In Vivo Biosynthetic Studies of the Dolichos biflorus Seed Lectin

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ABSTRACT

The in vivo biosynthesis of the Dolichos biflorus seed lectin was studied by pulse-chase labeling experiments using [35S] methionine and [14C]glucosamine. These studies demonstrate that each of the two mature lectin subunit types are derived by the processing of separate glycosylated precursors. The appearance of the precursor to subunit I before the precursor to subunit II supports the possibility raised by previous studies that both subunit types of this lectin may originate from a single gene product.

All legume seed lectins described to date are oligomeric proteins containing identical or nonidentical subunits (for review, see ref. 16). These lectins are synthesized on the rough endoplasmic reticulum where they undergo a cotranslational removal of their signal sequence and possible cotranslational glycosylation and then transit the Golgi en route to their final destination in the protein bodies (for review, see ref. 10). The members of this class of lectins have been grouped into three categories based on their structures (2). Differences among these categories have also been found in the biosynthetic pathways of the lectins.

The first category contains lectins, such as favin and the pea lectin, that are initially synthesized as a single high mol wt precursor that is subsequently cleaved to yield the large \( \beta \)-chain and small \( \alpha \)-chain of each of these lectins (17, 18). The processing of the pea lectin occurs in the protein bodies (19) and appears to be accompanied by the additional removal of four and six amino acids, respectively, from the carboxyl termini of the \( \alpha \)- and \( \beta \)-chains (18).

The second category is represented by Con A, which contains four identical polypeptide chains (16). Each of these chains arises from a glycosylated precursor that undergoes posttranslational proteolytic cleavage and a novel peptide ligation resulting in a sequence circularly permuted from the sequence of the precursor (3, 4). The central 15 amino acid segment containing the carbohydrate unit is lost during this processing (3).

The third category contains other legume seed lectins with subunits of similar or equal size. The biosynthetic pathway of only one of these lectins, the Phaseolus vulgaris lectin, has been studied in detail. The E and L subunits of this lectin have been found to arise from different genes (20) and to combine in different proportions to generate five isomers of the tetrameric lectin (24). No posttranslational proteolytic modifications of these subunits have been detected, but their oligosaccharide side chains are extensively modified during transit to the protein bodies (9, 31, 32).

The Dolichos biflorus seed lectin is a member of this third category of lectins. It is a tetrameric glycoprotein containing equal amounts of two different subunits, I and II, that have been found to differ only in the length of their carboxyl termini (6–8, 15, 25). Only a single mRNA and in vitro mRNA translation product have been found for this lectin (27). Sequence analysis of the lectin cDNA has shown that it contains the carboxyl terminal sequences of both subunits, suggesting that subunit II may be derived from subunit I by the removal of a 10 amino acid carboxyl terminal segment (26). The present in vivo study was undertaken in an effort to elucidate the processing events that occur during the biosynthesis of this lectin.

MATERIALS AND METHODS

Materials

L-[35S]Methionine (1125–1131 Ci/mmol) and EN3HANCE were purchased from New England Nuclear (Boston, MA). D-[1-14C]Glucosamine (59 mCi/mmol) was obtained from Amersham (Arlington Heights, IL), cyanogen bromide activated Sepharose 4B from Pharmacia (Uppsala, Sweden), and tunicamycin and TFMS from Sigma Chemical Co. (St. Louis, MO). Dolichos biflorus seeds were obtained from F. W. Schumacher Co. (Sandwich, MA). These seeds were germinated and grown in U. C. Mix I fertilizer II (c) (1) in a growth chamber at 25°C with a 16 h/8 h light/dark cycle.

Radioactive Labeling

Cotyledons from developing D. biflorus seeds were labeled by the procedure of Spencer et al. (28) using 22 \( \mu \)Ci of [35S] methionine or 4 \( \mu \)Ci of [14C]glucosamine per cotyledon. In experiments in which the radioactivity was chased, the cotyledons were further incubated in 20 \( \mu \)L of either 0.25 M L-methionine or 0.25 M D-glucosamine. One to two cotyledons were used for each experimental point. In some experiments, the cotyledons were incubated in 20 \( \mu \)L of either 1 mg/mL

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3 Abbreviation: TFMS, trifluoromethanesulfonic acid.
tunicamycin in 1 mM NaOH or in 1 mM NaOH alone for 90 min prior to pulse-chase labeling with [35S]methionine.

Preparation of Immunoaffinity Resin

_D. biflorus_ seed lectin was purified as previously described (11, 14) and denatured by heating at 80°C for 20 min. Rabbit antiserum was raised to this heat denatured lectin by the procedure previously described for the native lectin (30). This antiserum was chromatographed on a resin made by coupling heat denatured seed lectin to CNBr-activated Sepharose 4B according to the Pharmacia instructions. Antibodies binding to the resin were eluted with 0.2 M glycine (pH 2.2), and immediately dialyzed against 0.1 M NaHCO₃ containing 0.5 M NaCl. These isolated antibodies specific to the heat denatured seed lectin were then coupled to CNBr-activated Sepharose 4B to produce an immunoaffinity resin.

Tissue Extraction

Labeled cotyledons were homogenized (5% w/v) in PBS containing 1 mM PMSF (buffer A). The homogenates were centrifuged at 1000g for 5 min and the supernatants either stored at −20°C or chromatographed directly on an immunoaffinity column. Pellets stored at −20°C were in some cases further extracted with 0.5 M NaCl or 0.1% SDS in buffer A, and the solubilized material was chromatographed on an immunoaffinity column.

Immunoaffinity Chromatography and Analysis of Extracts

Supernatants from extracts of labeled cotyledons were chromatographed in PBS on the immunoaffinity resin containing antibodies against heat denatured seed lectin described above. Bound protein was eluted from the resin with 0.2 M glycine (pH 2.2) and immediately dialyzed against H₂O. Samples to be deglycosylated were treated with TFMS for 1 h on ice as described by Carp et al. (5). Protein samples were lyophilized, redissolved in sample buffer and subjected to SDS-urea PAGE as previously described (6). The gels were stained with Coomassie brilliant blue, impregnated with ENHANCE, dried and exposed to XAR-5 film (Eastman Kodak, Rochester, NY) which was prefocused (22).

RESULTS

Preliminary studies on immunoblots of extracts from developing _D. biflorus_ seeds showed that the seed lectin can first be detected at 22 DAF. Previous studies had shown that the lectin is present at maximum levels by 28 DAF (30). Consequently, 22 to 28 d old seeds were chosen for the present investigation of lectin biosynthesis. The weights of the seeds used for this study ranged from 20 mg at 22 d to 80 mg at 28 d.

Short-Term Labeling of _D. biflorus_ Cotyledons

Cotyledons of 28 d old seeds were labeled for 15, 30, 45 or 60 min with [35S]methionine. Fluorographs of electrophoretic gels of lectin isolated from these labeled cotyledons show the appearance of two labeled polypeptides, bands 1 and 2, each with a slightly lower electrophoretic mobility than the mature lectin subunits I and II, respectively (Fig. 1A). The relative amounts of radioactivity in each of these bands was determined by scanning densitometry of the fluorographs. These scans (Fig. 2) show that at the earliest labeling time, most of the label is associated with band 1 and that as the labeling time increases, the label becomes more evenly distributed between both bands. This conclusion is supported by pulse-chase labeling experiments in 22 d old cotyledons, where the label is predominantly associated with band 1 after 1 h of labeling and no chase (Fig. 1B, lane 1). After 3 h of chase, the label is more equally distributed between the two precursor bands (Fig. 1B, lane 2).

Pulse-Chase Labeling of _D. biflorus_ Cotyledons

Previous studies from our laboratory have established that both subunits of the mature seed lectin contain a single carbohydrate unit covalently attached by a N-glycosidic linkage (7, 8). To determine if the lectin precursor bands 1 and 2 are glycosylated, cotyledons from 28 d old seeds were labeled for 1 h with [35S]glucosamine, chased for various time periods with excess cold glucosamine, and extracted and chromatographed on the immunoaffinity column as previously de-
Figure 2. Determination of percent incorporation of radioactivity into lectin precursor bands with increasing times. Each lane of the fluorograph in Figure 1A was scanned with a Zineh scanning laser densitometer. The peaks from bands 1 and 2 were integrated and the area of each is presented as a percent of the total area.

Figure 3. Glucosamine pulse chase labeling of lectin polypeptides. Cotyledons from 28 d old seeds were labeled for 1 h with [35S]methionine and subsequently incubated with excess unlabeled glucosamine for various times. Extracts from the labeled cotyledons were processed as described earlier and analyzed by SDS-urea gel electrophoresis and subsequent fluorography. The different lanes show proteins obtained from chases of 1) 0 h; 2) 3 h; 3) 7 h; 4) 11 h; 5) 15 h; and 6) 24 h. Arrows indicate the mobilities of subunits I and II of the mature seed lectin.

scribed. The presence of label in each of the precursor bands 1 and 2, as seen faintly after no chase and more distinctly after 3 h of chase, as well as in the mature lectin subunits (Fig. 3), demonstrates that each of these polypeptides is glycosylated.

Cotyledons from 22 to 28 d old seeds were labeled for 1 h with [35S]methionine and then chased with excess cold methionine for various times. After extraction and isolation of lectin by immunofluorimetry chromatography, aliquots were deglycosylated with TFMS and compared with untreated samples by SDS-urea gel electrophoresis. As had been found in the initial labeling experiments (Fig. 1, lane 4), a pulse of 1 h with no chase results in the appearance of the two precursor bands, 1 and 2 (Fig. 4, lane 1). By 3 h of chase (Fig. 4, lane 3), a labeled band corresponding to the lectin subunit II of the mature lectin began to appear just below band 2, and by 7 h of chase (Fig. 4, lane 5), band 1 has become very diffuse with its lower edge corresponding in mobility to mature subunit I. By 24 h of chase (Fig. 4, lane 9), only the mature lectin subunits are labeled. A similar progression of label was observed for lectin isolated from pulse-chase labeled 22, 24 and 26 d old cotyledons (data not shown), except that in the case of the 22 d old cotyledons, band 2 appeared to be converted to subunit II prior to the conversion of band 1 to subunit I.

At all chase times in the above pulse-chase experiment on 28 d cotyledons, deglycosylation of the lectin by TFMS results in the conversion of the electrophoretic pattern to two bands of identical electrophoretic mobility to the deglycosylated mature lectin subunits (Fig. 4, lanes 2, 4, 6, 8, and 10). These results demonstrate that both the mature lectin subunits and their precursors are glycosylated and indicate that the processing of the two precursor bands 1 and 2 to subunits I and II, respectively, involves only the processing of the carbohydrate units.

Effect of Tunicamycin on Lectin Biosynthesis

To further examine the relationships among the precursor bands and the mature lectin subunits, 28 d old cotyledons were preincubated in 1 mg/ml tunicamycin in 1 mM NaOH for 90 min, labeled with [35S]methionine for 1 h and chased with cold methionine. Tunicamycin is an antibiotic that selectively inhibits N-glycosylation of newly synthesized proteins (29). Analysis of labeled lectin polypeptides showed that after a 7 h chase there are only two labeled bands, each with an electrophoretic mobility slightly faster than each of the mature lectin subunits (Fig. 5A, lane 1). After a 24 h chase, no change was noted in the mobility of these two polypeptides (Fig. 5A, lane 2). Control cotyledons preincubated in 1 mM NaOH alone showed the usual pattern of labeled precursor bands 1 and 2 with processing to subunits I and II (Fig. 5B). These results are in agreement with the results obtained with the TFMS treatment above.

DISCUSSION

Previous studies from our laboratory have shown that the D. biflorus seed lectin is a tetrameric glycoprotein composed of equal amounts of two types of structurally similar subunits, I and II (6, 7). These subunits contain N-glycosidically linked carbohydrate units consisting of mannose, N-acetylglucosamine, fucose and xylose (8, 21). Amino acid sequence analysis shows the presence of a single consensus N-glycosylation site per polypeptide chain (26). The present biosynthetic data show that the two types of mature subunits arise in two stages: (a) the early appearance of two glycosylated precursor bands and (b) the later conversion of these glycosylated precursors into the mature lectin subunits by alteration of their carbohydrate units.

The inhibition of glycosylation of the subunit precursors with tunicamycin is in agreement with evidence from previous in vitro biosynthetic studies that showed glycosylation to be a cotranslational event in the synthesis of this lectin (27). Such cotranslational glycosylation and subsequent posttranslational alteration of carbohydrate units have been found to be common features in the biosynthesis of many plant lectins.
ensure the differential (6, 7). that the tides or arrival appear for subunit found always (A) tides. with consistent associated with gel electrophoresis for subunit. This finding, multiple seed lectin subunit diversity by differential modification of a common precursor provides yet another variation in the biosynthetic schemes for these lectins. Although the biological significance of such subunit modifications is not yet known, it is of interest that subunits of both the pea lectin and wheat germ agglutinin have recently been shown to undergo carboxyl terminal modifications (18, 23) and that studies on the individual subunits of the D. biflorus lectin have shown that carbohydrate binding activity is associated only with subunit I (13).

It must be recognized that the present results do not yet completely rule out the possibility of origin of these subunits from different genes or by alternate splicing of a single mRNA. Final confirmation of a differential posttranslational proteolytic processing mechanism must await the identification of the putative processing protease. Such experiments are now underway in our laboratory.

LITERATURE CITED

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