

Post-translational peptide bond formation during concanavalin A processing *in vitro*

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Post-translational processing of concanavalin A (Con A) is complex, involving deglycosylation, proteolytic cleavage on the carboxy group side of asparagine residues and formation of a peptide bond *de novo*. This has been studied with the ¹²⁵I-labelled Con A glycoprotein precursor as a substrate for processing *in vitro*. Extracts of immature jackbean cotyledons and the commercially available purified preparation of asparaginylendo-

peptidase were able to catalyse the above processes. The processing resulted in the conversion of the 33.5 kDa inactive glycoprotein precursor into an active lectin. Processing activity was maximal at approx. pH 5.5. Evidence to support processing at authentic sites was obtained by observation of the release of ¹²⁵I at positions in the sequence where tyrosine residues were present.

INTRODUCTION

Many proteins are synthesized as inactive precursors and after transportation to their subcellular destinations are activated by a process such as proteolytic cleavage. In plant cells the vacuole is an acidic compartment that in seed tissues can fragment during maturation into the protein storage vesicles. Seed proteins found in this compartment might be used as a store of amino acids for the developing seedling or might have a role in defence. In dicotyledons, endoproteolytic cleavage at the peptide bond immediately C-terminal to an asparagine residue has been implicated in the biosyntheses of vacuolar and protein storage vesicle components such as storage proteins [1–8], defence-related proteins [9,10], ricin [11] and lectins [12–22].

Concanavalin A (Con A), the lectin of jackbean seeds, comprises up to 20% of the total protein in the mature seed [23]. Since its purification and crystallization [24], its physical properties and mitogenic effects towards animal cells have been extensively studied. However, the biological function of lectins in legume seeds remains unclear [25]. The complete primary structure of the mature protein, determined from amino acid sequencing by Edelman and co-workers [26,27], indicated that the protomeric unit of Con A is either a single 237-residue polypeptide or a form that would be derived from this polypeptide by a single proteolytic cleavage between Asn-118 and Ser-119. In the native protein, protomeric units associate to form dimers and tetramers [28,29].

Whereas the post-translational processing of some other legume lectins involves proteolytic cleavage at peptide bonds at the carboxy group side of asparagine residues, cDNA cloning and pulse-chase experiments [12,13,30] indicated that, in addition to this type of proteolytic cleavage, events occur in Con A biosynthesis that have no precedent. A proposed model of processing is shown in Figure 1 [12]. A signal sequence leading to segregation into the endoplasmic reticulum is co-translationally cleaved and the nascent polypeptide is glycosylated [31,32]. The

resultant 33.5 kDa glycoprotein precursor does not possess lectin activity [12,33]. The first step in post-translational processing might be deglycosylation [30], resulting in the acquisition of carbohydrate-binding activity [34]. Proteolytic cleavages follow that result in the formation of 15.1, 18.8 and 14.2 kDa polypeptides. However, comparison of the primary structure of the mature protein deduced from amino acid sequencing with its cDNA sequence indicates that a peptide bond is formed between Asn-118 and Ser-119 post-translationally. Two possibilities would account for the next step of processing. There could be a third proteolytic cleavage followed by a ligation giving rise to post-translational formation of a peptide bond between residues Asn-118 and Ser-119. If this were to occur there would be a requirement for input of energy for peptide bond formation in the ligation step. An alternative explanation is that energy from peptide cleavage could be directly transferred to peptide formation in a transpeptidation (splicing) reaction in which cleavage and ligation were to occur in a single concerted step. The final stage of processing, proteolytic cleavage of a tetrapeptide from the N-terminus of the 30.4 and 17.8 kDa polypeptides is thought to occur very slowly in the course of jackbean maturation. The two-chain form of Con A found in the mature seed would arise when the ligation/transpeptidation reaction did not take place.

All the proteolytic and transpeptidation/ligation events of Con A biosynthesis occur at peptide bonds immediately to the carboxy group side of asparagine residues. A protease has been purified from jackbeans and characterized with artificial and exogenous substrates [35–37]. It possesses a strong specificity, which suggests that the precursor and processing intermediates of Con A are likely to be physiological substrates. A previous study with a recombinant, non-glycosylated and renatured two-chain form of Con A has indicated that the enzyme seems to be able to catalyse peptide bond formation between residues Asn-118 and Ser-119 [38]. In this study we have used the glycoprotein precursor of Con A, purified from immature jackbeans, to show that it can be processed by the protease and have confirmed that

Abbreviation used: Con A, concanavalin A.

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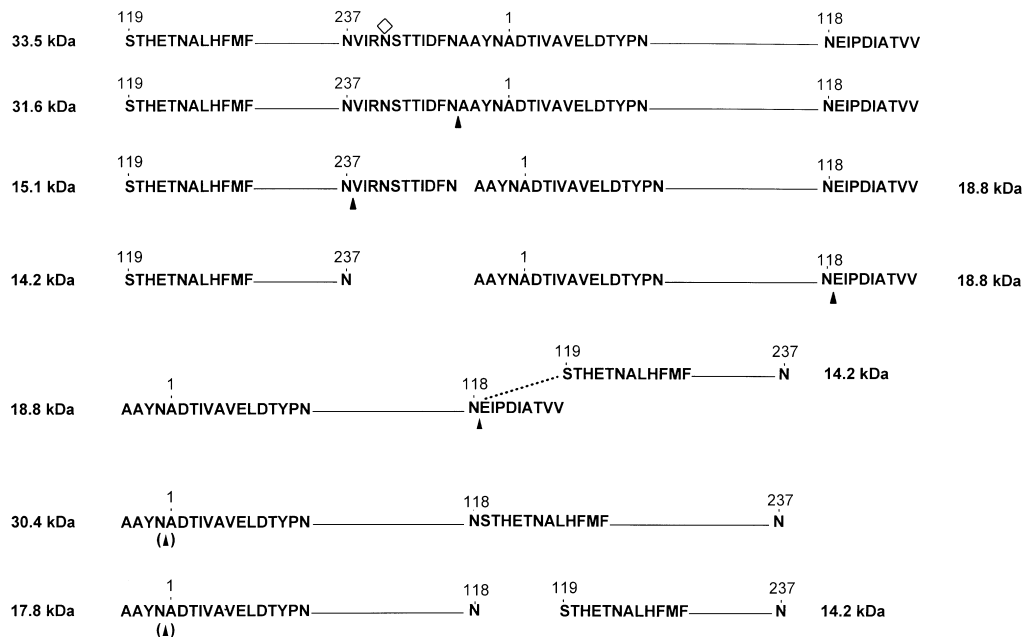


Figure 1 Model of Con A post-translational processing events

The figure is adapted from Bowles et al. [12]. Amino acid numbering uses the sequence of the intact subunit of mature Con A as a reference [26]. The formation of a peptide bond between Asn-118 and Ser-119 could occur either through a ligation or a transpeptidation mechanism. During seed maturation the tetrapeptide AAYN is slowly cleaved from the N-termini of the 30.4 and 17.8 kDa polypeptides to give mature Con A (not shown) consisting of 30.0, 16.2 and 14.2 kDa polypeptides.

processing occurs *in vitro* at the same sites as *in vivo* by protein sequencing of the processed forms.

EXPERIMENTAL

Purification and radiolabelling of the Con A glycoprotein precursor

After harvesting, immature jackbeans were frozen in liquid nitrogen and stored at -70°C . The Con A glycoprotein precursor was purified from an immature jackbean microsomal fraction as described previously [34] followed by an additional step of gel filtration with Ultrogel AcA44 (IBF Biotechniques, France) equilibrated in 10 mM sodium acetate (pH 5)/150 mM NaCl/1 mM CaCl_2 /1 mM MnCl_2 . Iodination was performed by the Enzymobead method (Bio-Rad, Watford, Herts., U.K.) in accordance with the manufacturer's instructions. The Con A glycoprotein precursor was labelled to a specific radioactivity of approx. 6.5×10^{12} c.p.m./g and stored frozen at -20°C in 10 mM sodium phosphate (pH 7.4)/150 mM NaCl.

Digestion of ^{125}I -labelled Con A glycoprotein precursor

Digests with crude extracts were performed with an immature jackbean (1.8 g fresh weight) ground to a powder in liquid nitrogen and stored at -70°C . A soluble extract was prepared by mixing the powder with 10 mM sodium phosphate (pH 7.4)/150 mM NaCl at 4°C with a seed-to-buffer ratio of 1:10 (w/v) followed by centrifugation (12000 g for 10 min). The digest mixture (30 μl) contained ^{125}I -labelled Con A precursor (1.3×10^5 c.p.m.), jackbean extract (15 μl ; 21 μg of protein) and 100 mM sodium acetate, pH 5.0. The digest was incubated at 25°C for 12 h.

Digests with the purified asparaginylendopeptidase preparation contained, unless otherwise stated, 100 mM sodium acetate, pH 5.5, 5 mM EDTA, 1 mg/ml BSA, ^{125}I -labelled Con A

precursor (approx. 1.5×10^5 c.p.m.), asparaginylendopeptidase (1 μl , 6.4 μunits [36,37]; TaKaRa Shuzo Co. Kyoto, Japan) in a total volume of 20 μl . Digestion was performed at 25°C for 15 h.

Measurement of carbohydrate-binding activity by ovalbumin–Sephacose chromatography

Each sample was made up to 400 μl by the addition of BSA to 1 mg/ml and column buffer [10 mM sodium acetate (pH 4.5)/150 mM NaCl/1 mM CaCl_2 /1 mM MnCl_2]. After removal of any precipitated material by centrifugation (12000 g for 5 min), the sample was applied at room temperature to an ovalbumin–Sephacose column that had been pre-equilibrated in column buffer. The column was eluted sequentially with column buffer alone, 0.2 M galactose in column buffer, column buffer alone and 0.2 M methyl α -mannoside in column buffer. The radioactivity in each fraction was measured.

SDS/PAGE, autoradiography and Edman degradation

SDS/PAGE and autoradiography were performed as described [12,34]. For Edman degradation with a solid-phase sequencer, after SDS/PAGE to separate the digestion products and a brief staining in Coomassie Blue R250, the radioactive processed polypeptides were excised from the gel with the use of stained non-radioactive Con A polypeptides as a positional guide.

Electroelution and Edman degradation with a solid-phase sequencer (Milligen 6600 Prosequencer) were performed as previously described [12]. For Edman degradation with an Applied Biosystems Model 477A liquid-pulse sequencer, after SDS/PAGE, processed radioactive polypeptides were transferred to a poly(vinylidene difluoride) membrane (Bio-Rad, Hemel Hempstead, Herts., U.K.) by electroblotting [10 mM 3-(cyclohexylamino)propane-1-sulphonate/ Na^+ (pH 11.0)/10% (v/v) methanol for 90 min at 7 V/cm]. The membrane was

washed thoroughly in deionized water, dried and autoradiographed. Radioactive polypeptides were excised from the membrane before sequencing. Instead of the usual analysis of phenylthiohydantoin amino acids from sequencing by HPLC, a modified machine cycle, comprising elements of the manufacturer's BLOTT and ATZ cycles, was used to allow collection of the entire fraction at each cycle and radioactivity was counted directly to identify the positions of [125 I]iodotyrosine residues in the sequence.

RESULTS AND DISCUSSION

Processing of Con A precursor by extracts from immature jackbeans

After application of the 125 I-labelled Con A precursor to ovalbumin–Sepharose, 95% of the applied radioactivity passed through the affinity matrix (Figure 2A), confirming that the precursor does not possess carbohydrate-binding activity as was shown previously [34].

After incubation of the precursor with an extract from immature jackbeans, a significant proportion (52%) of the applied radioactivity bound to the matrix and was eluted specifically by methyl α -mannoside, indicating that it had been converted into a carbohydrate-binding species (Figure 2B).

Analysis by SDS/PAGE and autoradiography of the fractions from ovalbumin–Sepharose are shown in Figure 3. In the fraction with carbohydrate-binding activity (lane 6), the 33.5 kDa gly-

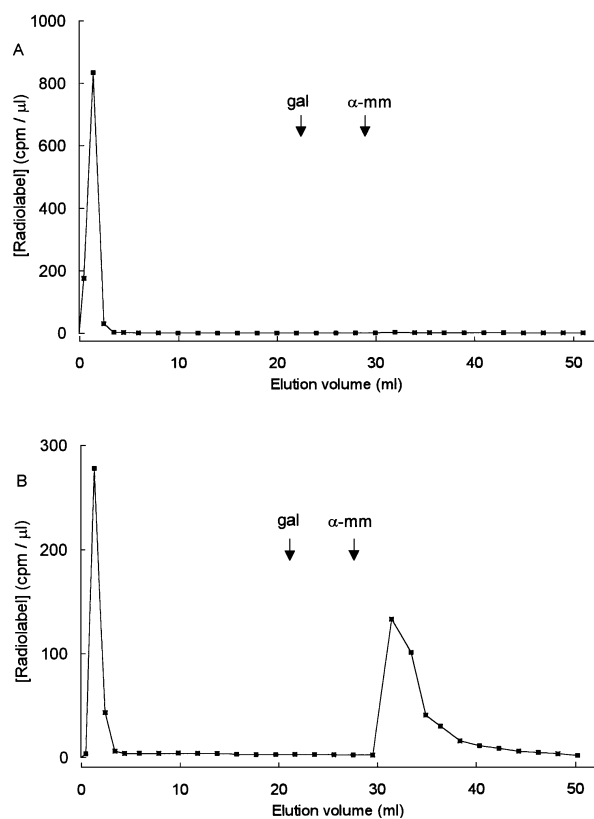


Figure 2 Conversion of Con A precursor to an active lectin

(A) Ovalbumin–Sepharose chromatography of untreated precursor; (B) ovalbumin–Sepharose chromatography of Con A precursor digested with extract from immature jackbean. Abbreviations: gal, galactose; α -mm, methyl α -mannoside.

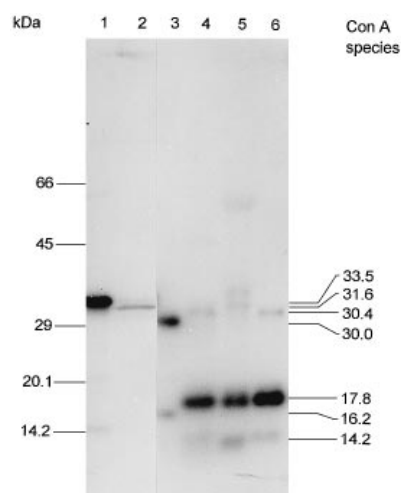


Figure 3 SDS/PAGE and autoradiography of 125 I-labelled Con A precursor before and after digestion with immature extract

Lane 1, Con A precursor (untreated); lane 2, deglycosylated Con A precursor; lane 3, mature Con A; lane 4, total digest of precursor by immature jackbean extract; lane 5, digest of precursor (fraction unbound to ovalbumin–Sepharose); lane 6, digest of precursor (methyl α -mannoside eluate from ovalbumin–Sepharose).

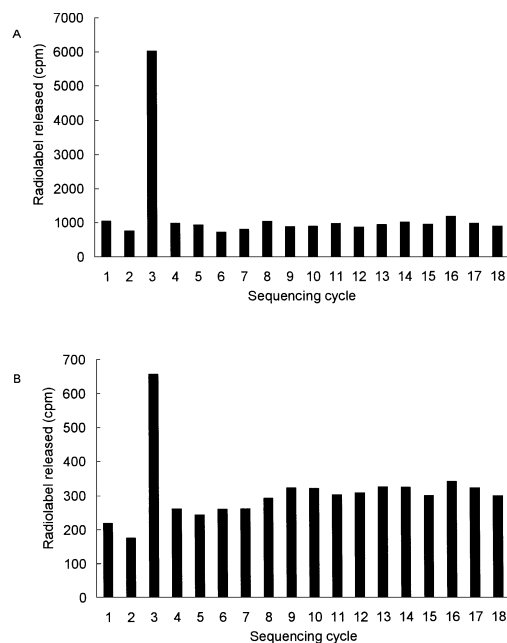


Figure 4 Release of 125 I during successive Edman degradation cycles of the 17.8 kDa (A) and 30.4 kDa (B) polypeptides derived from crude jackbean extract digests of Con A precursor

coprotein Con A precursor had been converted into polypeptides with mobilities identical with those of the 30.4, 17.8 and 14.2 kDa Con A polypeptides found in immature jackbeans. Because these polypeptides are intermediates in the synthesis of the mature protein [12], it seems that processing *in vitro* is occurring in a similar manner to processing *in vivo*. Although a certain amount of the 30.4 kDa polypeptide was produced by processing *in vitro*, suggesting that post-translational peptide bond formation oc-

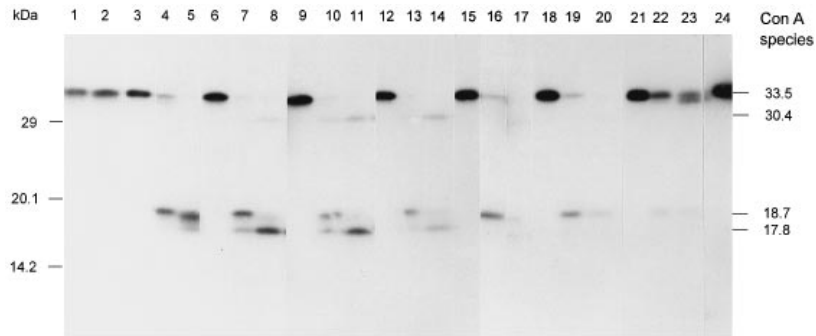


Figure 5 Dependence on pH of purified asparaginylendopeptidase digestion of Con A precursor

Con A glycoprotein precursor was incubated with asparaginylendopeptidase as described in the Experimental section, with the standard buffer being replaced by various buffers indicated below. After 4 h and 16 h, samples were taken and subsequently analysed by SDS/PAGE followed by autoradiography. Lanes 1, 4, 7, 10, 13, 16, 19 and 22, 4 h digests; lanes 2, 5, 8, 11, 14, 17, 20 and 23, 16 h digests; lanes 3, 6, 9, 12, 15, 18, 21 and 24, control digests (incubation in the absence of asparaginylendopeptidase for 40 h). Lanes 1–15, 100 mM sodium acetate; lanes 1–3, pH 4.0; lanes 4–6, pH 4.5; lanes 7–9, pH 5.0; lanes 10–12, pH 5.5; lanes 13–15, pH 6.0. Lanes 16–24, 100 mM sodium phosphate; lanes 16–18, pH 6.0; lanes 19–21, pH 6.5; lanes 22–24, pH 7.0.

curred, the principal products were 17.8 and 14.2 kDa polypeptides. The synthesis of these forms does not require post-translational peptide bond formation. The 14.2 and 15.1 kDa polypeptides, although thought to be present in equimolar quantities with the 17.8 and 16.2 kDa polypeptides, appear weakly on autoradiographs, reflecting the ratio of tyrosine residues in each polypeptide (14.2+15.2 kDa/17.8 kDa/16.2 kDa, 1:7:6).

According to the processing model outlined in Figure 1, there are tyrosine residues 3 and 16 amino acids from the N-termini of the 30.4 and 17.8 kDa polypeptides. Additional evidence that proteolytic cleavage *in vitro* was occurring at the same sites as *in vivo* was obtained by Edman degradation of these ^{125}I -labelled polypeptides formed from processing *in vitro*. Because radioiodination labels tyrosine residues principally, it was reasoned that radiolabel should be released after the third cycle of Edman degradation if processing occurred at the sites predicted. This was indeed so (Figure 4). A small amount of label might also have been released at position 16 where the second tyrosine was predicted but, as expected considering losses at each cycle of sequencing, this was not as clear.

Processing of Con A precursor by purified asparaginylendopeptidase

Digestion of the Con A glycoprotein precursor with the commercially available asparaginylendopeptidase at various pH values is shown in Figure 5. Owing to the radiolabelling properties described above, the 15.1 and 14.2 kDa polypeptides are not visible. At pH 4.0 (lanes 2 and 3), the enzyme seemed to be virtually inactive but as the pH was increased to 4.5 (lanes 5 and 6) partial processing of the precursor to two-chain forms was observed with the appearance of 18.7 and 15.1 kDa polypeptides as outlined in Figure 1. At pH 5.5 (lanes 10 and 11) processing seemed to be most extensive, resulting in formation of 17.7 and 30.4 kDa polypeptides. This is consistent with the reported pH optimum of 5.0–6.5 for the protease with dinitrophenyl-Pro-Glu-Ala-Asn-NH₂ as a substrate [37]. The presence of the 30.4 kDa polypeptide suggests that peptide bond formation had taken place *in vitro*. At higher pH values, radioactivity was lost, possibly as a result of the loss of solubility of Con A at higher pH [28,29].

Digestion of the precursor at an optimum pH of 5.5 resulted

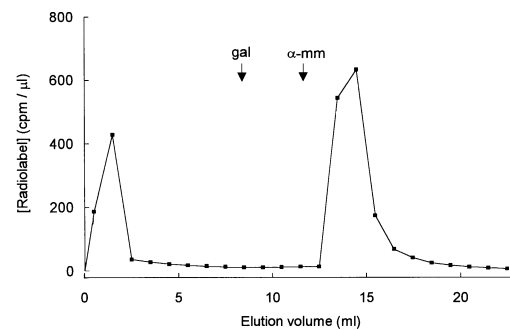


Figure 6 Ovalbumin-Sepharose chromatography of Con A precursor digested with asparaginylendopeptidase

Abbreviations: gal, galactose; α -mm, methyl α -mannoside.

in conversion of 63% of the protein to a form possessing carbohydrate-binding activity (Figure 6). Both the 30.4 and 17.8 kDa polypeptides appeared in the carbohydrate-binding fraction (results not shown).

Confirmation of the identity of the processed forms was provided by Edman degradation on an Applied Biosystems 477a sequencer (Figure 7). With the 17.8 kDa polypeptide, radioactivity was observed to be released after cycles 3 and 16, the expected positions of tyrosine residues. With the 30.4 kDa polypeptide a peak of radioactivity was observed at position 3 as expected, along with a possible peak at position 16. The large peak of radioactivity released at the first cycle might have been due to the loss of protein weakly bound to the membrane; the release of a significant amount of material in the first cycle is frequently observed in sequencing proteins.

Conversion of the precursor into an active lectin

Previous studies have indicated that post-translational processing of Con A *in vivo* involves deglycosylation, several endoproteolytic cleavages and formation of a peptide bond by an unknown mechanism. We have shown that crude jackbean extracts, which contain both N-glycanase and asparaginylendopeptidase activities, are able to catalyse processing of the Con A glycoprotein

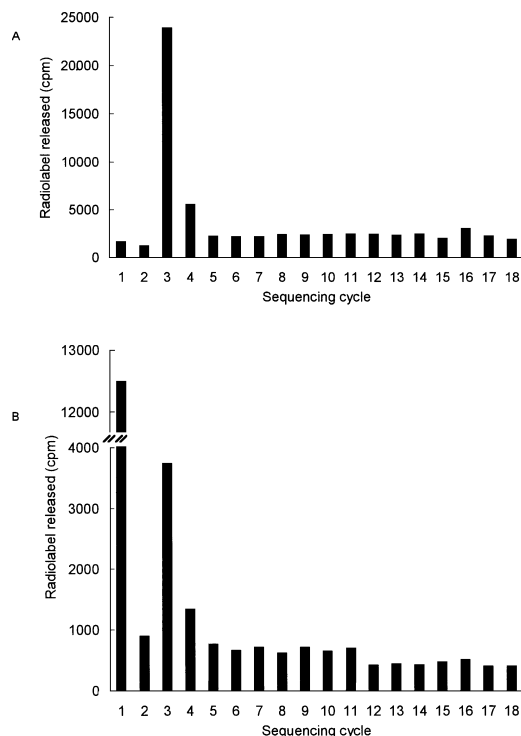


Figure 7 Release of ^{125}I during successive Edman degradation cycles of the 17.8 kDa (A) and 30.4 kDa (B) polypeptides derived from asparaginyl-endopeptidase Con A precursor digests

precursor *in vitro* and that peptide bond formation does occur to some extent during processing. During these events the Con A precursor is converted from a species that lacks ovalbumin-Sepharose-binding activity into a species that binds to the affinity matrix, suggesting that it has been converted into an active lectin.

The glycan has a crucial role in masking lectin activity of the Con A precursor [34,39]. Its removal, catalysed by N-glycanase, is thought to be the first step of processing *in vivo* and conversion of the precursor into an active lectin [12,30]. A non-glycosylated recombinant precursor can act as a substrate for processing by asparaginylendopeptidase *in vitro* [38]. We have now shown that a preparation of purified asparaginylendopeptidase is capable of performing precursor processing, apparently without a requirement for the prior removal of the N-glycan. One possibility is that lectin activity is gained on excision by asparaginylendopeptidase by removal of the glycan while it is still attached to the peptide. Alternatively, N-glycanase present in the asparaginylendopeptidase preparation would explain the formation of a polypeptide with a similar molecular mass to that of non-glycosylated precursor in a digest with endopeptidase at high pH (Figure 5, lane 23). When assayed with a CNBr-derived ovalbumin glycopeptide [40], a trace of N-glycanase activity was detected in the asparaginylendopeptidase preparation (9.2 nmol of NH_3 released from 100 nmol of substrate in 6 h using 2 μl of asparaginylendopeptidase).

Mechanism of peptide bond formation

Under the various conditions and times with which we have performed precursor digestions, we have always found that the 17.8 and 30.4 kDa polypeptides seem to be produced together at

the same time from the 18.7 kDa intermediate. This suggests that there is no separate ligation step between the 14.2 and 17.8 kDa polypeptides, because otherwise we should expect to see the 30.4 kDa polypeptide formed after the 17.8 kDa polypeptide. It is more likely that two possible fates exist for the 18.7 kDa intermediate. Either it can be spliced by transpeptidation with the 14.2 kDa polypeptide or there is simply a further endo-proteolytic cleavage that gives rise to the two-chain form of Con A (17.8 and 14.2 kDa polypeptides). In the seed, the splicing reaction seems to predominate, whereas under our conditions *in vitro* the cleavage is dominant.

Whereas deglycosylation and post-translational peptide bond formation seem to be events uniquely associated with Con A and closely related lectins, asparaginylendopeptidases have also been isolated from pumpkin [4,41,42] and soybean [43]. Judging from a comparison of the pumpkin cDNA sequence and jackbean N-terminal sequence, and also molecular masses, the two enzymes are very similar. This suggests that for Con A, post-translational peptide bond formation is a consequence of the shape of the Con A precursor and semi-processed intermediates rather than a result of a special processing enzyme in the jackbean.

In this respect there is a parallel with proteins maturing by an autocatalytic excision of a peptide, which include a yeast vacuolar H^+ -translocating ATPase [44–47], a bacterial DNA polymerase [48,49] and a RecA protein [50]. In these cases the excised polypeptides have sequence similarity to homing endonucleases and some have now been shown to contain this activity [51]. The spliced peptide bond in Con A occurs between Asn and Ser residues, whereas one of the peptide bonds to be broken in autocatalytic processing has always been shown to have the sequence Asn-Ser/Thr/Cys [52]. However, in Con A the topology of primary structure rearrangement is different and processing is clearly not autocatalytic.

We thank John Findlay, Hugh Jones and Mike Lord for helpful discussions. This work was supported by SERC grant GR E 83542 to D. J. B.

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