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The glycoprotein precursor of concanavalin A is converted to an active lectin by deglycosylation

P.S.Sheldon and D.J.Bowles[1]

Centre for Plant Biochemistry and Biotechnology, University of Leeds, Leeds, LS2 9JT, Great Britain

[1]Corresponding author

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We have previously shown that concanavalin A is synthesized as a glycoprotein precursor that is unable to bind to sugars and is processed through six intermediate forms before assembly of the mature active lectin. Since processing involves removal of the *N*-glycan, four proteolytic steps and a religation, the precise event that leads to carbohydrate binding activity was not known. We have now purified the glycoprotein precursor from microsomal membranes and show that deglycosylation *in vitro* is sufficient alone to convert the precursor to an active carbohydrate binding protein. This is the first demonstration of a novel role for *N*-glycans and *N*-glycanases in the regulation of protein activity.

Key words: deglycosation/glycoprotein/lectin/protein processing

Introduction

Concanavalin A (Con A) the lectin of jackbeans, is an abundant component of the plant seed. At seed maturity the lectin is located in protein bodies of the storage parenchyma cells of the cotyledons, where it constitutes 20% of the total protein (Agrawal and Goldstein, 1976; Bowles, 1990b). Accumulation of Con A in the seed starts during the mid-stages of development when the newly synthesized protein is transported from the lumen of the rough endoplasmic reticulum (RER) through the Golgi apparatus to the vacuolar compartment (Herman and Shannon, 1984; Herman *et al.*, 1985; Maycox *et al.*, 1988). Protein loading into the vacuole is accompanied by progressive fragmentation of the compartment to give rise to the protein bodies (Bowles 1990a). When a cDNA clone corresponding to Con A, was sequenced (Carrington *et al.*, 1985) and the predicted amino acid sequence of the translation product compared with the sequence of the mature lectin protein established by Edelman and coworkers (Cunningham *et al.*, 1975; Wang *et al.*, 1975), a surprising anomaly was discovered and it was suggested that a protein rearrangement must occur during lectin processing. This suggestion was confirmed by a detailed analysis of lectin biosynthesis that involved metabolic labelling, pulse-chase analysis, immunoprecipitation and N-terminal sequencing of six abundant molecular species that appeared during the assembly of the lectin (Bowles *et al.*, 1986). As suggested from the comparison of sequence data, the intact subunit of Con A was indeed formed by the reannealing of two fragments, since alignment of amino acid residues 1-118 and 119-237 was reversed in the final form of the lectin identified in the pulse-chase and the precursor first labelled.

The first precursor was known to be *N*-glycosylated (Herman and Shannon, 1984) and both studies from our laboratory (Bowles *et al.*, 1986) and those from Chrispeels *et al.* (1986) suggested the glycoprotein was inactive as a lectin. The pulse-chase had shown that the first step in the processing of Con A caused only a small reduction in size from M_r 33 500 to M_r 31 600 (Bowles *et al.*, 1986) and subsequently we showed that only the first precursor was glycosylated. This indicated a novel event in post-translational processing in which an *N*-glycan was removed in one step to convert a glycoprotein into a protein (Marcus and Bowles, 1988). Jackbeans are known to contain an *N*-glycanase that could be responsible for the observed processing event (Sugiyama *et al.*, 1983; Yet and Wold, 1988).

During biosynthesis of the lectin *in vivo*, proteolytic cleavage of the deglycosylated polypeptide into two fragments rapidly followed the removal of the *N*-glycan (Bowles *et al.*, 1986). The rapidity of this event made it impossible to determine precisely whether deglycosylation or proteolysis was the key step in converting the precursor into an active lectin. To address this issue we have now purified the glycoprotein precursor and reconstituted the first step of lectin processing *in vitro*. We show that removal of the *N*-glycan alone is sufficient to convert the inactive protein into a lectin.

Results

Purification of the glycoprotein precursor of Con A

It is known that the glycoprotein precursor is present only at trace levels in developing jackbean cotyledons for a limited time period (Marcus *et al.*, 1984; Bowles *et al.*, 1986). As a means of concentrating the precursor at early stages in the purification scheme, we took advantage of the known lability of the protein body compartment compared to that of the RER. As shown in Figure 1, fractionation of an extract from developing jackbeans by centrifugation at 100 000 *g*, led to the recovery of the bulk of the seed proteins in the soluble fraction since they are stored as lumenal products within the protein bodies that rupture on disruption of the tissue. Two species of Con A can be detected by anti-Con A in immunoblots of the soluble fraction, the mature lectin subunit M_r 30 000 and the intermediate of M_r 30 400 shown previously to give rise to the mature lectin *in vivo* (track 5). Sonication of the microsomal pellet released additional polypeptides, including a third species of Con A of M_r 33 500 (track 6), previously identified as the glycosylated precursor that remains membrane bound until released by sonication (Chrispeels *et al.*, 1986; Marcus, 1988). Further purification of the precursor by DE-52 cellulose chromatography is shown in Figure 2A and the final

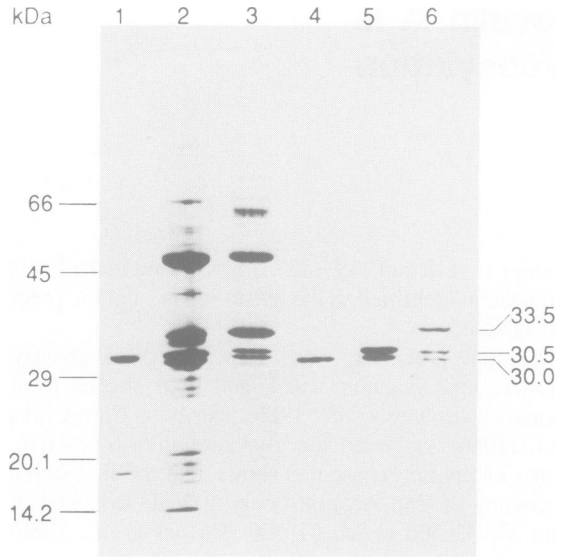

Fig. 1. Stages in the fractionation of jackbean cotyledon tissue analysed by SDS−PAGE and immunoblotting. Tracks 1−3 are visualized by Coomassie Blue and tracks 4−6 are Western blots stained with anti-Con A. Tracks 1 and 4, mature Con A; tracks 2 and 5, 100 000 *g* supernatant of a jackbean extract; tracks 3 and 6, lumenal products released by sonication of the 100 000 *g* pellet.

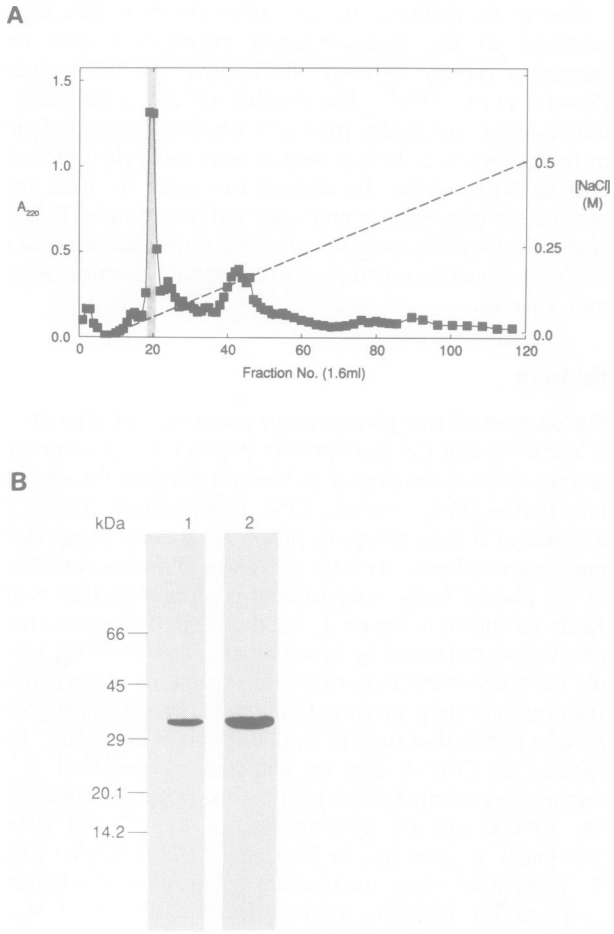

Fig. 2. Purification of the Con A glycoprotein precursor. **(A)** DE-52 cellulose chromatography of the lumenal products released by sonication of the 100 000 *g* pellet from immature jackbeans. The shaded area corresponds to pooled fractions containing the Con A glycoprotein precursor. **(B)** The purified precursor analysed by SDS−PAGE and immunoblotting. Track 1, stained with Coomassie Blue; track 2, stained with anti-Con A.

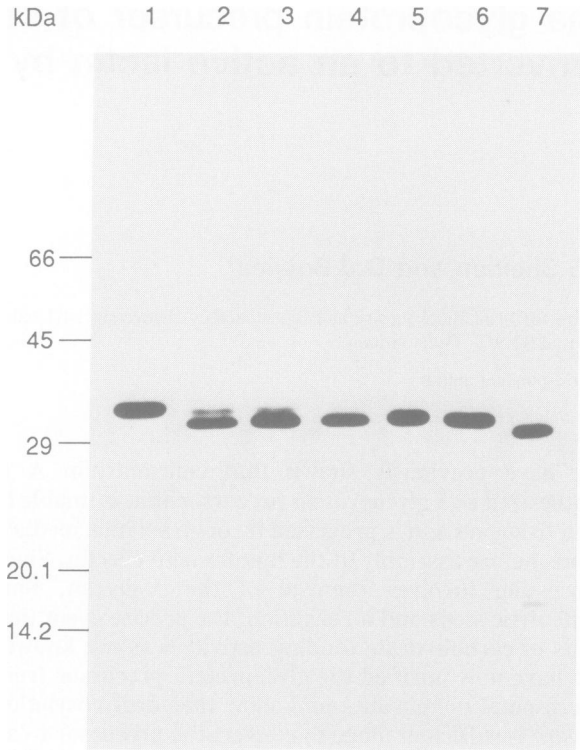

Fig. 3. Treatment of the glycoprotein precursor with *N*-glycanase. Following treatment of the precursor with *N*-glycanase at 25°C, samples were analysed by SDS−PAGE and autoradiography. Track 1, untreated precursor; tracks 2−5, 5, 10, 15 and 30 min incubation respectively; track 6, 24 h incubation; track 7, mature Con A.

preparation used in all subsequent procedures is shown in Figure 2B. The identity of the purified protein was confirmed by N-terminal sequencing and comparison with that known for the glycoprotein precursor as described earlier (Bowles *et al.*, 1986). The presence of an *N*-glycan on the purified protein was confirmed by lectin blotting with Con A (Bowles *et al.*, 1983; Faye and Chrispeels, 1985). A typical yield was 30 μg precursor/g fresh weight cotyledon. Since only trace quantities of the precursor were available, the polypeptide was radio-iodinated.

Conversion of the precursor to an active lectin by deglycosylation

A time course of the effect of enzyme hydrolysis with *N*-glycanase is shown in Figure 3. Deglycosylation occurred rapidly and without the prior requirement of denaturation of the glycoprotein. This suggests ready accessibility of the amide bond on the surface of the native precursor. We then used the interaction with ovalbumin−Sepharose to compare the carbohydrate binding activity of the precursor before and after deglycosylation; the results are shown in Table I. When the glycoprotein was applied to the affinity matrix 94.6% of the radioactivity was recovered in the flow-through and only trace levels were eluted by methyl α-mannoside or remained on the matrix (Table I, column 1). In contrast, after *N*-glycanase treatment a substantial proportion of the deglycosylated precursor bound to the matrix (38.4% of the applied sample) and was eluted specifically by methyl α-mannoside (Table I, column 2). When the fractions from these two applications were analysed by SDS−PAGE and autoradiography, the results shown in Figure 4 were

Table I. Application of a Con A species to ovalbumin–sepharose

Fraction	% Total radioactivity applied to column		
	Untreated precursor	*N*-glycanase treated precursor	Mature Con A
Applied to column	100 (1.2×10^6 c.p.m.)	100 (1.02×10^6 c.p.m.)	100 (1.37×10^6 c.p.m.)
not bound	94.6	43	13
galactose eluate	0.5	6.3	1.4
methyl α-mannoside eluate	1.4	38.4	56.1
irreversibly bound to matrix	1.4	4.8	15.0
total recovery	97.9	92.5	85.5

Samples were prepared as described in the methods and applied to the affinity matrix. Fractions correspond to those analysed as shown in Figure 4.

obtained. As expected, the unbound fraction from Table I column 1 contained the precursor of M_r 33 500, confirming earlier suggestions that the glycoprotein lacks the ability to bind carbohydrate. Interestingly, the trace level of radioactivity recovered in the methyl α-mannoside eluate from column 1 (1.4% of the total applied) was highly enriched in a polypeptide identical in size to that of the precursor following *in vitro* treatment with *N*-glycanase. This could represent the deglycosylated intermediate formed during processing *in vivo*, which is present in such trace quantities in the seed extract that it cannot be detected until enrichment by absorption and elution from the affinity matrix. Alternatively, some precursor may be deglycosylated during the course of its purification. Analysis of the fractions from Table I, column 2 indicated that both the effluent from the matrix and the methyl α-mannoside eluate contained polypeptides of identical size, equivalent to the *N*-glycanase treated precursor. Further studies showed that the *N*-glycanase treated protein progressively denatured and aggregated as time following enzyme treatment increased (data not shown). These observations are consistent with studies by Jones and coworkers (Min *et al.*, 1992) which indicate that recombinant pro-Con A, which is equivalent to the deglycosylated form of the native precursor described in this work, exhibits a low threshold of solubility. This tendency to aggregate may give rise to inactivity and be one reason why only a proportion of the *N*-glycanase treated protein bound to the affinity matrix.

It is also possible that iodination could modify specific tyrosine residues known to be located in the carbohydrate binding pocket of the lectin (Doyle and Roholt, 1968; Carver *et al.*, 1985; Derewenda *et al.*, 1989). When mature Con A was iodinated to the same specific activity as that of the precursor and applied to ovalbumin–Sepharose under identical conditions to those described above, the results in Table I column 3 were obtained. Of the radioactivity applied only 56.1% bound to the matrix and was eluted by methyl α-mannoside.

The metal ion requirement for binding to ovalbumin–Sepharose of the *N*-glycanase treated precursor was investigated using EDTA. For the chelating agent to be effective, chromatography was carried out at neutral/alkaline pH and the matrix buffer consisted of 10 mM Tris–HCl pH 7.4, 200 mM NaCl, 1 mM Ca^{2+} and 1 mM Mn^{2+}. Under these conditions, 24% of the applied radioactivity was recovered in the methyl α-mannoside fraction. The substitution of 10 mM EDTA for Ca^{2+} and Mn^{2+} reduced this proportion to 0.6%. These results indicate that deglycosylated precursor and mature Con A share the same divalent metal ion requirement for carbohydrate binding activity.

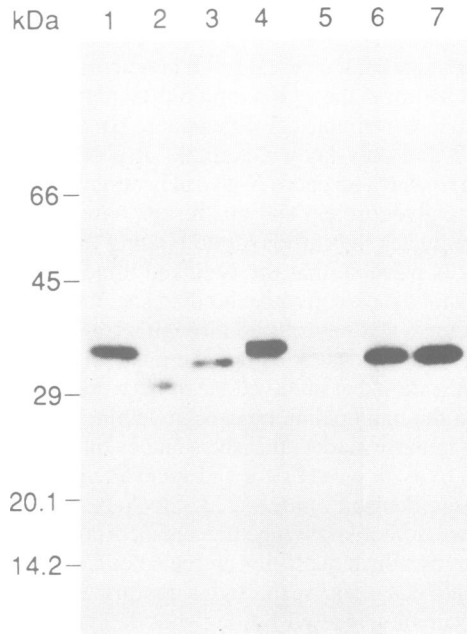

Fig. 4. Analysis of fractions from affinity chromatography of Con A species. The analyses by SDS–PAGE and autoradiography correspond to the fractions described in Table I, columns 1 and 2. Tracks 1–3, marker proteins, Con A precursor, mature Con A and precursor treated with *N*-glycanase respectively; tracks 4 and 5, affinity chromatography of untreated precursor, effluent and methyl α-mannoside eluate respectively; tracks 6 and 7, affinity chromatography of *N*-glycanase treated precursor, effluent and methyl α-mannoside eluate, respectively.

Discussion

We have shown that the inactive glycoprotein precursor of Con A can be converted to a carbohydrate binding protein by removal of the *N*-glycan *in vitro*. Earlier studies had delineated a complex series of processing events that occur during biosynthesis of the lectin *in vivo*, but the key step converting the protein into a carbohydrate binding lectin was not known (Carrington *et al.*, 1985; Bowles *et al.*, 1986; Chrispeels *et al.*, 1986). Our data now lead to the conclusion that the presence or absence of the *N*-glycan determines carbohydrate binding activity of the precursor and that further proteolytic processing and religation are not required for this activation.

This conclusion, based on deglycosylation of the native jackbean glycoprotein, is fully supported by the results of Jones and coworkers (Min *et al.*, 1992) who have used an alternative strategy to study the same problem. They have

expressed a cDNA for Con A in *Escherichia coli* and have shown that the recombinant protein is able to bind to carbohydrates. Since the recombinant pro-Con A is not glycosylated within the heterologous prokaryotic expression system and since peptide sequencing confirmed the anticipated lack of any protein rearrangement, their data precisely mirrors the results for the deglycosylated precursor described in this paper.

Earlier pulse–chase analyses (Bowles *et al.*, 1986; Marcus and Bowles, 1988) showed that deglycosylation is a normal occurrence during Con A processing *in vivo* and an *N*-glycanase activity in jackbeans has been described (Sugiyama *et al.*, 1983; Yet and Wold, 1988). Wheat germ and barley germ agglutinins are also known to be synthesized as glycoprotein precursors (Mansfield *et al.*, 1988; Lerner and Raikhel, 1989). However, in contrast to the events in Con A biosynthesis, the cereal lectin precursor is converted to the mature form through a single proteolytic event that removes the C-terminal glycopeptide. To date, Con A assembly is the only known example of post-translational processing in which an entire *N*-glycan is removed in a single step from a glycoprotein and our results now indicate that this step regulates the carbohydrate binding function of the protein. It is possible that the N-linked oligosaccharide is located within the carbohydrate binding site of the precursor and the lectin is self-neutralized through intra- and/or inter-protomeric interactions. Although there is one precedent for self-neutralization that involved the *in vitro* manipulation of glycans on the mammalian hepatocyte lectin, there was no indication in those studies that the same events occurred to regulate activity *in vivo* (Pricer and Ashwell, 1971; Hudgin *et al.*, 1974; Paulson *et al.*, 1977; Stockert *et al.*,1977).

As yet we do not know whether self-neutralization is the mechanism by which the *N*-glycan regulates Con A activity, since it could equally arise through a conformational change in the precursor triggered by deglycosylation.

However, this observation raises two crucial issues. The first concerns the role of protein activation within cells of the plant. Monensin is known to induce the re-routing of proteins destined for the protein bodies to the cell surface (Bowles, 1990a). Following ionophore treatment the newly synthesized Con A precursor that accumulated at the cell surface was found to be the glycoprotein (Bowles *et al.*, 1986). This implies either that monensin treatment inhibited *N*-glycanase activity or that the glycoprotein precursor switched traffic routes prior to the site of the processing event. If the latter, then activation of the lectin by deglycosylation may well occur in the *trans*-Golgi network, or following arrival within the protein body compartment. Protein bodies are known to be the site of proteolytic processing of many seed storage proteins and lectins. The second issue raised by our results concerns the possibility that modulation of protein activity by the removal of glycans may prove to be a widespread occurence. Although *N*-glycanase activity has been reported in eukaryotic and prokaryotic cells (Takahashi and Nishibe, 1981; Sugiyama *et al.*, 1983; Plummer *et al.*, 1984), the distribution of the enzyme may as yet reflect the number of times it has been investigated, rather than the generality of its occurrence.

Materials and methods

Purification of the glycoprotein precursor of Con A

Jackbeans were grown as described previously (Bowles *et al.*, 1986) and immature seeds (1–2 g fresh weight) were harvested and stored intact at −70°C until use. All operations in the purification were carried out at 0–4°C. Following removal of the testas, cotyledons were homogenized in PBS (10 mM potassium phosphate pH 7.4, 150 mM NaCl), 10% (w/v) sucrose (fresh weight:buffer ratio, 1:10 w/v). After removal of cell debris and walls (2000 *g* for 10 min), the extract was further centrifuged (100 000 *g* for 60 min) to give a supernatant and microsomal pellet. The pellet was washed through three cycles by resuspension in PBS–sucrose and centrifugation (100 000 *g* for 30 min) and then resuspended in PBS and sonicated (5×15 s, mark 7, Branson sonicator, micro-tip). Following further centrifugation (100 000 *g* for 30 min), the resultant supernatant contained lumenal products released by sonication from the microsomes. It was dialysed against 5mM 1,3-bis[tris (hydroxymethyl)-methylamino] propane (Aldrich)–HCl pH 7.0 and applied to a DE-52 cellulose (Whatman Biosystems, Ltd) column (0.7×6 cm) equilibrated in the same buffer. The matrix was eluted with a 0–0.5 M linear gradient of NaCl in the same buffer over 200 ml and the eluate fractions were monitored by SDS–PAGE (Bowles *et al.*, 1986) and immunoblotting (Towbin *et al.*, 1979; Hawkes *et al.*, 1982) with antibodies to mature Con A.

The fractions containing the Con A glycoprotein precursor were pooled, dialysed against 10 mM acetic acid–*N*-ethylmorpholine pH 5.5, lyophilized and stored in aliquots at −70°C until use. The precursor and mature Con A were iodinated using the Iodogen method in the presence of 0.2 M methyl α-mannoside (Salacinski *et al.*, 1981). After labelling the protein was separated from unreacted iodide by chromatography on Trisacryl GF05 (IBF Biotechnics) equilibrated in PBS. BSA was added to the eluates to a final concentration of 0.2 mg/ml.

Treatment with N-glycanase

Precursor, radiolabelled with ^{125}I (3 Ci/g) was incubated *in vitro* with *N*-glycanase (Genzyme Biochemicals Ltd) at a ratio of 0.5 units enzyme/3.2×10^6 c.p.m. precursor in a final volume of 40 μl. Following incubation at 25°C, aliquots were removed at time intervals and quick frozen in sample buffer, prior to heating at 95°C for 10 min and analysis by SDS–PAGE and autoradiography as described in Bowles *et al.* (1986).

Application of Con A Species to an Affinity Matrix

Three species of Con A were applied to ovalbumin–Sepharose: the glycoprotein precursor, the precursor treated with *N*-glycanase for 30 min and mature Con A. The affinity matrices (0.8 ml) were equilibrated in 0.1 M sodium acetate pH 4.5, 1 mM Ca^{2+}, 1 mM Mn^{2+} at room temperature and a new matrix was used for each application. Samples were applied in equilibration buffer containing 1 mg/ml BSA. The *N*-glycanase treated samples were applied immediately to the matrix. Radioactivity was determined in the following fractions: wash effluent (20 ml equilibration buffer), galactose eluate (10 ml 0.2 M galactose in equilibration buffer followed by 2 ml equilibration buffer), methyl α-mannoside eluate (20 ml, 0.2 M methyl α-mannoside in equilibration buffer), before radiolabel retained on the washed out Sepharose was also measured. No account was taken of label entrapped on the glass wool filter and column.

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