*Phytochemistry*, Vol. 26, No. 5, pp. 1435–1440, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00


# THE ISOLATION AND AMINO ACID SEQUENCE OF THE β- AND γ-SUBUNITS OF THE LECTIN FROM THE SEEDS OF *DIOCLEA GRANDIFLORA*

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(*Revised received* 16 *September* 1986)

**Key Word Index**—*Dioclea grandiflora*; Leguminosae; Diocleae; lectin; subunits; structural analysis.

**Abstract**—Although the two smaller β- and γ- subunits of the lectin from *Dioclea grandiflora* were clearly resolved by sodium dodecyl sulphate (SDS) gel electrophoresis, the concensus of other techniques including ultracentrifugation, isoelectric focusing in 8 M urea, size-exclusion chromatography in dissociating solvents and amino acid and sequence analysis indicated that they were similar in molecular size and that they had arisen either by a single enzymic cleavage at $Asn^{118}$–$Ser^{119}$ in the middle of the 237 residue-long mature α-subunit or by multiple cleavages occurring during post-translational processing of intermediates. The existence of minor forms of the β- and γ- subunits resulting from a cleavage at $Asn^{124}$–$Ser^{125}$ of the α-subunit was also recognized. The results indicated that the apparent difference in molecular size of the β- and γ-subunits deduced from SDS-gel electrophoresis could be explained by the anomalous behaviour of both subunits in this separation technique. The structural features of the *D. grandiflora* lectin are compared with those of concanavalin A obtained from seeds of the botanically related *Canavalia ensiformis*.

## INTRODUCTION

The lectin from the seeds of *Dioclea grandiflora* (Mart.) characterized recently [1] belongs to the general group of D-mannose (D-glucose)-binding lectins [2]. It requires the presence of $Ca^{2+}$ and $Mn^{2+}$ ions for full activity, it contains no covalently bound carbohydrate, and in the presence of sodium dodecyl sulphate (SDS) it splits into three subunits, α, β and γ, of approximate $M_r$ 25–26 000, 13–14 000 and 8–9000, respectively [1]. The results of the full amino acid sequence of the major α-polypeptide subunit of this lectin containing 237 amino acid residues indicated a close homology with concanavalin A despite differences in 53 amino acid positions [3] between the two lectins. The results also suggested that the smaller β- and γ- subunits had sequence homology with the respective N-terminal and the C-terminal halves of the intact α-subunit and that they might have arisen as the result of hydrolysis of the $Asp^{118}$–$Ser^{119}$ peptide bond of the α-subunit. However the low $M_r$ indicated by SDS-gel electrophoresis for the γ-subunit was not compatible with such a polypeptide containing, from $Ser^{119}$, the entire C-terminal part of the intact α-subunit without the further proteolytic modification which has previously been suggested to occur for concanavalin A [4].

In the following, the isolation and characterization of the β- and γ-subunits of the *D. grandiflora* lectin and their amino acid sequences are described. It is shown that indeed the β-subunit is homologous with the sequence of amino acid residues 1–118 of the α-subunit and that despite its high mobility in SDS-gel electrophoresis the γ-subunit contains the remaining 119 amino acid residues (119–237) of the C-terminal portion of the intact α-subunit.

## RESULTS

### *Preparation and properties of the β- and γ-subunits*

The separation of the γ- and β-subunits by preparative SDS-gel electrophoresis is shown in Fig. 1a. Because of an asymmetrical distribution of aromatic amino acids in the two polypeptides the extent of the separation is visualized more easily from the results of analytical SDS-gel electrophoresis of the eluted fractions of the preparative run (Fig. 1b).

The amino acid compositions of the two separated subunits were distinctly different; however, the sum of the contents of the amino acids in the two polypeptides was close to that determined for the α-subunit (Table 1). The results of sedimentation experiments in 5 M guanidine HCl also indicated that while the α-subunit had an $S_{20,w}$ value of 1.27 S, the two fragmented polypeptides were smaller, with $S_{20,w}$ values of 0.99 S for β and 0.98 S for γ, but very similar in size. In isoelectric focusing experiments in 8 M urea-containing gels, the polypeptide bands found in different preparations of *D. grandiflora* lectins (Figs 2a and 2b) could be accounted for in the patterns exhibited by the isolated subunits, α, β and γ.

### *Amino acid sequence of the β-subunit*

Microsequence analysis of the β-subunit by the DABITC method yielded the sequence of amino acids 1–20 as shown in Fig. 3. This sequence is exactly the same as that found previously at the N-terminus of the intact α-subunit [3]. Analysis of the chymotryptic peptides of the β-subunit gave the sequences shown in Fig. 3. All the major peptides recovered corresponded with the sequen-

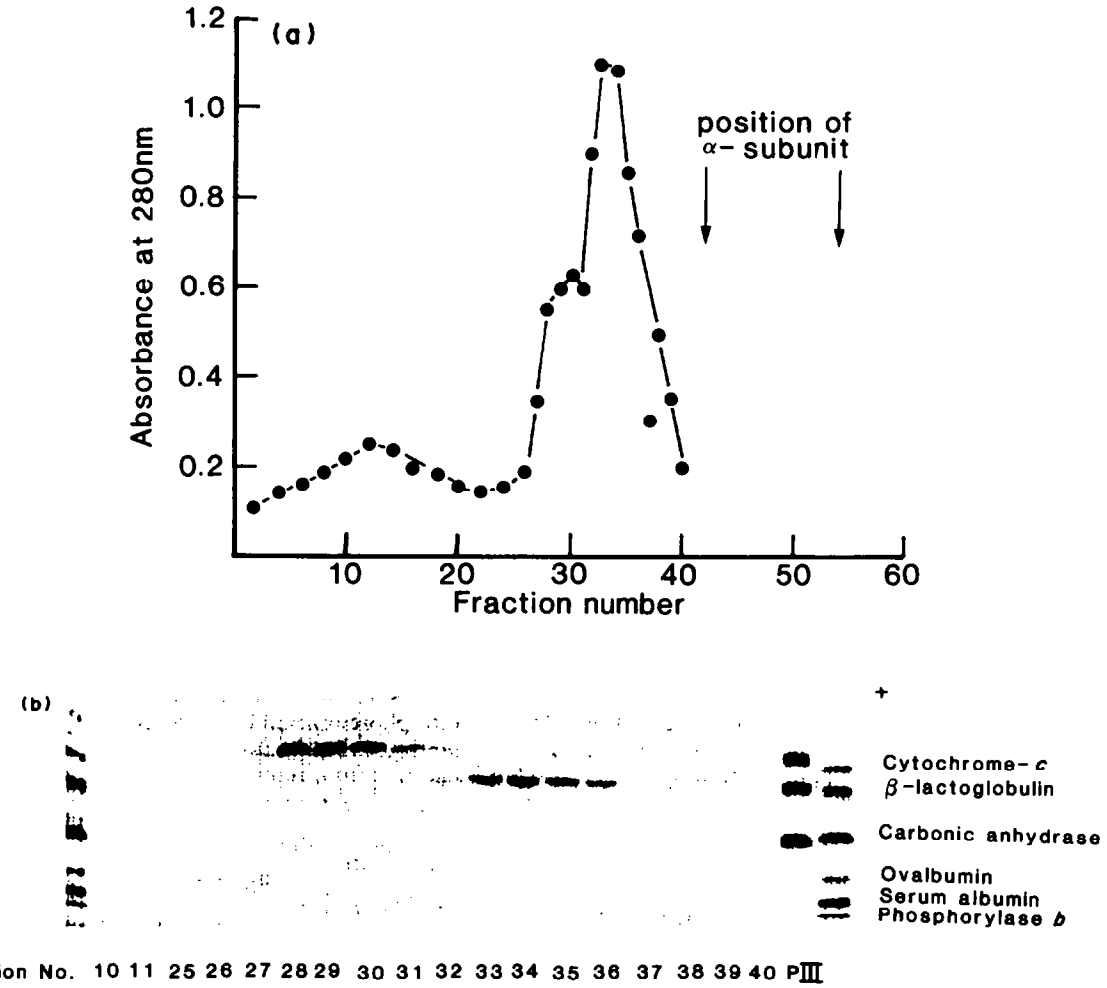


Fig. 1. Fractionation of the subunits of *D. grandiflora* lectin by preparative SDS-gel electrophoresis. (a) Electrophoresis was carried out at 27 V and 12 W. Fractions of about 4 ml were collected at 20 min intervals and their absorbance was read at 280 nm. (b) Location of subunits in fractions obtained by preparative SDS-electrophoresis. Samples of about 10 μl were examined by analytical SDS-gel electrophoresis on 0.5 mm thick gels at 50 mA for 80 min. Numbering of the fractions is the same as in (a) and the separation of $M_r$ marker proteins is shown.

ces previously observed in the residues 1–118 of the α-subunit [3]. However, in addition, low levels of peptide $\beta C_{11}$ were observed, the sequence of which corresponded with residues 119–124 of the α-subunit. Digestion of the β-subunit with carboxypeptidase A for various periods released only Asn and Thr in major amounts, these residues being those found in positions 117 and 118 of the α-subunit [3]. Thus these results indicate that the major form of the β-subunit has the same sequence as residues 1–118 of the α-subunit and that a minor form corresponding to residues 1–124 is also present. Also, as found previously with the intact α-subunit [3], heterogeneity exists in the structure of the β-subunit at amino acid residue 10. The extent of this heterogeneity appears to depend on the method of purification of the subunits. In the α-subunit, when prepared by molecular-sieve chromatography in SDS, the major form contained Asn in this position. However, when the α- or β-subunits were prepared by SDS-gel electrophoresis a considerable proportion of this was replaced by Asp.

*Amino acid sequence of the γ-subunit*

Microsequence analysis of the γ-subunit gave an amino acid sequence for the first 13 residues which corresponded with that previously observed between residues 119 and 131 of the α-subunit [3]. However, traces of amino acids observed previously in positions 126–131 of the α-polypeptide were also found. This indicated that small amounts of a shorter γ-subunit also existed. The sequences of the tryptic peptides derived from the γ-subunit also corresponded with the tryptic peptides expected from residues 119–237 of the α-subunit. The presence of Asn and Ala at the C-terminus of the γ-subunit confirmed that the major form of this polypeptide is identical with residues 119–237 of the intact α-subunit. More heterogeneity was found in the structure of the γ-subunit than in the β-subunit. Only the heterogeneities in residues 132 and 204 previously observed in the α-protein were confirmed during the sequencing of the γ-subunit, but it is possible that the other heterogeneities which occurred in

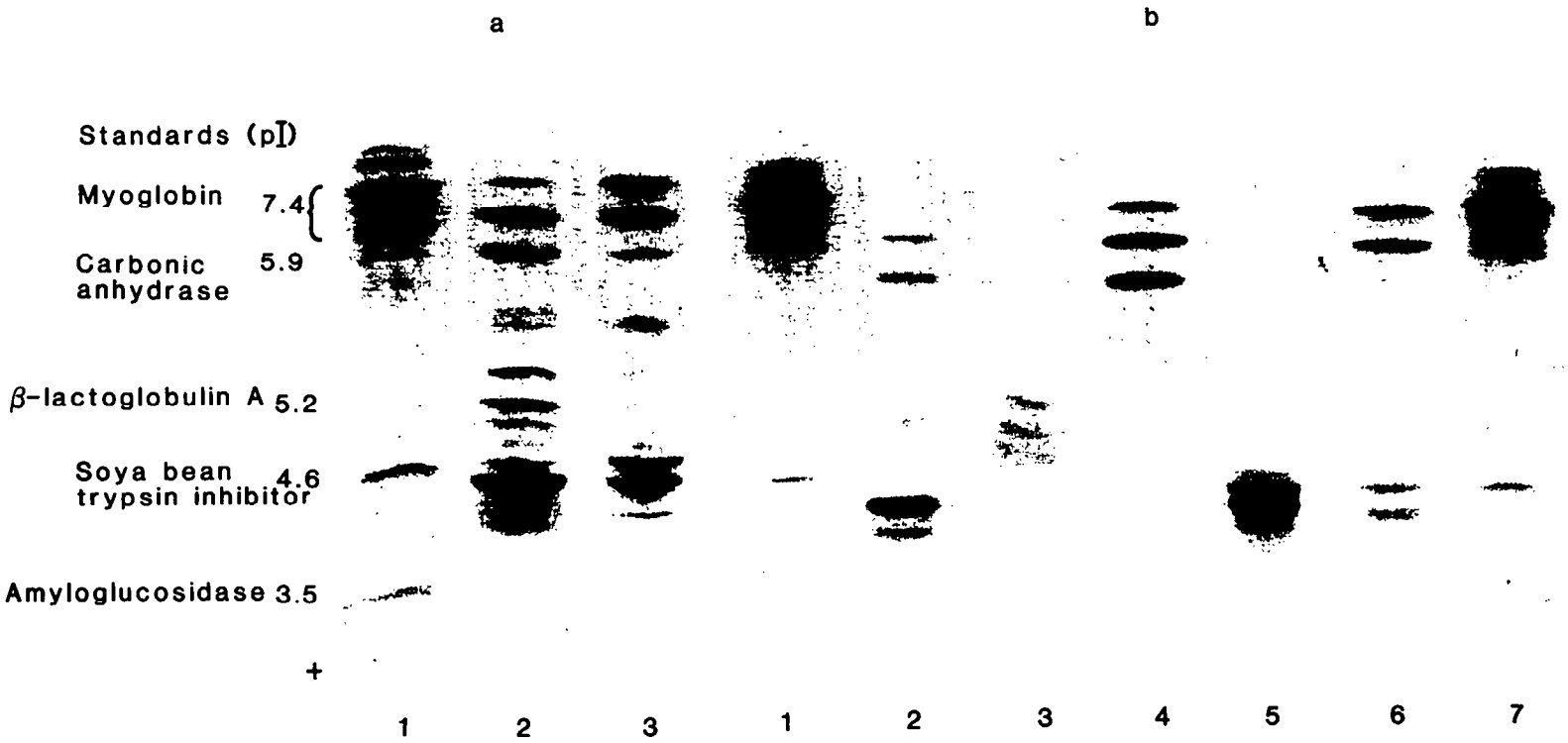


Fig. 2. Isoelectric focusing patterns of *D. grandiflora* lectin and its isolated subunits on polyacrylamide gels containing 8 M urea. (a) Patterns showing variation of electrofocused bands in two different batches of PIII *D. grandiflora* lectin. Lane 1, standard proteins; lanes 2 and 3, two different preparations (a and b) of PIII lectin. (b) Banding patterns of the isolated subunits of PIII lectin used for the amino acid sequencing studies. Lane 1, standard proteins; 2, PIII, lectin a; 3, α-subunit; 4, β-subunit; 5, γ-subunit; 6, PIII lectin b; 7, standard proteins. All lanes, except the standards, were loaded with about 10 μg of protein.

Table 1. Amino acid composition of *D. grandiflora* lectin subunits*

| Amino acid | β-Subunit | γ-Subunit | α-Subunit |
|---|---|---|---|
| Asp | 15.0 | 16.8 | 31.3 |
| Thr | 11.3 | 7.4 | 17.7 |
| Ser | 16.9 | 15.0 | 33.1 |
| Glu | 6.1 | 7.5 | 13.1 |
| Pro | n.d. (4)† | n.d. (7)† | n.d. (11)† |
| Gly | 7.6 | 9.6 | 17.4 |
| Ala | 6.9 | 9.6 | 17.4 |
| Val | 11.0 | 6.0 | 16.1 |
| Met | 1.0 | 0 | 1.0 |
| Ile | 8.5 | 6.0 | 14.1 |
| Leu | 7.7 | 9.2 | 18.0 |
| Tyr | 5.6 | 1.6 | 7.0 |
| Phe | 1.9 | 9.5 | 10.7 |
| His | 1.9 | 2.8 | 4.4 |
| Lys | 6.2 | 5.2 | 12.3 |
| Arg | 3.5 | 2.6 | 7.4 |
| Trp | 3.0 | 2.0 | 4.5 |
| Cys | 0 | 0 | 0 |

*The values are shown as molar ratios based on 118 residues for the β- and 119 residues for the γ-subunit. For comparison, the molar ratios previously determined [3] for 237 residues of the intact α-subunit are also given. The values were obtained from analyses carried out in triplicate of samples hydrolysed for 24, 48 and 72 hr. The values for Thr and Ser were obtained from linear extrapolation to zero time and the values of Val, Ile and Leu from the 72 hr results. The Trp value was obtained after hydrolysis with 4 N methane sulphonic acid. n.d., not determined.

†Results in parentheses were obtained from sequence data.

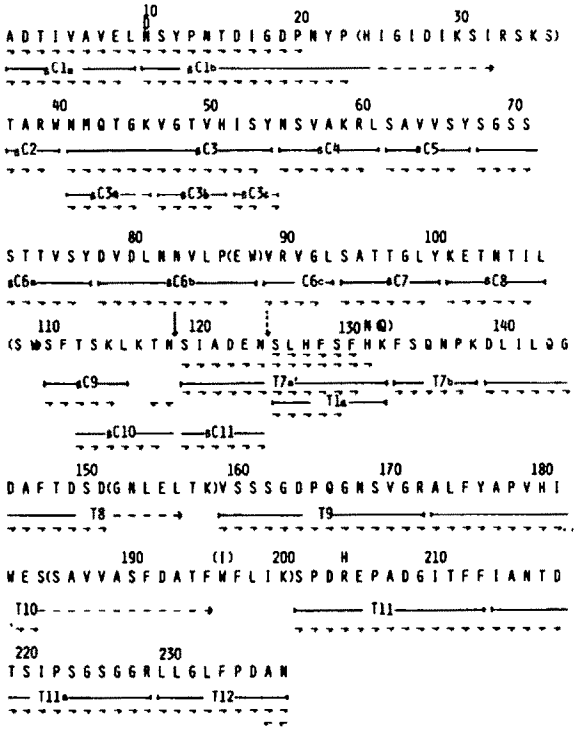

Fig. 3. Amino acid sequence of the β- and α-subunits of the lectin from the seeds of *D. grandiflora* and its relationship to the sequence of the intact α-subunit [3]. Peptides were obtained by digestion with chymotrypsin, designated C, from the β-subunit and with trypsin, T, from the γ-subunit. Their numbering, for easier comparison with the structure of the α-subunit, is kept close to that assigned to the peptides obtained for the α-subunit previously. Solid lines indicate the length of the peptides obtained and the individual residues in the peptide are underlined (——) if their sequence was determined by the DABITC method. Residues in peptides whose sequence was not determined are given in parentheses and underlined by a broken line. The actual sequence in these regions is taken from the amino acid sequence of the α-subunit and is consistent with the amino acid composition (Table 1). The C-terminal amino acid residues determined by digestion with carboxypeptidase-A are underlined (——). The major and minor cleavage points in the α-subunit sequence, giving rise to the β- and γ-subunits, are indicated by a solid and a broken arrow, respectively. Heterogeneities are indicated by the insertion of the alternative amino acid over the main form in the sequence. The identity of those in parentheses was not established in sequencing the β- or γ-subunits but was taken from the work on the α-subunit. In addition, $Asn^{132}$ occurred in small amounts only.

positions 133 and 196 of the α-subunit were present also in the γ-protein.

## DISCUSSION

Although analytical SDS-gel electrophoresis suggested a significant difference in the molecular size [1], the attempted separation of the two smaller subunits of the *D. grandiflora* lectin by column chromatography in the presence of dissociating solvents was unsuccessful. Gel supports of a wide range of porosities in SDS or in 5 M guanidine HCl were tried. Although the α-subunit was clearly separated from the two smaller subunits [3] these last two always emerged together from such columns. Their separation was finally achieved by preparative SDS-gel electrophoresis (Figs 1a and 1b). Sedimentation velocity runs on the separated smaller subunits confirmed that their molecular size was very close. Furthermore, the sum of the amino acid residues of the β- and γ-subunits was also very similar to that of the intact α-subunit. All these results suggested that the properties of *D. grandiflora* lectin resembled closely those of concanavalin A [5, 6] which, in addition to intact subunits, also contained variable amounts of fragmented subunits of nearly identical molecular size but of different mobility in SDS-gel electrophoresis [7]. It has been suggested previously that in SDS-gel electrophoresis binding of less than 1.4 g of SDS per g of protein leads to a reduced electrophoretic mobility [8]. Indeed, such anomalous behaviour has been observed frequently with glycoproteins, highly disulphide-crosslinked proteins [8] or proteins containing localized, highly negatively charged structures, for example, $\alpha_{S1}$-casein [9]. An unusual amino acid composition in the carboxy terminal part of the neurofilament

proteins has also been suggested to lead to reduced mobility in SDS-gel electrophoresis [10]. Moreover, the substitution of even a single cysteine for an arginine in a histidine-binding protein ($M_r$ 25000) of a *Salmonella typhimurium* mutant causes it to migrate in SDS-gel electrophoresis as if its $M_r$ were greater by as much as 2000 [11]. On the other hand, the binding of more than 1.4 g of SDS per g of protein leads to increased electrophoretic mobility [12]. It is thus possible that the anomalously high mobility of the γ-subunit is brought about by increased SDS binding due to the unusual aromatic amino acid distribution in the two halves of the *D. grandiflora* lectin and that it does not reflect a real difference in the size of the two subunits.

The results of sequencing studies on the β- and γ-subunits of the *D. grandiflora* lectin would be consistent with their derivation by cleavage of $Asn^{118}$–$Ser^{119}$ of the α-subunit [3], as suggested for concanavalin A [7] in which the bond is exposed to proteolytic modification in a loop linking the lower two chains of the rear β-structure [13]. Two independent groups [14, 15] have recently educed evidence for an interesting alternative biosynthetic route to the α-, β- and γ-subunits of concanavalin A whereby the primary translational product, a single polypeptide chain containing the ultimate β- and γ-sequences of the mature lectin in reversed order, is extensively cleaved by a specific endopeptidase acting on asparaginyl peptide bonds. The mature α-subunit is proposed to result from a novel ligation step [14] or, more probably, a concerted transpeptidation [15] giving rise to the $Asn^{118}$–$Ser^{119}$ band of the α-subunit sequence. Such a protease with specificity for cleavage on the C-terminal side of an asparagine residue may indeed occur widely [5, 14, 16]. Although a number of Asn linkages to Ser or other amino acids have been identified in the concanavalin A sequence, many of these are probably not sufficiently accessible for enzyme attack [14]. Interestingly, however, our sequence studies of the β- and γ-subunits of the *D. grandiflora* lectin have provided evidence of an alternative cleavage of the $Asn^{124}$–$Ser^{125}$ bond with the production of small amounts of a slightly longer $\beta_1$- and a complementary shorter $\gamma_1$-subunit. The C-terminal sequence of the $\beta_1$-polypeptide is identical to the sequence of residues 118–124 of the intact α-subunit [3], indicating that it is derived by the action of the hypothetical asparaginyl endopeptidase *after* the ligation step and not by trimming of an intermediate corresponding to the 18.8 k polypeptide depicted by Bowles *et al.* [15]. A similar split has not been reported for concanavalin A where no homology exists with the *D. grandiflora* lectin sequence between residues 120–123 [3] although an asparaginyl residue is present at position 124 in both lectins.

The determination of the amino acid sequence for the subunits also supported the findings of the isoelectric focusing experiments in 8 M urea (Fig. 2) and showed that all variants of the γ-subunits had a net negative charge. According to the amino acid sequence, the γ-subunit had a potential maximum of 15 (or a minimum of 14) acidic residues while the number of basic amino acids varied between 7 and 11 in the variants (Table 1). However, without clear quantitative results on the frequency of amino acid substitutions in the variants it is very difficult to relate the number of bands with different isoelectric points to precise sequences. The results are somewhat easier to interpret with the β-subunit in which the two major variants differ by one charge and contain either $Asn^{10}$ or $Asp^{10}$ in their sequence. Indeed, they may correspond to the two major isoelectric bands in Fig. 2. The two minor bands may possibly be related to the additional minor variants originating from the split at $Asn^{124}$–$Ser^{125}$. However, there are also natural variations in the different preparations of PIII of *D. grandiflora* lectin, as shown by isoelectric focusing in 8 M urea-containing gels (Fig. 2), and these may also add to the complexity of the banding patterns of the subunit.

In conclusion, the main forms of the two smaller subunits were shown to be derived from the major intact α-subunit by an almost symmetrical cleavage of the polypeptide chain at residues $Asn^{118}$–$Ser^{119}$. The resulting polypeptides showed anomalous behaviour on SDS-gel electrophoresis, the mobility of the γ-subunit being greater than warranted by its molecular size, while the β-subunit is probably somewhat retarded. Although most of the structural features of the *Dioclea* lectin resemble those described previously for concanavalin A, another lectin from a plant belonging to the same tribe of Diocleae, the two minor polypeptides arising from a break at $Asn^{124}$–$Ser^{125}$ were not observed with concanavalin A, while the additional cleavage points which have been suggested to occur with concanavalin A [4] did not occur with the *Dioclea* lectin. The extended sequence determined for the minor $\beta_1$-subunit suggests that asparaginyl endopeptidase (s) is still active in the later stages of evolution of the mature lectin.

## EXPERIMENTAL

The initial purification of the *D. grandiflora* lectin was carried out as described previously [1]. Although the α-subunit was separated from the other two subunits by gel filtration in 0.1% SDS and this material was used for sequencing studies [3], the two smaller subunits emerged together from all the columns investigated even in the presence of 5 M guanidine HCl. The size exclusion materials included Ultrogel (LKB Instruments Ltd., U.K.) AcA-34, AcA-44, AcA-54 and AcA-202 gels covering a great range of porosities for such a separation. Finally, the separation of the β-and γ-subunits was accomplished by preparative SDS-gel electrophoresis of PIII lectin preparations [1] in an apparatus (Birchover Instruments Ltd., U.K.) based on the design of ref. [17]. Briefly, a gel of 9 cm diameter and 5 cm height was formed in a gel-former by polymerizing a mixture of recrystallized acrylamide and methylene bis-acrylamide in a 39:1 (w/w) ratio to give 11% (w/v) final concentration. The gel buffer was 0.033 M Na-phosphate, pH 7.5, containing 0.2% (w/v) SDS. The polymerized gel was transferred to the running-tube and ionic impurities were removed from it by electrophoresis in the same phosphate buffer at 27 V and 23° for 4 hr. The electrode buffer was then replaced with fresh buffer and a clear sample soln of 8 ml was layered on to the gel. Typically the sample was prepared by dissolving 60–70 mg of lectin or partially purified subunits from previous runs in 0.033 M Na-phosphate, pH 7.5, containing 2.5% (w/v) SDS and 1% (w/v) 2-mercaptoethanol and heating at 100° for 20 min. Electrophoresis was carried out at 27 V and about 12 W. Fractions of about 4 ml were collected at 20 min intervals and their absorbance was read at 280 nm. Suitable aliquots were also run on analytical SDS-gels to locate more precisely the recovered subunits. The fractions corresponding to the γ- and β-subunits were separately pooled, dialysed thoroughly against $H_2O$ and recovered by freeze-drying. Residual SDS was removed from the samples by dissolving them in a small vol. of $H_2O$ and precipitating the polypeptide by

adding $Me_2CO$ to the solns to give a final concentration of 90% (v/v) at 0–4°. Finally the precipitated protein material was taken up in $H_2O$ and recovered by freeze-drying.

Analytical SDS-gel electrophoresis was performed using a discontinuous buffer system [18] employing the following gel parameters [19]: stacking gel, T, 3.95% and C, 1.42%; separating gel, T, 17.6% and C, 0.45%. The runs were carried out in a Multiphor (LKB Instruments Ltd., U.K.) apparatus on gels of 0.5 mm thickness at 50 mA for 1 hr 20 min.

Isoelectric focusing in urea-containing gels was performed on thin (0.7 mm) polyacrylamide gels at 4% total acrylamide. Final concentration of the carrier ampholyte in the gel was 2.5% (v/v) in a ratio of 1.5:1.0 of pH 3–10 and 4–6 Ampholines (LKB Instrument Ltd., U.K.) in 8 M deionized urea. The samples were dissolved in 9.5 M urea and applied by paperwicks on the gel. Runs were performed initially at 0.07 W and the power was increased gradually to 0.18 W during 3 hr 45 min. In the runs W × min varied between 27.2 and 29.8 with prefocusing for 5.7–5.8 W × min before sample application.

Sedimentation velocity runs on the isolated subunits were done in a Spinco Model E analytical ultracentrifuge. The polypeptides were dissolved in 5 M guanidine HCl to give concentrations between 10 and 12 mg/ml and these solutions were run in an An-D rotor, at 59 780 rev/min and 20°. Calculations of the results were made with a computer program as described before [20].

Amino acid analyses were carried out using a Rank-Hilger Chromaspek analyser on samples hydrolysed with constant boiling HCl under $N_2$ at 105° for 24, 48 and 72 hr. Tryptophan was estimated using a Locarte amino acid analyser on samples hydrolysed *in vacuo* with 4 N methane sulphonic acid containing 0.2% (w/v) 3-(2-aminoethyl)-indole at 115° for 22 hr [21].

*Digestion of the β-subunit with α-chymotrypsin.* A sample of 1.7 mg of the polypeptide was dissolved in 0.8 ml 0.2 M *N*-ethyl morpholine-acetic acid buffer, pH 8.5, and digested with chymotrypsin (2% w/w enzyme–substrate ratio; Type $A_4$, Boehringer-Mannheim GmbH) for 3 hr at 37°. After freeze-drying, the digest was dissolved in 0.1 ml 0.1% trifluoroacetic acid (TFA) and subjected to reverse phase HPLC on a μ-Bondapak C-18 column (0.5 × 25 cm, HPLC Technology Ltd.) in a Varian Model 5000 HPLC with a linear gradient of 0–70% MeCN (HPLC grade S, Rathburn Chemical Co.) in 0.1% TFA as described previously [3]. Peptides were detected by measuring the absorbance of the eluted fractions at 214 nm.

*Digestion of the γ-subunit with trypsin.* A sample (3.8 mg) was dissolved in 2 ml 0.05 M $NH_4HCO_3$ and digested with trypsin (2% w/w enzyme–substrate ratio; trypsin treated with L-(1-tosylamide-2-phenyl)-ethyl chloromethyl ketone (Worthington Biochem. Corp.) for 4 hr at 37°. After freeze-drying, the peptides in the digest were dissolved in a minimum vol. of 6 M guanidine HCl in 0.1% TFA and separated by reverse phase HPLC as described above.

Peptides and the intact subunits were subjected to micro-sequence analysis by the manual DABITC–phenylisothiocyanate double coupling method followed by the identification by TLC of the amino acid derivatives liberated [22]. The qualitative amino acid composition of the peptides was determined by TLC of the dansylated amino acids on polyamide sheets [22]. The C-terminal residues of the subunits and of some of the peptides were determined by the use of carboxypeptidase A (type II, treated with diisopropylfluorophosphate, Sigma Chemical Co.) in 0.2 M *N*-ethylmorpholine acetic acid buffer, pH 8.5, in combination with the dansyl technique as described previously [23].

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