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ConA-BINDING POLYPEPTIDES IN JACKBEAN COTYLEDONS

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Key Word Index—*Canavalia ensiformis*; Leguminosae; Jackbean; concanavalin A; α-mannosidase; lectin binding polypeptides.

Abstract—Concanavalin A (ConA) binding components of jackbeans have been studied using two techniques: affinity chromatography on immobilized ConA, and lectin overlays using ^{125}I-ConA. The results indicate that the seed polypeptides recognized by ConA in each technique are different. The heavy subunit of the enzyme α-mannosidase (M_r 66 000) is recognized by ConA in a sugar-specific interaction in ^{125}I ConA overlays but when seed extracts are applied to ConA-Sepharsoe under native conditions the enzyme is not bound. Only a minor polypeptide found in the soluble fraction after cotyledon extraction is bound and eluted specifically from the ConA affinity matrix by methyl α-mannoside. Although the size of this polypeptide (M_r 65 000) is very similar to that of the α-mannosidase subunit, it does not react with anti-mannosidase after Western blotting suggesting it is not antigenically related to the enzyme. The polypetide purified by affinity chromatography does not bind ^{125}I-ConA in the overlay procedure.

INTRODUCTION

There have been several studies in which jackbean proteins have been analysed for their ability to intract with the endogenous lectin, concanavalin A (ConA) [1–4]. Using purified α-mannosidase, we showed that the heavy subunit of the enzyme (M_r 66 000) was glycosylated and after heat/SDS denaturation was recognized by ConA using the technique of ^{125}I-ConA overlays [4]. The glycan was removed by treatment with endo H and this result, together with GC analysis of the oligosaccharide, confirmed that the enzyme was an *N*-linked glycoprotein [5]. In earlier studies from our laboratory, we had shown that a single polypeptide could be purified from jackbean extracts using ConA as an affinity matrix [2]. The polypeptide had a similar M_r to that of the heavy subunit of α-mannosidase. In order to clarify the relationship between these two ConA-binding components we have undertaken the present study.

RESULTS

A post-1000 *g* supernatant, prepared in saline phosphate bufferred (PBS) was applied to ConA-Sepharose. Reapplication of elution buffer did not desorb additional material from the matrix (Fig 1A, B), implying that elution of proteins by each buffer was completed by one application. Also, a 'non-specific' sugar, such as D-galactose was unable to desorb any protein from the immobilized ConA. Protein was eluted both by methyl α-mannoside and detergent, irrespective of the sequence of the two buffers. Dialysis of the extract, prior to application onto the affinity matrix did not alter the yields of protein in the eluates (results not shown). The eluted material shown in Fig. 1A and B was assayed for α-mannosidase activity. Enzyme activity was high in the extract applied to the matrix (0.15 units/mg protein) and this was at unchanged levels in the effluent that passed through the column; no enzyme activity was found in any of the eluate fractions.

An SDS-PAGE analysis of the eluate fractions and visualization using Coomassie dye is shown in Fig. 2. The major polypeptide in the methyl α-mannoside eluate had M_r 65 000 (track B). Minor components consisted of a diffuse band of M_r 47 500, electrophoresing in the region of the canavalin monomer, and a polypeptide at M_r 27 000, which electrophoresed in the same region as the ConA intact subunit. The pattern of polypeptides in the detergent eluate were very different (track C). When an equivalent analysis to that shown in Fig. 2 was stained with periodate–Schiffs reagent, a diffuse band ahead of the tracking dye in the detergent eluate was observed and probably represented glycolipid (results not shown). Results of immunoblotting (Fig. 3) confirmed the identity of canavalin (track B) and ConA (track C). Although anti-mannosidase recognized both the heavy and light subunits of a purified enzyme sample, there was no interaction between the antiserum and any polypeptide in the eluate from the ConA matrix (track D).

Further experiments to define the nature of the detergent eluate, indicated that identical yields could be obtained if a membrane vesicle suspension was applied to Sepharose or ConA-Sepharose, implying that non-specific physical entrapment of vesicles was responsible for the components recovered in the detergent eluate (results not shown).

As a further way of studying the relationship between the α-mannosidase heavy subunit and the polypeptide of M_r 65 000 in the methyl α-mannoside eluate, Elders' method of 2-D peptide mapping was used. The results are shown in Fig. 4 in which 4A is the map of the α-

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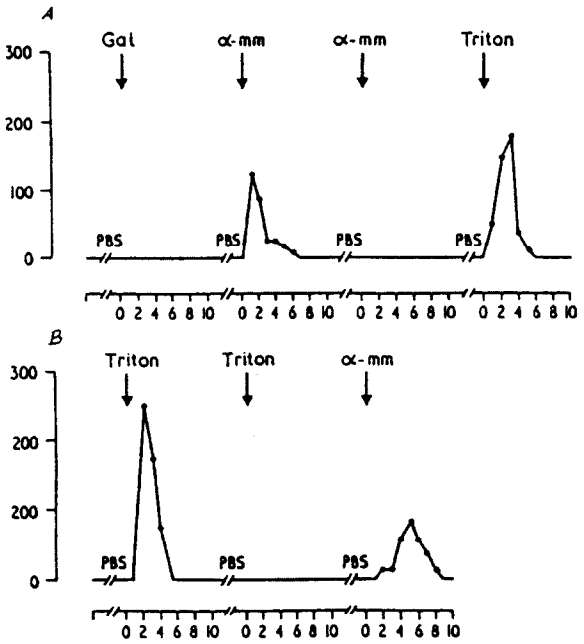

Fig. 1. Application of jackbean extracts to ConA-Sepharose. Post-1000 *g* supernatants were prepared from cotyledon homogenates as described in the experimental. After application of the extracts to ConA-Sepharose, the matrices were eluted sequentially and protein in the eluates measured. In 1A, the matrix was eluted with galactose, methyl α-mannoside and Triton X-100. In 1B, Triton X-100 was applied prior to the sugar.

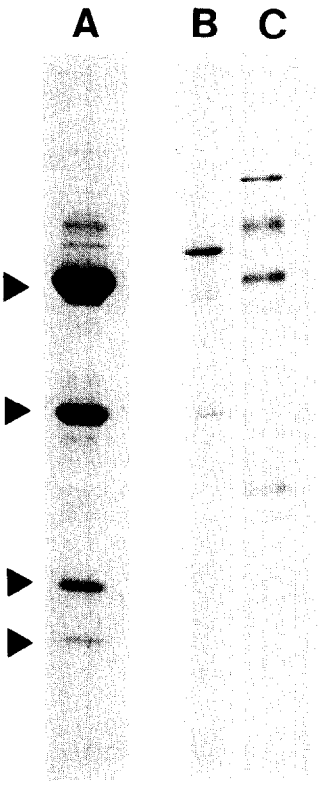

Fig. 2. SDS-polyacrylamide gel electrophoresis of eluates from ConA-Sepharose after application of jackbean extracts. Polypeptides from the post-1000 *g* supernatant and those in eluates from the ConA matrix were separated by SDS-PAGE and visualized by Coomassie Blue as described in the experimental; 60 μg of protein was loaded per track. Track A = total extract applied to the matrix; track B = material eluted by methyl α-mannoside; track C = material eluted by Triton X-100. B and C correspond to protein peaks in Fig. 1A. Arrows correspond to canavalin subunit M_r 49500; ConA subunit M_r 27000 and ConA-related fragments M_r 13000 and M_r 11000.

mannosidase subunit M_r 66000 and 4B is that of the polypeptide eluted from the ConA affinity matrix. For comparison, 4C is the map of the polypeptide isolated in 1980 and shown in Fig. 2D of [2]. Although clearly the two polypeptides eluted from the ConA matrices are identical, there is very little similarity between them and the α-mannosidase subunit.

Polypeptides in the post-1000 *g* supernatant and methyl α-mannoside eluate from the affinity matrix were separated by SDS-PAGE and overlaid with ^{125}I-ConA (Fig. 5). The heavy subunit of α-mannosidase in the cotyledon extract was recognized by ^{125}I-ConA (track A). In contrast, the ^{125}I-lectin did not detect any components in the fraction eluted by methyl α-mannoside (track B).

DISCUSSION

ConA has often been used as a tool to investigate glycoconjugates both as an immobilized affinity absorbent and as an ^{125}I-labelled probe [10]. In general, numerous glycosylated proteins in cellular extracts interact with the lectin and can be displaced by a hapten inhibitor such as methyl α-mannoside. In the present study, the two techniques of lectin affinity chromatography and lectin overlays have been used in parallel to study the range of jackbean components potentially able to bind to ConA. The results indicate that the polypeptides recognised by ConA in each technique are different.

ConA is able to recognise the mannosylated subunit of α-mannosidase after SDS/heat denaturation of the enzyme [4, 5] but data in this report show that the native glycoprotein cannot be isolated from jackbean extracts by affinity chromatography on immobilised ConA. Earlier studies had indicated a minor polypeptide found in soluble fractions after jackbean extraction, bound to ConA Sepharose and could be eluted with methyl α-mannoside [2]. The present report shows that this polypeptide does not bind ^{125}I-ConA in overlays.

The similarity in M_r and apparent affinity for ConA, suggested that the heavy subunit of α-mannosidase and the polypeptide isolated on ConA affinity matrices might be structurally-related. However, the lack of cross-reactivity of anti-mannosidase with the polypeptide of M_r 65000, and the dissimilarity in peptide maps of the two proteins, has now discounted this possibility.

EXPERIMENTAL

Seed extraction. Jackbeans, obtained from EMBRAPA, Brazil, were dissected dry to provide cotyledons which were ground to a fine powder in a coffee-grinder and homogenized in a pestle and mortar (dry wt: buffer ratio of 1 g/20 ml) at 4°. The homogenisation buffers consisted of 10 mM K-Pi, pH 7.2 containing

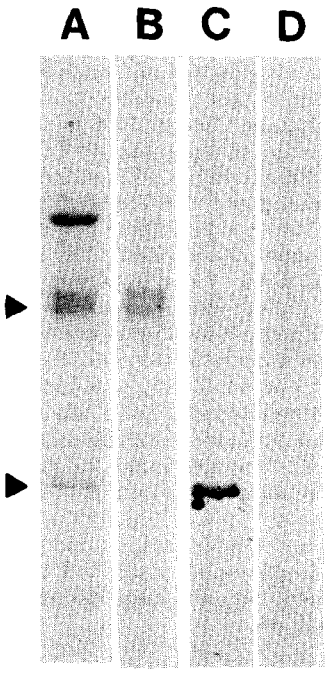

Fig. 3. Comparison of proteins eluted from ConA-Sepharose with jackbean components of known identity. Following SDS-PAGE of the methyl α-mannoside eluate from ConA-Sepharose, polypeptides were transferred to nitrocellulose, probed with antisera to jackbean proteins and products visualized by a double antibody-peroxidase as described in experimental; 60 μg protein was loaded per track. Track A, shows polypeptides in the eluate visualized by Coomassie Blue; tracks B, C and D show equivalent tracks to A, but blotted and probed with antisera: track B, anticanavalin; track C, anti-ConA; track D, anti α-mannosidase.

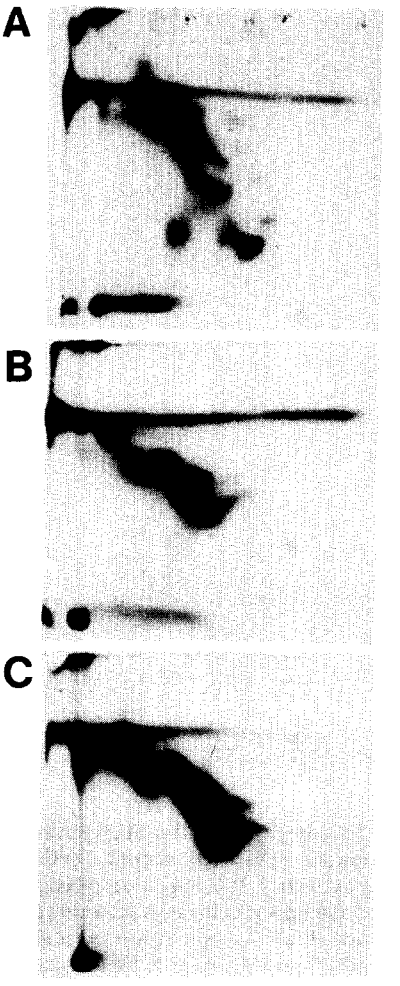

Fig. 4. ^{125}I-labelled tryptic peptide maps of jackbean components. The heavy subunit of α-mannosidase (M_r 66 000) (track A); the polypeptide of M_r 65 000 in methyl α-mannoside eluates from ConA matrices (track B); and the polypeptide shown as Fig. 2D in [2] (track C), were subjected to peptide mapping as described in the Experimental.

145 mM NaCl (PBS). The homogenates were sonicated using a microprobe (4 × 10 sec Dawe sonicator) and centrifuged at 1000 *g* for 5 min at room temp. The post-1000 *g* supernatant was further processed (i) centrifuged at 100 000 *g* for 2 hr at 4° or (ii) dialysed extensively against PBS at 4° (48 hr, 4 changes, 4 l).

Affinity chromatography. Fresh batches (2 ml) of ConA-Sepharose (Pharmacia) in disposable mini-columns were used for each expt; when the affinity matrices were developed in PBS containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ at 4°, the capacity of 2 ml immobilized ConA was in excess of 4 mg ovalbumin (Sigma) or transferrin (Sigma). For soluble extracts (post-1000 *g*/post-100 000 *g* supernatants) prepared in PBS, 250 mg cotyledon protein was applied in each expt to the ConA-Sepharose column equilibrated at 4° in the respective buffer. The affinity matrices were washed extensively at 4° with the respective buffers until protein in the effluents was < 10 μg ml as determined by protein assay. The columns were then eluted sequentially using several different elution buffers. Between each change of buffer, the matrix was re-equilibriated with 10 column volumes of PBS. Elution buffers included: 200 mM galactose, 200 mM Me α-D-mannoside, and 0.1% (w/v) Triton X-100, all final concn in PBS. For all expts, 1 ml eluate fractions were collected.

General techniques. ConA, α-mannosidase and canavalin were purified from Brazilian jackbeans and monospecific polyclonal antisera raised to the proteins as described in ref [6]. α-Mannosidase assays were carried out on jackbean extracts and eluates from immobilized ConA, using 4-nitrophenyl-α-D-mannoside as substrate [5]. SDS-polyacrylamide gel electrophoresis was performed using 10–15% (w/v) polyacrylamide gradients gels under reducing conditions [7]. M_r markers for SDS-PAGE were purchased from Boehringer and M_r of jackbean proteins determined using log % (w/v) polyacrylamide vs log M_r plots. ^{125}I lectin overlay procedures of polypeptides separated by SDS-polyacrylamide gel electrophoresis were carried out using ^{125}I-ConA (sp. act. 1.32×10^9 cpm/mg) [5]. Protein was determined using a modified method of Lowry [8] with BSA as std. Elders method [9] of 2D mapping of ^{125}I-labelled peptides was carried out as described in ref. [7].

Protein was transferred to nitrocellulose using a Biorad transblot apparatus; controls with ^{125}I-labelled proteins indicated >95% transfer from the gel to the nitrocellulose. The nitrocellulose containing the transferred polypeptides was maintained in Tris–saline until used for overlay. The sheets were then (i) incubated overnight at 4° in Tris–saline containing 2 mg/ml BSA, (ii) rinsed 10 min in Tris–saline (iii) overlaid with monospecific IgG raised in rabbits to the jackbean proteins; 1:100 dil

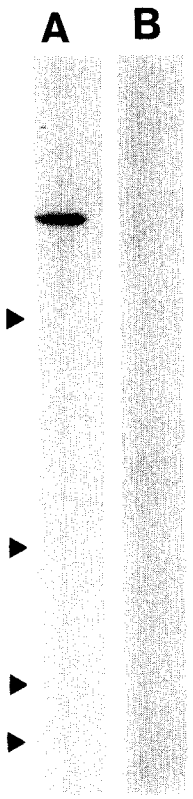

Fig. 5. ^{125}I-ConA overlays of jackbean polypeptides. Polypeptides were separated by SDS-PAGE, stained with Coomassie Blue and then overlaid with ^{125}I-ConA as described in the experimental. An autoradiograph of the gel is shown; 60 μg protein was loaded per track. Track A = post-1000 g supernatant; track B = material eluted from ConA matrix by methyl α-mannoside. Arrows correspond to canavalin subunits M_r 49 500, ConA subunit M_r 27 000 and ConA-related fragments M_r 13 000 and M_r 11 000.

of IgG was made in 10 mM Tris–HCl, 500 mM NaCl pH 7.4, 2 mg ml BSA and incubated with the nitrocellulose sheets in sealed-bags at room temp for 1 hr with agitation (iv) the sheets were then washed for 5 min, 2 changes with Tris–saline (v) 5 min, 2 changes with Tris–saline, 0.5% (v/v) Tween 20 (vi) 5 min, 2 changes with 10 mM Tris, 500 mM Nacl, 0.5% (v/v) Tween 20 (vii) 5 min, 2 changes with Tris–saline.

The binding of the specific rabbit antibody to cross-reactive material on the nitrocelulose sheets was then visualised using an indirect second antibody method. The sheets were incubated (i) in 1:250 diln of goat anti-rabbit IgG (Miles) made in Tris–saline containing 2 mg/ml BSA for 1 hr room temp. (ii) 5 min, 2 changes in Tris–saline (iii) 5 min, 2 changes in Tris–saline (iv) 5 min, 2 changes in Tris–saline, 0.5% (v/v) Tween 20 (v) 5 min, 2 changes in 10 mM Tris–HCl 500 mM NaCl, 0.5% (v/v) Tween 20 (vi) 5 min, 2 changes in Tris–saline (vii) 1:250 dil of peroxidase rabbit anti-peroxidase (Miles) in Tris–saline containing 2 mg/ml BSA for 1 hr at room temp (viii) wash steps as described in (ii–vi). The enzymic reaction, was then carried out as follows: 20 mg 3-amino-9-ethyl-carbazole was added to 2.5 ml of DMF. On solubilization of the carbozol, 50 ml of 10 mM Tris–HCl, pH 7.4 was added to form a turbid soln. The nitrocellulose sheets were placed in this soln, and 25 μl of 30% (v/v) H_2O_2 was added and the enzymic reaction allowed to develop to completion over 3–5 min. The reaction was stopped by removing the sheets and washing extensively in H_2O before drying.

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