pH 6.5; 11, histidine/HCl pH 7.0; 12, phosphate/Na^+ pH 6.0; 13, phosphate/Na^+ pH 6.5; 14, phosphate/Na^+ pH 7.0; 15, phosphate/Na^+ pH 7.5; 16, phosphate/Na^+ pH 8.0; 17, markers Con A glycoprotein precursor (33.5 kDa) and mature Con A (30 and 17.5 kDa).

One of the products of N-glycanase digestion is an amino oligosaccharide which is unstable and on hydrolysis releases ammonia [25]. Formation of this product is characteristic of an N-glycanase and it is not produced for example by digestion with Endo H. Using the CNBr glycopeptide from ovalbumin as a substrate, the purified N-glycanase preparation was found to be highly enriched with the activity causing ammonia-release from the glycopeptide (Table 1).

MS of the Con A precursor, before and after treatment with bacterial PNGase F or the jackbean N-glycanase preparation from this study, is shown in Figure 6(B). Peaks with a mass/charge ratio of 27000–30000 appear to represent species carrying a single electrical charge, whereas peaks with a mass/charge ratio of 13000–15000 are likely to represent species carrying a double electrical charge. The results indicate that

the enzyme activities from both sources catalyse a reduction in molecular mass of about 1550 Da. The presence of a $GlcNAc_2Man_7$ glycan on the precursor would give a theoretical molecular mass change of 1541 Da on deglycosylation, which lies within the predicted accuracy of the measured values. This value of 1550 Da, corresponding to an oligosaccharide composition of $GlcNAc_2Man_7$, contrasts with a previous report that the N-glycan on the Con A precursor had a composition of $GlcNAc_2Man_9$ [7]. This discrepancy suggests that trimming by α-mannosidase of outer mannose residues has taken place, either during seed development, in precursor purification or in storage.

It can therefore be concluded that the increased mobility observed on SDS/PAGE of the Con A glycoprotein precursor after treatment with the purified enzyme is the result of N-glycanase catalysing cleavage of the amide bond between the asparagine and N-glycan.

Dependence of activity on pH

The enzyme shows activity over a wide range of pH, the optimum being in the region of pH 5.0 (Figure 7). A pH optimum of 4–5 was reported for N-glycanase from *Raphanus sativus* [28].

DISCUSSION

The jackbean lectin, Con A, is synthesized as an inactive N-linked glycoprotein, which undergoes a number of processing events involving deglycosylation, proteolysis and post-translational ligation of peptides to yield the mature protein [3–12]. Previously it has been shown that the essential step in converting the inactive precursor into an active lectin is deglycosylation [13]. The aim of the present study has been to purify the N-glycanase responsible for the activation of Con A, as a means to understand further the relationship between the enzyme and its substrate, and as a foundation for gene cloning.

In earlier work, an N-glycanase activity from jackbean was identified [29] and partially purified by chromatofocussing and gel-filtration chromatography [30]. The native enzyme was reported to have a molecular mass of 48 kDa on gel filtration, with the final preparation estimated as only 70% homogenous, with no sizes of polypeptides from SDS/PAGE analyses provided. In two studies on almond peptide N-glycanase (PNGase A), the enzyme was reported to consist of either two polypeptides of 55 and 25 kDa [31] or a single polypeptide of 50 kDa [32]. N-Glycanases have also been found in a range of animal cells [33–35] and prokaryotes [36].

We have used the Con A glycoprotein precursor as a substrate to follow the jackbean N-glycanase activity through a sequence of purification steps. Ultimately, we purified the enzyme to homogeneity, as demonstrated using acidic or basic native gel electrophoresis systems, when the activity corresponded to a single molecular species. On SDS/PAGE analysis under denaturing conditions the single component could be reproducibly resolved into a number of polypeptides, principally those of 78, 74, 54, 32 and 30 kDa. These polypeptides contain elements of related primary structure, as indicated by peptide mapping using *S. aureas* V8 protease. Specifically, they can be divided into two groups: 78, 74 and 54 kDa give common digestion products, which are distinct from those derived from the 32 and 30 kDa polypeptides. Gel filtration of the jackbean N-glycanase indicates a native molecular mass of $\sim$ 69 kDa. It is possible to rationalize the complexity of polypeptides on SDS/PAGE analysis if the initial translation product(s) is proteolytically processed in the lytic compartment of the protein bodies; such a complexity of molecular species is observed in Con A.

Whereas PNGase F deglycosylated the jackbean N-glycanase, Endo H had no effect. PNGase F will generally cleave Asn-linked oligosaccharides from a protein, provided that the innermost GlcNAc is not modified by the addition of α-1,3- or α-1,6-linked fucose residues [36,37]. Endo H has greater specificity and will not cleave complex-type sugar chains [38,39]. If the oligosaccharide(s) of the jackbean N-glycanase was of a complex tri- or tetra-antennary type, the structure would not be recognized by Con A and, in any case, these structures are not known to occur in plant glycoproteins. Certainly, none of the polypeptides corresponding to the N-glycanase activity was recognized by Con A in affinity blots following denaturation and SDS/PAGE. Nevertheless, native N-glycanase activity did bind to a Con A matrix and was eluted specifically with methyl α-mannoside. It is possible that the interaction between the native enzyme and Con A is between elements of protein structure rather than through a classical lectin–glycan complex. It is known that a conformational change is induced in Con A by occupancy of the sugar-binding pocket [40–43]. Such a conformational change could regulate the interaction between Con A and the jackbean N-glycanase. Preliminary analyses of N-glycanase activities from *Raphanus sativus* [28] and *Silene alba* [44] have shown they also bind to immobilized Con A matrices, although, as yet, characterization of the enzymes has not been carried out.

One role of N-glycanase in jackbeans is clearly to deglycosylate Con A and convert the precursor into an active form of the lectin [6,13]. The total number of Con A-binding polypeptides in jackbean cotyledons decreased markedly during development [8,27], and it is possible that in addition to protein turnover and synthesis in the cotyledon cells, the N-glycanase was also functioning to remove oligosaccharides and convert glycoproteins into their protein counterparts. The existence of a deglycosylated form of another seed glycoprotein, bean α-amylase, that can be observed during processing, may also be indicative of the action of an N-glycanase [45].

In addition to the production of deglycosylated proteins, N-glycanase may also have a role in the production of free oligosaccharides which possess biological activity. It has been suggested that oligosaccharides in cell suspension cultures could be formed as a result of N-glycanase activity [46,47]. Glycopeptides produced from yeast invertase are elicitors of phenylalanine ammonia lyase and ethylene biosynthesis in tomato callus suspension cells [48]. The corresponding oligosaccharides produced as a result of N-glycanase or Endo H treatment of the glycopeptides can act as suppressors of the elicitors from which they have been derived [49]. It was suggested in the latter study that such elicitors and suppressors could be derived from fungal pathogens of plants and that these might play a role in mediating plant–pathogen interactions. Certain endogenous oligosaccharides also stimulate tomato fruit ripening [50–52]. Whereas a plant-derived glycan or glycopeptide resulting from Con A processing may not possess the structural requirements for these particular biological activities, the N-glycanase in jackbean seeds could also act on additional glycoconjugates of pathogen or plant origin, releasing free glycans which do possess biological activity.

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