The Purification, Properties, and Localization of an Abundant Legume Seed Lectin Cross-Reactive Material from Spartium junceum

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ABSTRACT

The seeds of Spartium junceum contained a large quantity of lectin-like protein that did not appear to be either a hemagglutinin or active lectin. The cross-reactive material (CRM), like most legume seed lectins, was a tetrameric glycoprotein of about 130,000 M. The single-sized subunits of about 33,000 M, were not covalently associated. The amino acid composition was typical of legume lectins and was rich in hydroxy-amino acids and poor in sulfur-containing amino acids. The Spartium CRM contained about 3.5% covalently associated carbohydrate, most likely of the high-mannose type, since the CRM was precipitated by concanavalin A. The CRM was localized by electron-microscopic immunocytochemistry and found to be exclusively in protein-filled vacuoles (protein bodies). Because this protein was so similar immunologically, structurally, and in its physiology, to classic legume seed lectins, it is most likely a lectin homolog. Similar seed lectin CRMs appear to be both common and widespread in the Leguminosae.

Although the seeds of many legume species contain large quantities of lectin, there appears to be an equal number of legume species that are devoid of lectin activity (15, 17). These seed lectin “negative” legume species, however, in many cases, contain lectin-like proteins—that is, proteins that are immunologically cross-reactive with legume lectins.

Even though seed lectin CRMs are very widely distributed within the legume family and often appear to be abundant seed proteins, as far as we know, none has been purified and characterized. Whereas legum CRM from other plant tissues such as stems, leaves, and roots, have been studied, these CRMs generally appear to be present in very low amounts, and even though closely related to seed lectins, they often possess significantly different structures. We believe that the low-abundance, apparently non-vacuolar, lectin CRMs, perhaps typified by those from Dolichos (2, 16) and peas (1), are functionally distinct from the high-abundance vacuolar localized lectins that have been found in many legume seeds, as well as in bark (5, 11) and leaves (6). We wondered whether the seed lectin CRMs that we had previously observed were forms of seed lectins or similar to other lectin CRMs that have been described or perhaps if they might be a new class of lectin-related protein. For this reason, we have begun to study these proteins in more detail.

In the study reported here, we employed Western blots to show the presence of lectin CRMs in numerous nonhemagglutinating legume seed extracts and have described the purification, localization, and some properties of a specific lectin CRM from the seeds of the legume Spartium junceum.

MATERIALS AND METHODS

Hemagglutinin assays, enzyme assays, and Ouchterlony double diffusion were as described previously (7, 12, 15). Spartium junceum seeds were from F. W. Schumacher (Sandwich, MA). The quantity of CRM in extracts was estimated by determining the highest serial twofold dilution (titer) that could be made and still detect a precipitin band by Ouchterlony double-diffusion using a standard antiserum.

Seed flour (200 g) was prepared with a Wiley mill and then extracted overnight at 4°C with 600 mL of extraction buffer (0.05 M KPO4 [pH 6.8], 0.4 M NaCl, 1 M 2-mercaptoethanol). The suspension was squeezed through two layers of cheesecloth and the residue rinsed with an additional 600 mL of extraction buffer. The crude extract was then centrifuged for 20 min at 10,000 rpm (GSA rotor; Sorvall) after which the pellets were discarded. A 40% (NH4)2SO4 fractionation was made by adding powdered (NH4)2SO4 to 243 g/L over a 1-h period while stirring on ice. The precipitate was removed by centrifugation (as before) and discarded. The supernatant was then made 60% in (NH4)2SO4 by adding another 132 g/L of (NH4)2SO4. After stirring 1 h on ice, the precipitate was collected by centrifugation (as above) and the supernatant discarded. The 40 to 60% (NH4)2SO4 precipitate was suspended and 40 mL of (NH4)2SO4. After stirring 1 h on ice, the precipitate was collected by centrifugation (as above) and the supernatant discarded. The 40 to 60% (NH4)2SO4 precipitate was suspended and dialyzed against pH 5 buffer (0.025 M KPO4 [pH 5.0], 1 M 2-mercaptoethanol, 0.1 M PMSF, 10% [v/v] glycerol). The dialyzed extract was then passed over a 3 × 30 cm column of CM-cellulose (CM-52; Whatman) equilibrated with dialysis buffer. The CRM does not bind to the CM-cellulose column and is present in the early void fractions. The void fractions with highest CRM titer were pooled and passed over a second CM-cellulose column as before. The early void fractions containing significant CRM were pooled.

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2 Present address: Plant Molecular Biology Laboratory, U.S. Department of Agriculture, Agriculture Research Service, Beltsville, MD 20705.
3 Abbreviation: CRM, cross-reactive material.
and dialyzed against pH 8.0 buffer (same as pH 5 buffer except pH 8) and loaded onto a DEAE cellulose column (DE-52; Whatman) that was equilibrated with pH 8 buffer. The 1.5 × 50-cm DEAE column was washed with pH 8 buffer then eluted with a linear increasing gradient of NaCl in pH 8 buffer (750 mL buffer × 750 mL buffer + 1 m NaCl). Fractions containing CRM were examined by SDS-PAGE and the very peak and trailing edge fractions were pooled. The leading edge fractions, although containing considerable CRM, were more highly contaminated compared with those pooled. The DEAE pool (about 100 mL) was concentrated by ultrafiltration to 10 mL using a large-pore membrane (XM-50, Amicon). The concentrated CRM preparation was then chromatographed on a Sephacryl S-200 (Pharmacia) column (1.5 × 60 cm) using pH 8 buffer containing 0.4 M NaCl. The column was run at about 1.5 mL/min and 5-mL fractions were collected. The peak fractions, which appeared nearly pure by SDS-PAGE, were pooled, concentrated by ultrafiltration as before, and then rechromatographed on the S-200 column. Methods as previously described were antisera preparation (7, 12), immunogold cytochemistry (8), and SDS-PAGE Western blotting (5). Amino acid compositions and N-terminal sequence analyses were performed by the Biotechnology Instrumentation Facility in the Department of Biochemistry, University of California-Riverside.

Late maturation *Spartium* seeds were obtained from locally grown plants. The cotyledons were excised from the seeds and fixed and embedded in Lowicryl K4M as described by Herman and Shannon (8). Immunogold labeling with anti-*Spartium* CRM sera and anti-rabbit IgG-colloidal gold (EY Biochemicals, San Mateo, CA) was also as described previously (8). Control immunogold assays were accomplished by substituting preimmune sera for the specific antisera to label parallel grids. The labeled grids were examined and photographed with a Phillips 400 electron microscope.

**RESULTS AND DISCUSSION**

As indicated earlier, the seeds of many legume species contain lectin CRM even though they do not appear to display typical hemagglutinin activities. In a number of cases, we have observed strong immunological cross-reactions by Ouchterlony double-diffusion, suggesting the presence of substantial amounts of CRM. We examined several of these types of legumes by SDS-PAGE and Western blotting as shown in Figure 1. Note that although crude extracts of these legume seeds displayed highly variable and complex polypeptide patterns (Fig. 1A), most of them contained a single major lectin CRM (Fig. 1B), often at a Mr around 30,000. In several cases, there was a CRM band at about twice the size of the major CRM. We have found that many legume lectin subunits are difficult to totally dissociate, partially due to disulfide linkages between subunits, and they frequently showed dimers and even higher aggregates on reducing SDS gels. We suspect some of the extra (large Mr) CRM bands in these extracts to be the result of similar phenomena. The major CRMs in most of these seeds appeared to correspond to specific polypeptides that were visible by Coomassie staining and, thus, were fairly abundant. Note that *S. junceum* seed extracts contained a particularly strong and abundant CRM. It was for this reason that we chose it for further study.

*S. junceum* seeds have been reported both as containing a hemagglutinin (17) and as lacking any hemagglutinin activity (17). In an earlier survey (15), we found that fresh concentrated crude seed extracts possessed an extremely weak hemagglutinin activity using trypsinized rabbit or human type “O” erythrocytes. This activity, if in fact due to a lectin, was very fleeting and disappeared from the extract within 1 h on ice. We have not observed any hemagglutinin activity in *Spartium* extracts at any other stage of CRM purification. Hemagglutinin assays were done at medium and high ionic strength, at pH 5, 7, and 9, and with or without metal ions. Crude extracts as well as samples at several stages of purification were also dialyzed overnight against various buffers (both with and without 1 and 10 mM DTT and 10 and 50 mM 2-mercaptoethanol) and then examined for activity. Extracts were tested using either trypsinized or normal erythrocytes from rabbits and humans (A, B, and O types). If *Spartium* seeds contain a hemagglutinin, it is either extremely labile or possesses a specificity quite distinct from other known legume seed lectins.

Cruite *Spartium* extracts gave strong precipitin lines on
Ouchterlony double diffusion (Fig. 2) when challenged with antisera against several legume seed lectins. The extracts did not react with control sera or with sera made against wheat germ agglutinin or ricin agglutinin. The reaction with antisera to Bauhinia lectin was particularly strong, and therefore we used anti-Bauhinia sera to assay for the Spartium lectin CRM, either by Ouchterlony double diffusion, or on Western blots.

Table I summarizes the CRM purification described in “Materials and Methods” and shows that a substantial amount of CRM can be obtained from Spartium seeds even though the purification is conventional and considerable CRM is sacrificed along the way. We estimate that about 5 to 10% of the extractable protein in Spartium seeds is the lectin CRM and thus it is present in amounts comparable with those seen for most legume seed lectins.

Figure 3A shows SDS-PAGE patterns of crude and purified CRM extracts. As can be seen, the material obtained from the final step in the purification is essentially homogeneous.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Residues/Subunit</th>
<th>CRM</th>
<th>Arcin*</th>
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<tbody>
<tr>
<td>Asx</td>
<td>44</td>
<td>15.7</td>
<td>14.9</td>
</tr>
<tr>
<td>G1x</td>
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</tr>
<tr>
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<td>4.7</td>
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<tr>
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<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Cys</td>
<td>ND*</td>
<td>ND</td>
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</tr>
<tr>
<td>lle</td>
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<td>4.6</td>
<td>4.7</td>
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<tr>
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<tr>
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</table>

* Data from (13). ^ Analysis indicates about one residue but peptide is not fragmented by cyanogen bromide treatment. ^ Not determined.
and appeared to contain a single sized subunit of about \( M_r \)
33,500. From Figure 3A it is clear that the CRM subunit was
one of the major Coomassie brilliant blue staining polypep
ptides in seeds, and Figure 3B shows that it was also a major
glycoprotein. Figure 3C depicts a Western blot equivalent of
3A and 3B and shows that the CRM subunit was the major
CRM (to Bauhinia lectin) present.

When chromatographed on calibrated gel filtration col
ums (Sephacryl S-200 or S-300; Pharmacia), the Spartium
seed lectin CRM gave a single symmetrical peak of protein at
a position corresponding to a native mol wt of about 135,000
(data not shown). Therefore, the CRM, like most legume
lectins, appeared to have a tetrameric structure.

The purified CRM possessed an amino acid composition
(Table II) that is very typical of legume lectins, that is, high
in hydroxy and low in sulfur-containing amino acids. It is
possibly devoid of methionine since, in addition to very low
methionine in the analysis, the protein is not cleaved by
treatment with cyanogen bromide. Interestingly, the compo
sition of the CRM was remarkably similar to that of arcelin,
the insecticidal lectin from certain wild beans (13) (whose
composition we have included in Table II for comparison).
Whether or not Spartium CRM is actually more closely
related to arcelin than to other legume lectins must await
further study. Spartium CRM is about 3.5% carbohydrate as
determined by microquantitative phenol-H\( \text{H}_2\text{SO}_4 \) assay (6).
Additionally, the carbohydrate may be of the high mannos
type since the CRM was precipitated by Con A in agar doubl
diffusion tests.

As expected, in addition to being related to Bauhinia lectin,
the CRM was related to other legume lectins as seen in Figure
2. Major immunological cross-reactions were seen on double
diffusion with antiserum against Bauhinia, Ulex, and Lotus
lectins. Weaker cross-reactions were seen with several other
legume lectin antiserum (not shown). All sera that cross-reacted
on double diffusion also gave positive and specific reactions
on Western blots. Also, antiserum prepared against the pure
Spartium CRM cross-reacted with several legume lectins (Fig.

Figure 4. A, An indirect immunogold labeling experiment on late maturation Spartium storage parenchyma cells is shown. The protein storage vacuoles (PSV) are densely labeled with colloid gold particles. Other subcellular organelles including the nucleus (N) and oil bodies (OB) are sparingly labeled with nonspecific gold particles. Magnification \( \times \)12,000; bar = 1 \( \mu \)m. B, A parallel control labeling experiment using preimmune serum. Note that only sparse immunogold labeling is present on the PSVs in the control assay. \( \times \)12,000.
including those from *Griffonia* (BS II), *Lotus*, *Phaseolus*, *Sophora*, *Ulex*, and *Bauhinia*. Immunologically, the CRM appeared to be about as related to the galactose-specific legume lectins as many of them were to each other.

We wondered if the CRM might display carbohydrate binding properties, even though it appeared to lack a hemagglutinin activity, and therefore tested a number of immobilized carbohydrates for their ability to adsorb the CRM. The matrices tested were: Sephadex G-50, Sepharose 4b and Sepharose 4b-linked galactose, glucose, mannos, fucose, melibiose, N-acetyl galactosamine, and N-acetyl glucosamine. All of these affinity matrices were prepared as before (6) and tested for their ability to adsorb lectins with appropriate specificity. Fetuin-Sepharose (Sigma) was also tested. We did not observe any interaction between the CRM and any of these matrices. Columns were tested at several pHs, in buffers of low and high ionic strength, and in the presence of metals (Ca\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\)). Although it is possible that the CRM possesses a carbohydrate binding activity, it clearly did not have one that was comparable with any of the well-characterized legume lectins. Additionally, the purified CRM was tested for several enzyme activities including \(\alpha\)-galactosidase, \(\beta\)-galactosidase, \(\alpha\)-mannosidase, \(\beta\)-hexosaminidase, \(\beta\)-fucosidase, and \(\beta\)-glucosidase, but none was found.

The classic seed lectins of legume seeds have been demonstrated to be accumulated in the protein storage vacuoles or protein bodies of the storage parenchyma cells (3). Nonseed legume lectins have been reported to be localized in either the cell wall (4, 14) or in vacuoles of roots (19) and in protein storage vacuoles of leaves and bark (9). The intracellular distribution of lectin-like proteins arcelin and amyase inhibitor has not been determined although it is likely that these will also prove to be vacuolar proteins. To determine the localization of the *Spartium* CRM, thin sections of lowicryl-embedded late maturation seed cotyledons were indirectly labeled with anti-*Spartium* CRM and goat anti-rabbit colloidal golds. This experiment resulted in intense labeling of the protein storage vacuoles (Fig. 4A). The label was consistently observed to be homogeneously distributed, indicating that there are no apparent region differentiations of the *Spartium* protein storage vacuole. Parallel control grids incubated in preimmune serum resulted in sparse nonspecific labeling of the protein storage vacuoles (Fig. 4B), thereby demonstrating the specificity of the immunocytochemical label. Therefore, the *Spartium* CRM, like the classic legume seed lectins, is accumulated in the vacuole.

The *Spartium* CRM appears to have a blocked N-terminus since multiple attempts at automatic Edman degradation failed to yield a sequence and no amino acid sequence data are yet available. However, it possesses so many similarities to the legume seed lectins that if it is not a homolog from that class of seed protein, it is most certainly closely related evolutionarily. If one assumes that many of the hemagglutinin "negative" legume species like those shown above contain CRMs similar to that in *Spartium*, then this type of lectin CRM is both very abundant and very common. It is also possible that some of these seed lectin CRMs are homologous to the lectin-like protein from *Phaseolus vulgaris* (10), although the lectin-like protein is present together with a seed lectin (phytohemagglutinin) and appears to be a monomeric protein (18). Likewise, it is possible that some of these CRMs could be homologous to arcelin, a lectin that confers resistance to beetles in certain wild bean accessions. Arcelin appears to exist primarily as a dimeric protein, however, and is thus different from *Spartium* CRM in this structural feature. Whether the abundant seed lectin CRMs are homologous to seed lectins, lectin-like proteins, arcelin, or represent yet another evolutionary class (or classes) of lectin-related proteins, must await further study.

If these lectin CRMs are naturally devoid of carbohydrate binding properties, then one might ask which is evolutionarily older, the form with a carbohydrate binding site or that without one. This is a reasonable question because it bears on both the function and evolution of lectins and should be answerable once a sufficient base of protein and gene structural data is available.

If the lectin CRMs are actually "active" and we simply have yet to find their activity, then for this reason alone they deserve further study. Clearly, if they are carbohydrate binding proteins, then they possess binding specificities or characteristics that are different from known legume lectins and thus may have very unique and perhaps useful properties.

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**LITERATURE CITED**

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