

## Lectin in the bark of *Sophora japonica* L<sup>\*1</sup>

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### Introduction

Lectins are defined as non-immune (glyco)proteins which bind carbohydrate(s) to agglutinate cells and/or to precipitate glyco-conjugates<sup>1)</sup>. This definition implies that lectins are multivalent, that is, they possess at least two sugar binding sites. Because of their hemagglutinating activities and sugar binding properties, lectins are one of the very useful tools to determine the blood group, to detect sugar residue, to identify the kind of carbohydrate, and to isolate glyco-conjugates. Hence many lectins have been purified and characterized<sup>2)</sup>, and some of them are now utilized as such reagents. Lectins have been found in various tissues and organs of higher plants<sup>3,4)</sup>.

Despite much information on purified plant lectins, their physiological roles in the plant body are still unclear. Within last 40 years, a number of hypotheses for the

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physiological roles of lectins were proposed<sup>4)</sup>; such as the involvements in defense mechanism of the plant, cell recognition, specific attractants for rhizobial symbiosis, cell wall elongation, carbohydrate catcher, and storage protein itself. Defense mechanism is mainly based on the interactions between several lectins and pathogens. For example, wheatgerm agglutinin, which has a specificity for chitin oligomers<sup>5, 6)</sup>, binds to chitin-containing cell walls of hyphal tips, septa and young spore of the fungi<sup>7, 8)</sup>, and inhibits the growth and spore germination<sup>7)</sup>. Similar effects were also found in the lectins of potato<sup>9)</sup>, peanut and soybean<sup>10, 11)</sup>. The symbiosis hypothesis is based upon the interaction between bacteria of the genus *Rhizobium* and leguminous plants. Importance of this idea was revived by Hamblin and Kent<sup>12)</sup>, who reported the agglutination of *Rhizobium phaseoli* by seed extracts of *Phaseolus vulgaris*, a plant nodulated by this bacterium. They found that erythrocytes bound to the roots of this plant and could be agglutinated by the root extracts. Evidence on the possible involvement of lectins in cell wall elongation was reported by Roberts and Etzler<sup>13)</sup>. They showed an association of the highest levels of the lectin (CRM) with the most rapidly growing internodes within 19-day-old plants of *Dolichos biflorus*. A significant level of this lectin appears to be associated with the cell wall<sup>14)</sup>. "Carbohydrate catcher" means the aid of lectins in the transport of carbohydrates and their immobilization in the seeds. These hypotheses are based on the results with materials different from each other. Lectins have been found in the whole biological world, and even in a plant body, found in several tissues. Because the biochemical and physico-chemical properties of lectins are different among the sources, the physiological role(s) should be elucidated distinctly in each source material.

Lectin in tree stems was first reported by Krüpe and Ensgraber<sup>15)</sup> as having hemagglutinating activity in crude extracts from *Laburnum alpinum*, *Cytisus praecox*, *Sophora japonica*, *Robinia pseudoacacia* and *Evonymus europaea*, and has since been confirmed in many other tree species. Giel et al.<sup>16)</sup> surveyed hemagglutinating activity in 53 dicotyledonous tree species. Some of these tree-stem lectins have been purified and characterized<sup>16-23)</sup>. In *Sambucus nigra* and *Robinia pseudoacacia*, the bark lectins were reported to fluctuate annually, their amounts increasing in autumn and decreasing in spring<sup>24)</sup>.

The bark lectin of *Sophora japonica* accounts for about 30% of the total soluble protein<sup>22, 23)</sup> and is the most abundant bark lectin thus far characterized. It is a tetramer constructed of about 30-35 kDa subunits, and has binding specificity for galactose derivatives<sup>22)</sup>. The bark lectin differs from the lectins of seeds<sup>25)</sup> and leaves in *Sophora japonica*<sup>26)</sup>, with regard to molecular size and subunit composition, indicating that the lectins are coded by distinct genes<sup>22, 26)</sup>. This bark lectin was demonstrated to be localized in the vacuole<sup>27)</sup>, but it is unknown which cells contain the lectin,

whether the amounts fluctuate annually, and how they relate to the annual increments of the phloem tissues. It will be important to discuss the physiological role(s) of the lectins in the bark of tree stem. In Chapter 1, the studies were designed to resolve these problems.

The bark lectins are not only scientifically interesting, but also commercially valuable. As described in the first paragraph, some of the lectins are already utilized as reagents. If the bark lectin is capable of being used as those reagents, the bark which is regarded as wasted parts in wood industry will have a commercial value. In Chapter 2, the lectin was purified from the trunk bark, which is more abundant than that of branch in a tree, and characterized. It was examined whether lectin is abundant also in the trunk bark as well as in the branch bark. All of the information on the bark lectin of *Sophora japonica* has so far been obtained from the branch bark<sup>15, 22)</sup>.

#### 1. **Localization and developmental changes in the bark lectin of *Sophora japonica***

There are few studies on the localization of bark lectin. Greenwood et al.<sup>28)</sup> demonstrated that the bark lectin of *Sambucus nigra* was stored in protein bodies, as observed with seed lectins of other species. Herman et al.<sup>27)</sup> demonstrated that lectin in bark cells of *Sophora japonica* was localized in vacuoles. However, the exact deposition of bark lectins in different cell types has yet to be determined. In the tree stem, cells which are living and may contain lectin are cells in cambial zone, sieve tubes, companion cells, and ray and axial parenchyma cells of xylem and phloem. Clarification of the exact localization of lectin in the bark is important for understanding its function in the tree.

Nsimba-Lubaki and Peumans<sup>24)</sup> reported that the bark lectins of *Sambucus nigra* and *Robinia pseudoacacia* have an annual rhythm, their amounts increasing in autumn and decreasing in spring. However, there is no information on other tree species and on the relation of this phenomenon to the development of phloem tissue or to lectin localization. In temperate regions, the cambium of the stem, generally, produces phloem tissue from the beginning of spring to the middle of summer and adds it to the bark from the inside, the new tissue pushing the older outwards. This process is repeated year after year with the result that the older tissues are orderly arranged in annual rings from cambium to outer bark. There are many different patterns of the annual increment of phloem tissue and the formation of annual rings of inner bark<sup>29)</sup>. In addition, because of the tensile stress induced by the enlargement of the stem in circumference, the parenchyma cells in the outer region of the inner bark enlarge their tangential diameter, and divide radially, thus increasing their number; the process is called "dilatation"<sup>29)</sup>. To understand the function of lectin in the phloem tissue, it is important to clarify how biochemical changes are correlated with

these histological changes.

The present study was designed to determine the exact lectin localization in different bark cell types of *Sophora japonica*, and to demonstrate how the lectin distribution and localization is correlated with the development of the phloem tissue. The pattern of annual growth and tissue accumulation of the bark were characterized histologically, and determined the changes in the bark lectin during the life of phloem parenchyma.

### 1.1 Lectin localization in the stem

As illustrated in Figure 1.1, the stem of *Sophora japonica* consists of wood (xylem), cambial zone and bark. The bark consists of an inner bark (phloem), a living tissue, and an outer bark, a dead tissue. A cross section of the stem, corresponding to the rectangular area in the diagrammatic illustration, shows that ray parenchyma (*rp*) aligned radially from cambium (*C*) to xylem (*X*) and phloem (*P*). In the phloem, axial elements are aligned as follows. Bands of axial parenchyma (*ap*), bast fibers (*bf*), axial parenchyma (*ap*) and sieve tubes (*s*) are repeated from the cambium to the outer bark. In the xylem of this field of view, most of the axial elements consists of fibers, with some axial parenchyma (*ap*) and vessels (*v*) were also present. Completely differentiated bast fibers, xylem fibers and vessels consist only of cell walls.

Lectin was localized by immunofluorescence microscopy<sup>10)</sup>. A cross section near the cambium is shown in Figure 1.2 at somewhat greater magnification than the field

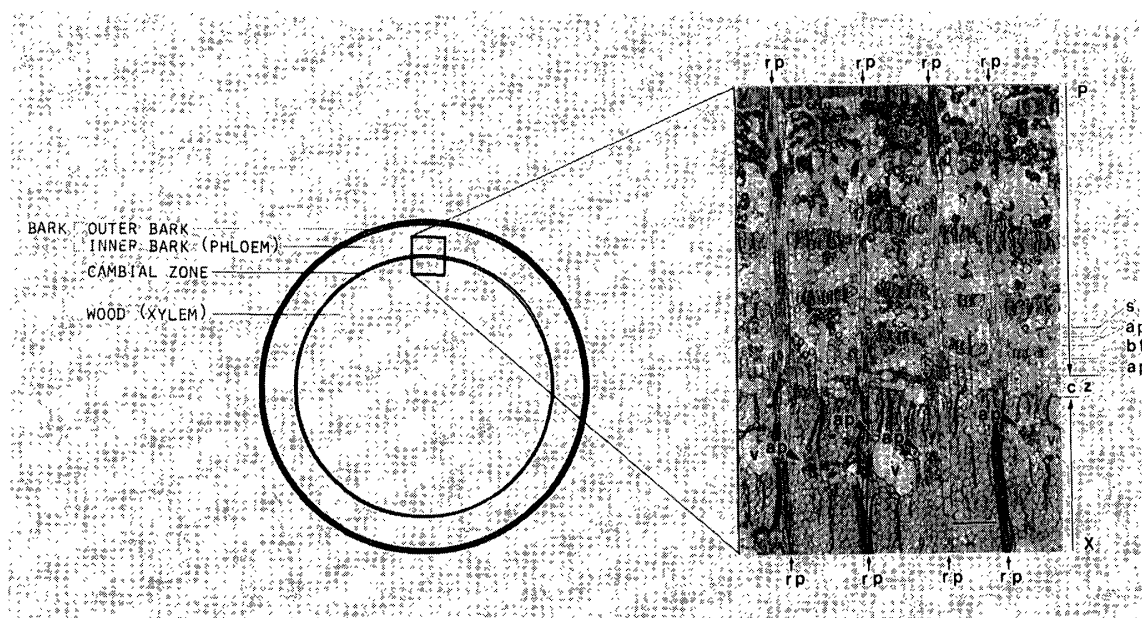


Fig. 1.1 Light microscope image of a cross section of the cambium from a stem of *Sophora japonica*. The section was prepared from a sample harvested from the portion illustrated as a rectangle in the diagram on the left, embedded in Spurr's resin and stained with toluidine blue O. CZ, cambial zone; X, xylem; P, phloem; *rp*, ray parenchyma; *ap*, axial parenchyma; *bf*, best fibers; *s*, sieve tubes. Bar=100  $\mu$ m.

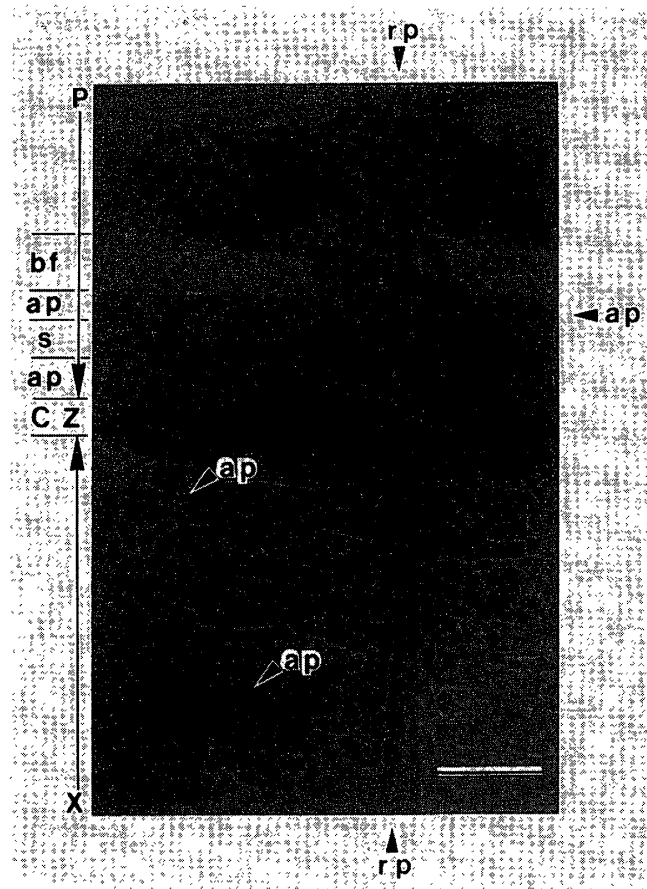


Fig. 1.2 Immunofluorescence localization of lectin in the stem of *Sophora japonica*. Lectin can be detected in ray parenchyma (*rp*) and axial parenchyma (*ap*) of both the phloem and the xylem. *CZ*, cambial zone; *X*, xylem; *P*, phloem; *bf*, bast fibers; *s*, sieve tubes. The sample was harvested in December. Bars=100  $\mu$ m.

shown in Figure 1.1. Lectin was detected as green fluorescence. The cell walls of bast fibers and xylem cells appeared yellow because of autofluorescence of lignin. Lectin was located mainly in both axial and ray phloem parenchyma. No green fluorescence was observed in the cells of cambial zone, the bast fibers, vessels and xylem fibers. The parenchyma cells in the xylem also appeared to contain lectin, but in low intensity of the fluorescence. Even when the area between bast-fiber bands was observed under higher magnification (Fig. 1.3), sieve tubes (*s*) and companion cells (arrowheads) were found to contain no lectin (Fig. 1.3A) although protein was observed by staining with Coomassie brilliant blue and KI/I<sub>2</sub> (Fig. 1.3B). These localization patterns were not changed in any other season (data not shown).

Lectin was found not only in the phloem but also in the xylem<sup>48)</sup>. Xylem lectin of *Sophora japonica* has not been reported so far. In order to determine whether the lectin in the phloem and in the xylem are identical, they were analyzed by immuno-

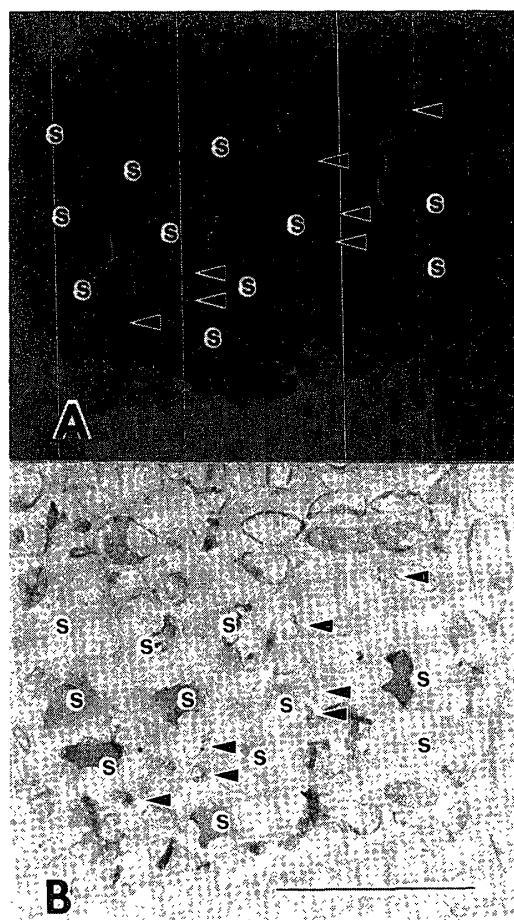


Fig 1.3 A, B Immunofluorescence and Coomassie-blue-staining images of sieve tubes and companion cells in the bark of *Sophora japonica*.  
A: Immunofluorescence localization of lectin; it can not be detected in the sieve tubes (*s*) and companion cells (*arrowheads*).  
B: Protein and starch stained with CBB and KI/I<sub>2</sub> respectively. The sample was harvested in December. Bars=100  $\mu$ m.

blotting (Fig. 1.4). While the phloem lectin showed apparent molecular sizes of 32, 33.5 and 35 kDa, which were consistent with findings in the other report<sup>23)</sup>, the xylem lectin showed a different molecular size of 40 kDa. Comparing the phloem lectin with the xylem lectin on SDS-PAGE, the former was dominant, but the latter was minor in the proteins. These results suggest that the xylem lectin might be a different species from the phloem lectin. Further it have been demonstrated that their intracellular localization. Figures 1.5 and 1.6 represent respectively electron micrographs of the phloem and the xylem ray parenchyma cells. They were quite different from each other with regard to cell content (Figs. 1.5A and 1.6A). The phloem cell had many vacuoles of various sizes of 1–8  $\mu$ m in diameter, containing many electron-dense clumps. Furthermore, the phloem cell was characterized by the presence of many

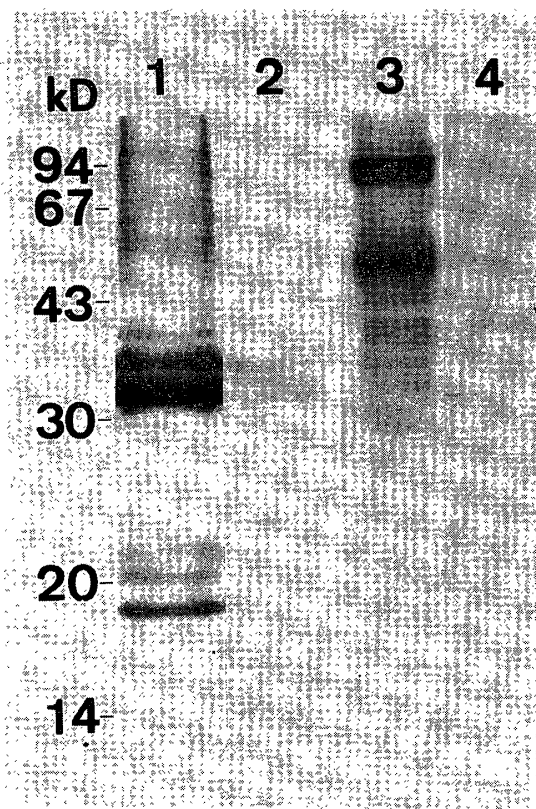


Fig. 1.4 SDS-polyacrylamide gel electrophoresis and immunoblotting of the proteins extracted from the phloem and xylem of *Sophora japonica*. Lanes 1 and 2 are proteins from crude extracts of the phloem. Lanes 3 and 4 are proteins from crude extracts of the xylem. Lanes 1 and 3, CBB staining. Lanes 2 and 4, immunoblotting detected with anti-SJA-antibodies. Numbers on the left represent apparent molecular sizes.

oil droplets and a few small starch grains, approx.  $1.5 \mu\text{m}$  in diameter. On the other hand, the xylem cell was distinguished by vacuoles of similar size, approx.  $3 \mu\text{m}$  in diameter, containing no electron-dense clumps and many large starch grains, approx.  $3 \mu\text{m}$  in diameter. Lectin localization in these two different types of ray parenchyma cells was demonstrated by using immuno-gold staining method. In the phloem cell, gold particles were found only in the electron-dense protein clumps in the vacuole (Fig. 1.5B), while in the xylem cell, the gold particles were also distributed within the vacuoles, except for many gold particles observed on the endoplasmic reticulum in the cytoplasm (Fig. 1.6B).

## 1.2 Histological characterization of the annual growth of the bark tissue of *Sophora japonica*

Annual growth of the bark (the secondary phloem) of *Sophora japonica* was studied histologically. Transverse sections near the cambium of the trunk, collected from March to July, are shown in Figure 1.7. In March, the cambium was still dormant.

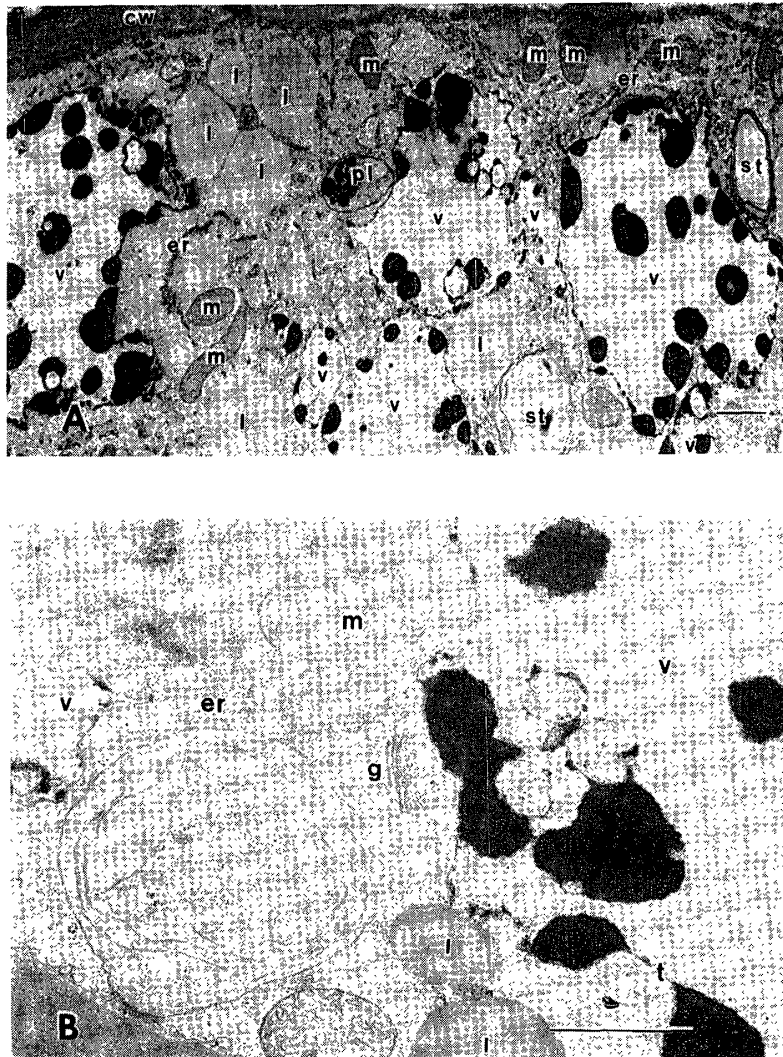


Fig. 1.5 A, B Immuno-gold electron micrographs of a phloem ray parenchyma cell of *Sophora japonica*.

A: An electron micrograph by conventional method.

B: Immuno-gold staining of lectin. Gold particles were observed on electron-dense cluster in vacuole.

*cw*, cell wall; *er*, endoplasmic reticulum; *g*, Golgi apparatus; *l*, lipid or oil droplet; *m*, mitochondrion; *pl*, plastid; *st*, starch; *v*, vacuole. The sample was harvested in December. Bars = 1  $\mu\text{m}$ .

In April, the cell number and radial diameter of the cells in the cambium increased, indicating that the latter had begun to divide. From May onwards the new tissue, derived from the cambium, began to differentiate, and sieve tubes in the tissue of the previous year began to undergo destruction. By June the first bast fiber and of the year was differentiated, and sieve tubes in the old tissue were now completely destroyed. Afterwards, bands of axial parenchyma, sieve tubes, axial parenchyma and bast fibers were repeated, and three or four fiber bands were produced until the end of summer.



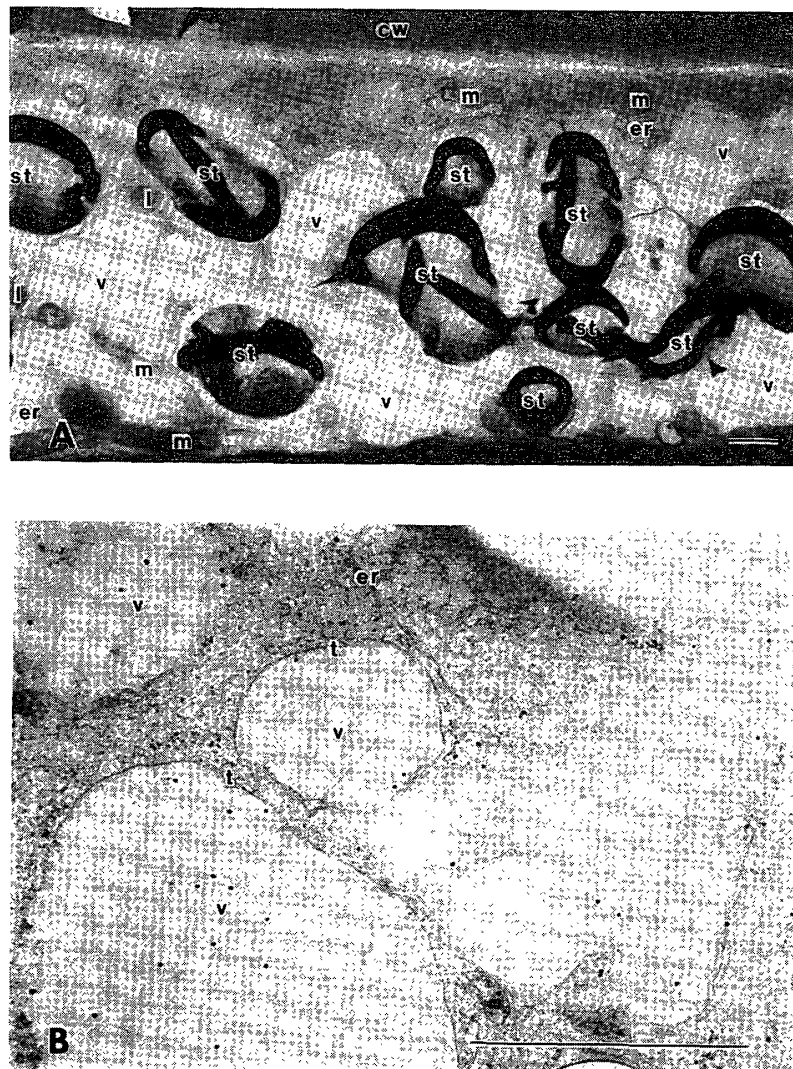


Fig. 1.6 A, B Immuno-gold electron micrographs of a xylem ray parenchyma cell.

A: An electron micrograph by conventional method.

B: Immuno-gold staining of lectin. Gold particles are observed mainly within vacuole.

*cw*, cell wall; *er*, endoplasmic reticulum; *l*, lipid or oil droplet; *m*, mitochondrion; *pl*, plastid; *st*, starch; *v*, vacuole. The sample was harvested in December. Bars=1  $\mu$ m.

Ray parenchyma cells were produced continuously.

The view of a whole bark sample is shown in Figure 1.8. Distortions of the alignment (arrowheads) occurred on each ray parenchyma at almost equal spaces (small arrows). Obviously in the July panel of Figure 1.7, the earliest interval between fiber bands of the year was wider and there were more sieve tubes present in it than in the other later intervals. Hence these distortions, arising because of the

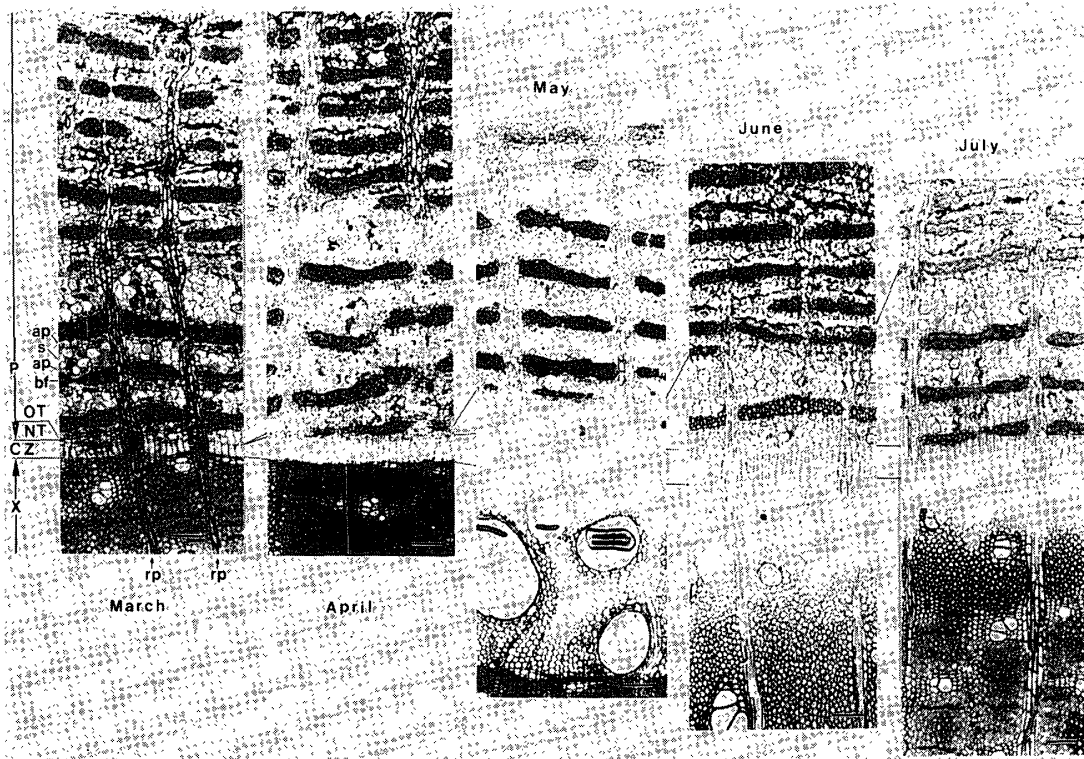


Fig. 1.7 Annual growth of secondary phloem of *Sophora japonica*. These transverse sections were prepared from the samples embedded in Spurr's epoxy resin and stained with toluidine blue O. CZ, cambial zone; P, phloem; X, xylem; NT, new tissue of the current year; OT, old tissue; bf, bast fibers; s, sieve tubes; ap, axial parenchyma; rp, ray parenchyma. Bars=100  $\mu$ m.

destruction of sieve tubes, indicate the early tissue of each year, and the tissue between one distortion and the next indicates that produced in a year. Therefore, this bark (Fig. 1.8) was determined to contain the living tissues formed in the past seven years at least. The tangential diameter of the cells was enlarged at the border of five-year-old and six-year-old tissue (large arrow in Fig. 1.8), indicating that dilatation had been initiated, and the cells positioned exterior to that point were more randomly arranged than those which were interior.

### 1.3 Annual changes in the amount of the bark lectin

The amount of lectin was measured throughout a year on a monthly basis by ELISA (Fig. 1.9). Lectin levels were at a peak in December, and a high level, about 80% of the peak, was maintained until March. The amount of lectin rapidly decreased to about 35% from March to May. It appeared to increase slightly from May to August, with some fluctuation, and then increased rapidly from September to November. The average amount of lectin in spring and summer, from May to August, amounted to about 50% of that in winter, from November to March.

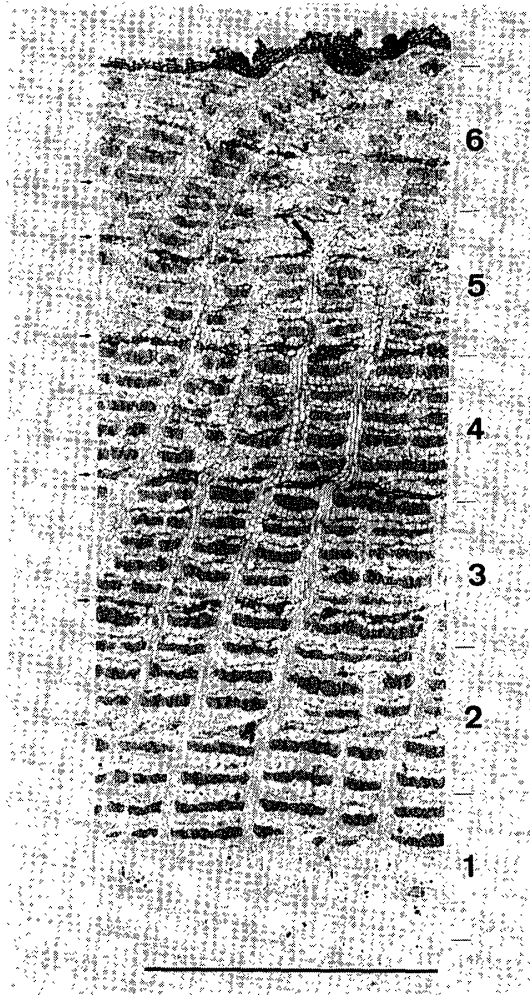


Fig. 1.8 A transverse section through a bark sample from a main stem of *Sophora japonica*.

This bark sample was harvested in June. The bottom end is cambium and the top end is the outer bark. Distortions occurred on the align of the ray parenchyma (*arrowheads*). The *small arrows* on the left indicate the position of the distortions which are the borders of the tissue age. The cells positioned by a *large arrow* increased their radial diameter, indicating the initiation of dilatation. The numbers of 500- $\mu\text{m}$  sections on the right correspond to the sample numbers on immunoblots and ELISA data of Fig. 1.10 Bar=1 mm.

In order to determine whether the changes described above occurred uniformly within the inner bark, bark samples were stripped off in June and February, dissected from the inside to the outside into 500  $\mu\text{m}$  sections, and the proteins in each section were immunoblotted (Fig. 1.10A). The section numbers in Figure 1.10 correspond to those in Figure 1.8. While the amounts did not differ greatly among the February sections (Fig. 1.10A, "F"), the amount of lectin in section 1 of June was markedly less than that in the other sections (Fig. 1.10A, "J").

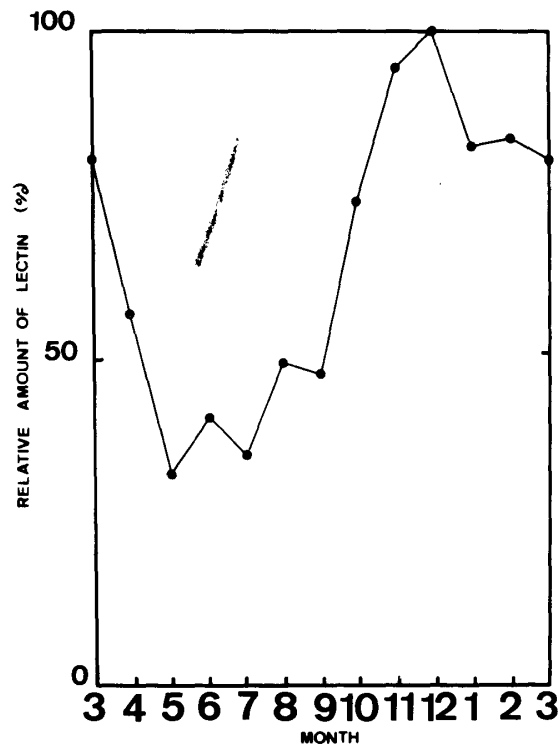


Fig. 1.9 Annual changes in the amount of bark lectin in *Sophora japonica*. The amounts were determined by ELISA, and are shown as percentages of the value in December, at which time lectin is most abundant.

The lectin content, on a fresh-weight basis, of each section was measured by ELISA (Fig. 1.10B), and the relative amounts are shown as the heights of the columns. A notable finding in the June sample, which had never been observed by immunoblotting, was that the lectin level of the outermost section (Fig. 1.10B, "J", No. 6) was markedly lower than that of the middle sections. This difference among sections was not seen in the February sample. As shown by immunoblotting (Fig. 1.10A), section 1 of June had a very low lectin level of only about 1/40–1/60 of that in the other sections. The lectin in the other sections appeared to increase slightly from the inside to the outside, both in June and February. The levels in the middle-aged tissues of June (Figs. 1.8, 1.10 No. 2–5) increased less than those of February, but the difference was only 20–25%. The total amount in June was 50% of that in February, which is in accord with the ELISA results in Figure 1.9.

#### 1.4 Annual changes of lectin localization in phloem ray parenchyma

Lectin was made visible on the radial sections of phloem ray parenchyma by immunofluorescence microscopy<sup>10</sup>. Lectin was recognized as green fluorescence. The newly formed parenchyma cells in June contained no lectin (Fig. 1.11A), but in September lectin was located mainly in the cytoplasmic layer (Fig. 1.11C), and only

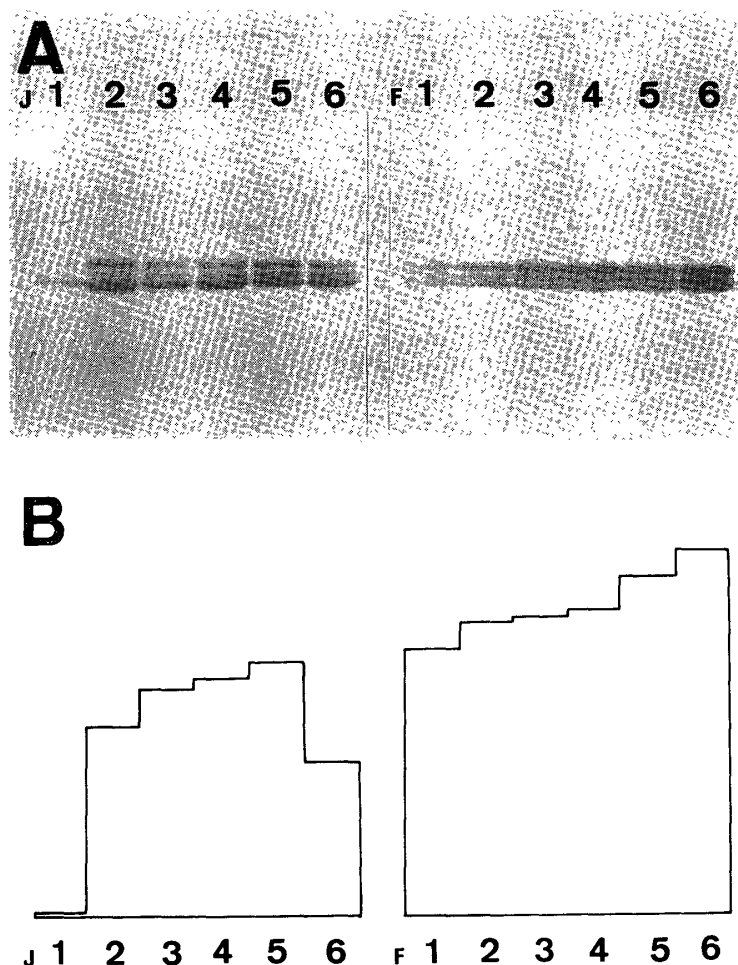


Fig. 1.10 A, B Immunoblotting and ELISA of sectioned bark of *Sophora japonica* in June and February.  
 in June and February.  
 The sample numbers correspond to the tissues of the same numbers in Fig. 1.8.  
 A: Immunoblotting of sectioned bark.  
 B: Relative lectin amount of each section determined by ELISA.  
 J=June; F=February.

in the vacuole as clumps in February (Fig. 1.11E). On the other hand, lectin was located in the vacuoles as clumps throughout all seasons in one-year-old tissue (Fig. 1.11B, D, F). The cytoplasmic layer of some cells in one-year-old tissue contained lectin in September (arrowheads in Fig. 1.11D), although the intensity was much less than in new tissue (Fig. 1.11C). The tissues older than those in September also contained lectin in the cytoplasmic layer (data not shown), but at even lower levels.

As shown in Figure 1.10B, the amount of lectin in the outermost section (No. 6) in June was less than that in the middle sections. In order to investigate the changes in localization patterns, the outermost region (Fig. 1.8, No. 6) was also observed by

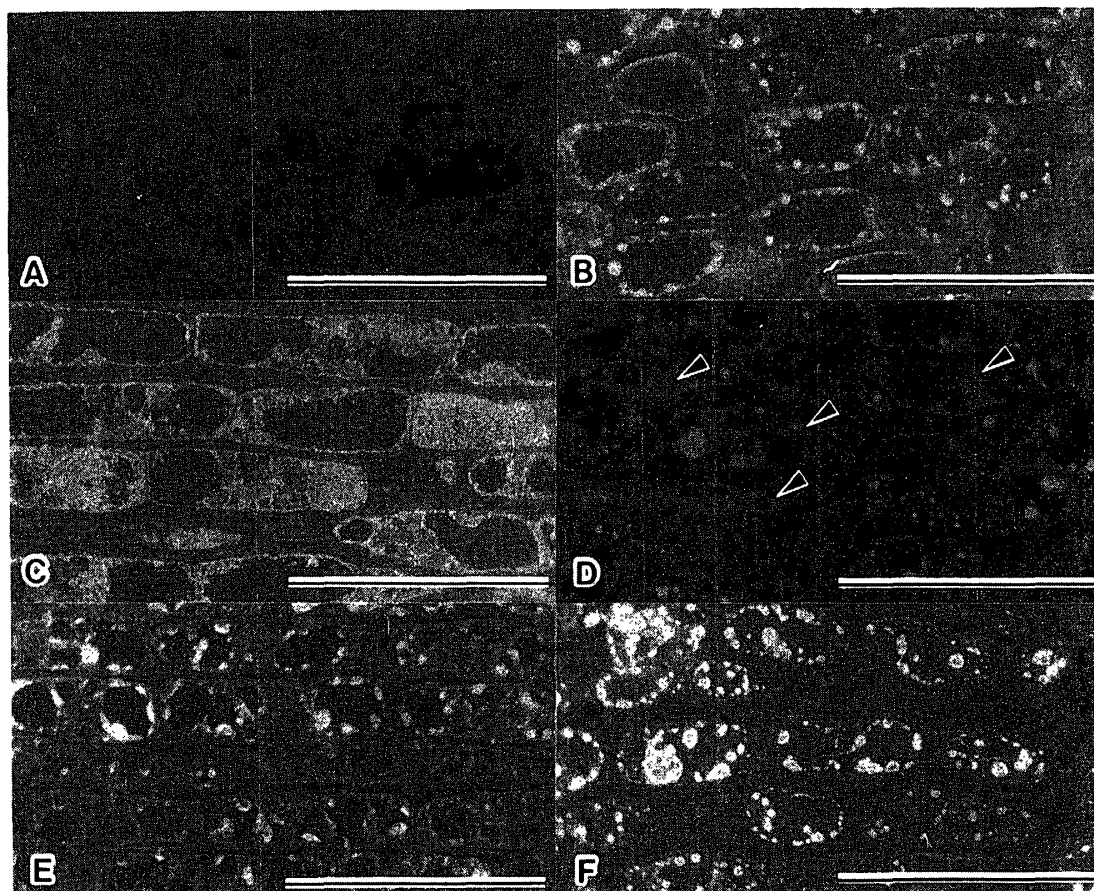


Fig. 1.11 A-F Immunofluorescence micrographs of radial sections of the phloem ray parenchyma of *Sophora japonica*. Lectin is detected as the fluorescence. A, B, harvested in June; C, D, harvested in September; E, F, harvested in February. A, C, E, tissue formed in the current year; B, D, F, tissue formed in previous year. Arrowheads=cells containing lectin in both vacuolar clump and cytoplasmic layer. Bars=100  $\mu$ m.

immunofluorescence microscopy (Fig. 1.12). Although some cells gave a reaction, most contained no lectin (arrowheads in Fig. 12). Especially important is the fact that the cells which bordered the outer bark did not contain any lectin (arrow in Fig. 1.12). These cells can be regarded as cork cambium or phellodermal cells.

In order to localize lectin within the cytoplasmic layer in September, ray parenchyma cells in the new tissue were observed by immuno-gold electron microscopy (Fig. 1.13). Gold particles were seen in the ER lumen (arrows in Fig. 1.13), the vesicles (arrowheads in Fig. 1.13), and the vacuoles, but hardly observed in the cytoplasmic matrix. The particle density in the cytoplasmic layer was significantly higher than in the two negative controls, although the value was about one-third of that in the electron-dense clumps in the vacuoles (Table 1.1). Hence the gold particles in the cytoplasmic layer indicate lectin localization, and that the amount

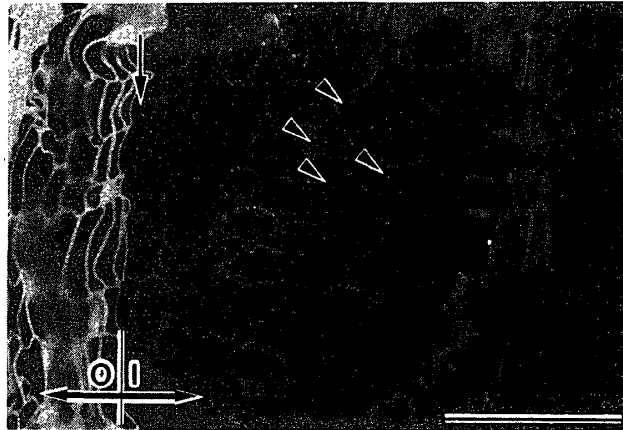


Fig. 1.12 Immunofluorescence micrograph of the radial section of the outermost region of living bark of *Sophora japonica*.  
*I*, inner bark (living bark); *O*, outer bark; *arrowheads*, cells containing no lectin; *arrow*, cells bordering on outer bark. Bar=100  $\mu$ m.

Table 1.1 Densities of gold particles on the phloem ray parenchyma cells in new bark tissue of *Sophora japonica* embedded in hydrophilic methacrylate.

	No. gold particles $\text{cm}^{-2}$ (mean $\pm$ S)
Control 1	0.21 $\pm$ 0.06
Control 2	0.046 $\pm$ 0.018
Immuno-gold	
V	7.2 $\pm$ 2.2
C	2.5 $\pm$ 0.5

Control 1=treatment with only gold-labeled protein A, no antibody treatment. Control 2=Treatment with preimmune rabbit serum instead of the antibody. Immuno-gold=immunogold electron microscopy; V=the electron-dense clumps in the vacuole; C=cytoplasmic layer. Each value was calculated by using ten micrographs of approx. 50  $\text{cm}^2$ .

in cytoplasmic layer is lower than that in the vacuoles. These results indicate that the lectin is located in the endomembrane system such as ER and vesicles in autumn and is evidently transported into the vacuole before winter.

### 1.5 The deposition route of the bark lectin

Lectin was localized in the ER lumen in autumn, and in the vacuole in winter. The route from the ER to the vacuole was studied by using immuno- and conventional electron microscopy. On the sections prepared from  $\text{H}_2\text{O}_2$ -etched-sample harvested in September, gold particles, indicating lectin localization, were observed only in the electron-dense clumps of the vacuole (Fig. 1.14). The gold particles were not ob-

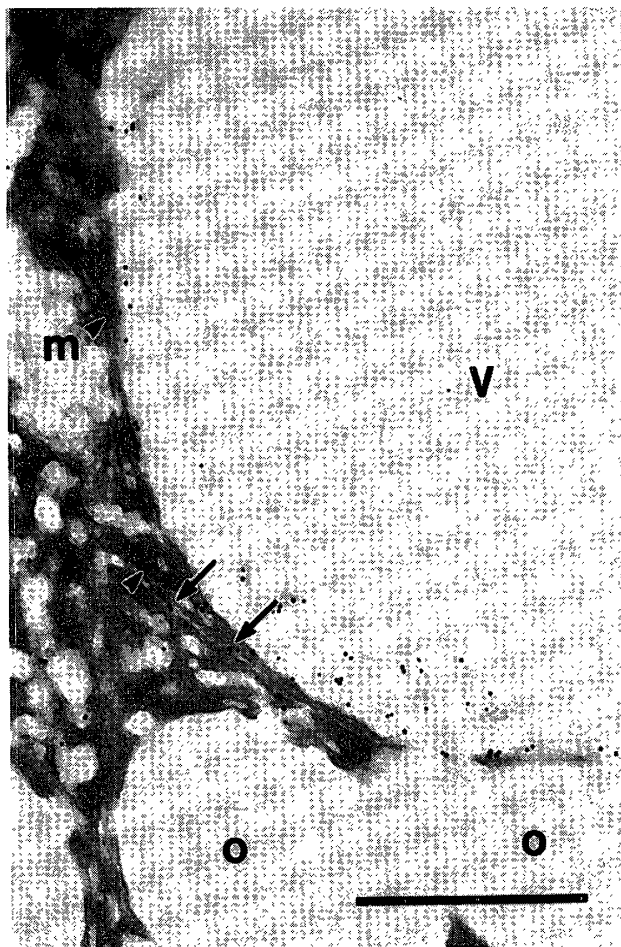


Fig. 1.13 Immuno-gold electron micrograph of the section of phloem ray parenchyma of *Sophora japonica* in September. Gold particles are seen on materials in vacuole, ER (arrows), and vesicles (arrowheads). v, vacuole; m, mitochondrion; o, probably trace of oil droplet extracted because it is not fixed by osmium. Bar=1  $\mu$ m.

served on any other organelle. However, Golgi apparatus was often seen at just back of the electron-dense clumps (Fig. 1.14). Moreover, the tonoplast between the Golgi cisternae and the clump formed several small invagination (arrows in Fig. 1.14B), and the electron-dense material was observed between the invagination and the clumps (arrowhead in Fig. 1.14B). On the sections prepared from methacrylate sample gold particles were observed in the lumen of the Golgi apparatus (arrows in Fig. 1.15) and its vesicles (arrowheads in Fig. 1.15), and of course in the vacuole. On the other hand, in the observation by the conventional electron microscopy, electron-dense materials were observed in the lumen of the ER-like endomembrane system (Fig. 1.16A), and moreover, it was sometimes observed that the ER-like endomembrane containing the electron-dense materials appeared to fuse to the tonoplast directly



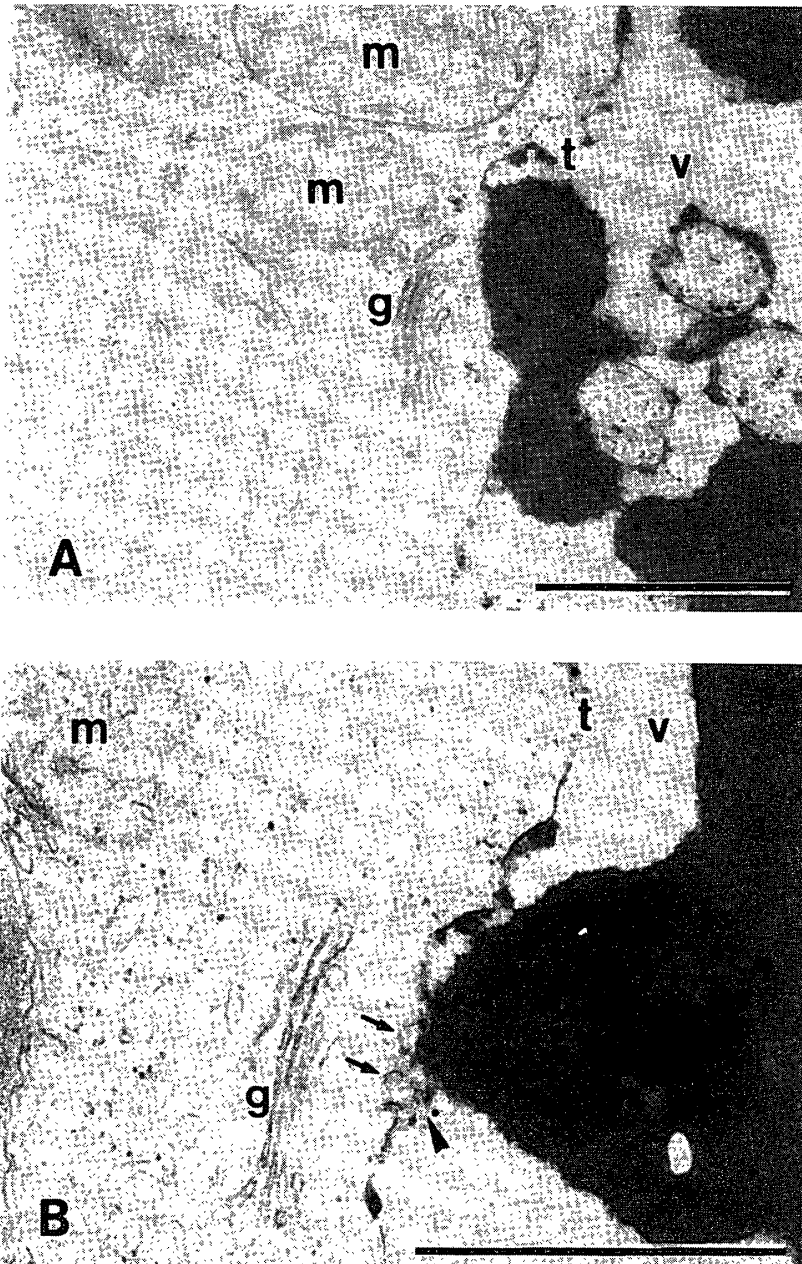


Fig. 1.14 A, B Immuno-gold electron micrographs of the sections of the phloem parenchyma cell of *Sophora japonica* etched by  $H_2O_2$  after embedding into Spurr's epoxy resin.

A: Gold particles are observed on the electron-dense materials in the vacuole, and the Golgi apparatus was observed at just back of the electron-dense clump.

B: The tonoplast between the electron-dense clumps and the Golgi apparatus forms invagination (*arrows*), and electron-dense materials were observed between the invagination and the clumps (*arrowheads*), *g*, Golgi apparatus; *m*, mitochondrion; *t*, tonoplast; *v*, vacuole. The sample was harvested in September. Bars = 1  $\mu$ m.

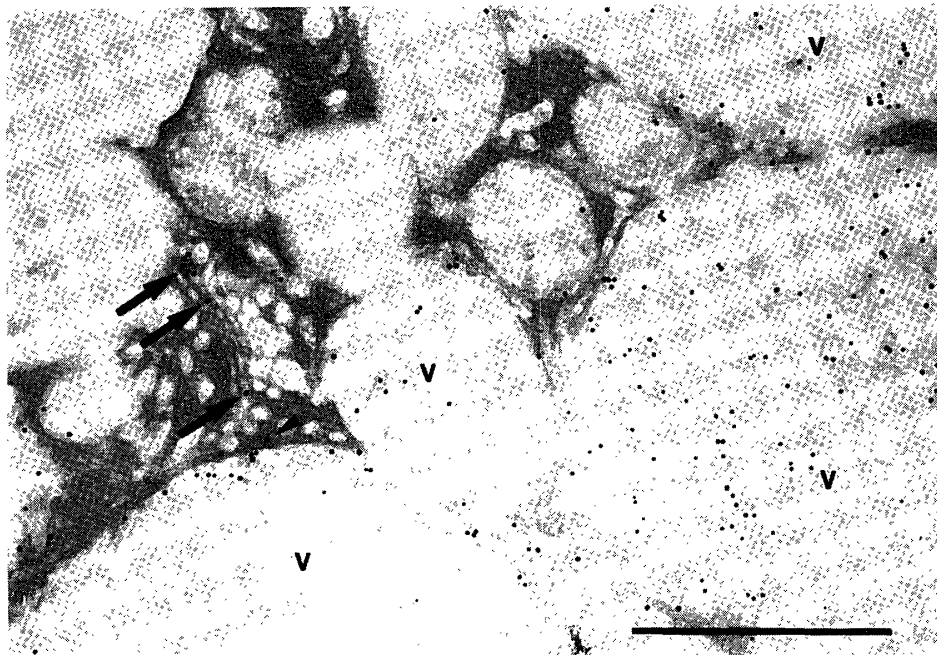


Fig. 1.15 Immuno-gold electron micrographs of the sections of the phloem parenchyma cell of *Sophora japonica* embedded in hydrophilic methacrylate resin. The sample was harvested in September. Gold particles are seen on the materials in vacuole, Golgi apparatus (arrows) and vesicles (arrowhead). v, vacuole. Bars=1  $\mu$ m.

(Fig. 1.16B).

## 1.6 Discussion

The bark lectin of *Sophora japonica* changed in the course of the year relative to the season, with the amount in spring and summer, from May to August, being about 50% of that in winter from November to March (Fig. 1.9). However, this annual change of lectin content did not occur uniformly throughout the bark. Whereas the amount in winter was similar from the inside to the outside of the bark, the inner- and outermost regions contained less lectin than the middle region of the bark in summer (Fig. 1.10B). These changes are mainly explained by proposing that, in spring and summer, new tissue, which contains no lectin, is added to the bark from the inside while the cells in the outermost part degrade lectin, so that the total amount of lectin decreases; in autumn the new tissue synthesizes lectin while the outermost tissue, in which lectin has been exhausted, dies and becomes outer bark, so that the total lectin amount in the inner bark increases. The present immunocytochemical data support this interpretation. In spring and summer, the new tissue contained no lectin (Fig. 1.11C), and lectin completely disappeared in some cells of the outermost region and in all the cells bordering on the outer bark (Fig. 1.12). In autumn lectin was located in the lumen of the ER and vesicles in the cells of the new tissue (Fig. 1.13). The latter ob-

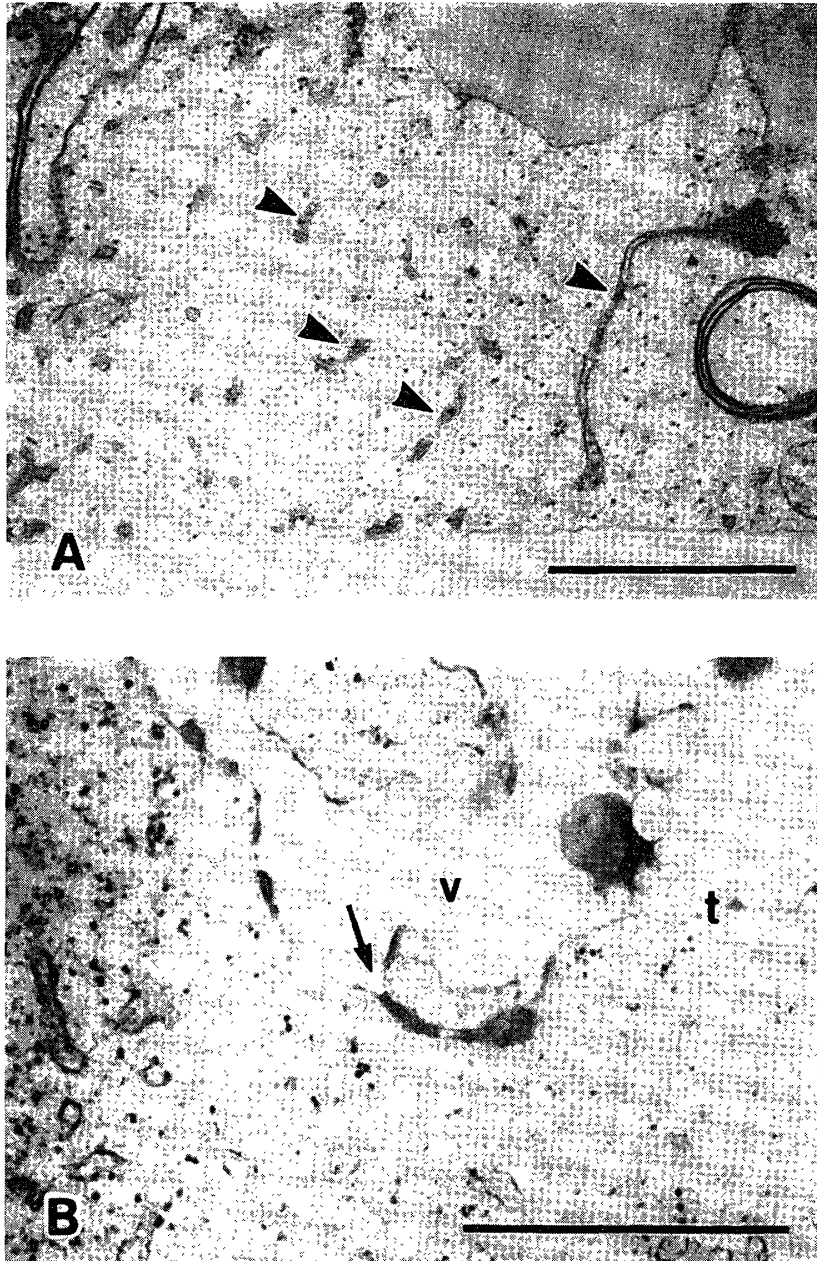


Fig. 1.16 A, B Conventional electron micrographs of the phloem ray parenchyma cells of *Sophora japonica*.

A: The ER-like endomembrane was filled with the electron-dense materials (*arrowheads*).

B: The ER-like endomembrane containing the electron-dense materials appeared to fuse to tonoplast (*arrow*). *t*, tonoplast; *v*, vacuole.

Bars = 1  $\mu$ m.

servation is consistent with the findings for several seed storage proteins in developing cotyledons<sup>30-33</sup>). Biochemical studies have demonstrated that these proteins are synthesized on ER-attached ribosomes and sorted into the ER lumen<sup>34-39</sup>), and it may

be assumed that the bark lectin is also synthesized on ER-attached ribosomes. Because in the middle-aged tissue the cytoplasmic layer of the cells also contained lectin in autumn (Fig. 1.11D), these tissues appear to retain the potential for lectin synthesis, although it is not as high as in the new tissue. This may explain why the amount of lectin increases slightly from the inside to the outside of the bark. The lectin in the middle part in June comprises only 75% of that in February. This may be because tissues of every age mobilize small amounts of lectin, or that samples could not be harvested from exactly the same position.

Concerning with the deposition route of the lectin, the results suggest two route models. The results on the immuno-gold electron microscopy (Figs. 1.14, 15) suggest that the bark lectin may pass through Golgi apparatus. This deposition route is also demonstrated in the other storage proteins in seeds of dicotyledons<sup>37,40,41</sup>. In contrast, the results on the conventional electron microscopy (Fig. 1.16) suggest that ER may be directly involved in the secretion of electron-dense materials to vacuole. It is possible that only some type(s) of lectin molecules or some subunit(s) may be transported through the Golgi and the others directly from the ER to the vacuole. The bark lectin of *Sophora japonica* is tetrameric molecules constructed with three or more types of subunits<sup>22, 23</sup>, and is mixture of the five molecular species which are able to be separated with ion-exchange chromatography<sup>22</sup>. The two-route models, that some type is transported via Golgi apparatus and the other type bypassing the Golgi within the certain protein species, was also proposed on the secretion of  $\alpha$ -amylase in the barley germination<sup>42</sup>.

Lectin was located in both axial and ray parenchyma cells of the phloem of *Sophora japonica*. The other living cells, i.e., cambium, sieve tubes and companion cells, hardly contained any lectin. Therefore, like the annual changes this lectin localization indicates that bark lectin has no direct relation to secondary growth in thickness or to nutrient transport, but is a storage or storage-related protein, as was proposed by Greenwood et al.<sup>28</sup> and Nshimba-Lubaki et al.<sup>21</sup>.

It was further proposed that the lectin is utilized primarily by the phloem-parenchyma cells themselves rather than being transported to the leaves in the next year. The nutrients required by leaves are supplied through the tracheids and/or vessels of the xylem. Of the carbohydrates stored in the stem, starch in the contact cells (the members of ray parenchyma in the xylem which provide the connection between the the living ray system and the vessels) is mobilized prior to that in the other ray cells<sup>43</sup>. In contrast, the bark lectin decreased mostly in the outermost region of the bark, and it appear unlikely that the resulting degradation products can be transported to the xylem, across the cambium. On the other hand, dilatation (see above) in the outer part of the inner bark occurs during the active period of secondary growth<sup>29</sup>, and cork-

cambium differentiates and is active in the same region<sup>44</sup>). The bark lectin may be assumed to be consumed *in situ* in these two processes. Because the amount of bark lectin decreased rapidly from March to May, it may be further assumed that the lectin is involved in these processes mainly at their beginning, when the supply of assimilates from other parts of the tree is not yet sufficient. There remains a possibility that some bark lectin is degraded from spring to summer and the resulting amino acids are transported to the cambium through the ray parenchyma and utilized in cambial growth. The observed loss of lectin in middle-aged tissues in summer, although small, may be the consequence of such a utilization.

## 2. Purification and characterization of the bark lectin of *Sophora japonica*

Some of well-characterized lectins, mainly obtained from the seeds of Leguminosae, are now available as reagents to identify carbohydrates, isolate glycoconjugates and so on. Lectins were also found in the barks of trunks or branches described as in introduction. If such lectins are able to be isolated by simple and economical procedures, inner barks and branches, which are the waste parts of trees, could become more valuable for the wood industry.

The seed lectin of *Sophora japonica* is well-known as SJA (*Sophora japonica* agglutinin)<sup>25, 45, 46</sup>), and have been already utilized as a reagent. Recently, lectin was purified from the branch bark of *Sophora japonica*<sup>22</sup>). But to some extent, the branch bark is anatomically different from the trunk bark. The trunk bark looks promising

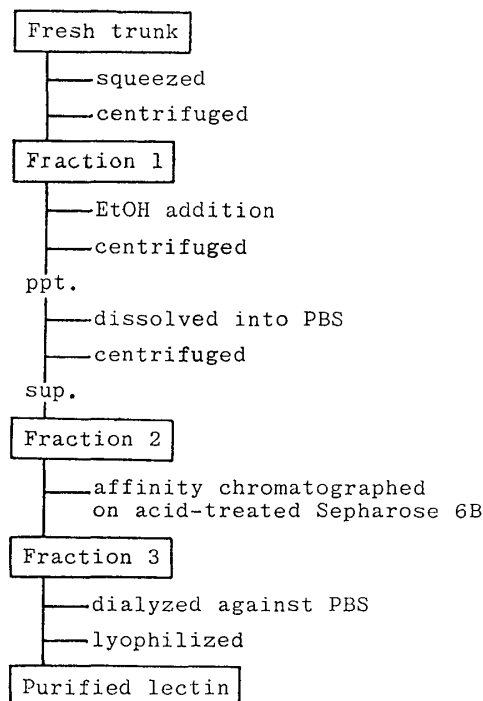


Fig. 2.1 Purification scheme of *Sophora japonica* bark lectin.

as another important lectin and partially characterized and compared it with SJA as a reference.

## 2.1 Purification of the lectin

The trunk bark lectin of *S. japonica* was purified by the following procedures: extraction of crude juice, ethanol precipitation and affinity chromatography (Fig. 2.1). Volume, protein amounts and agglutinating activity were measured at every stages (Table 2.1). The yield was 27% of total protein in the extracts. Comparing with the other tree species, in which lectin ranged from 5 to 10% of the total proteins<sup>16 20 21)</sup>, the trunk bark of *Sophora japonica* abounds in lectin. This result is accord with the report on the branch bark of this species<sup>22)</sup>. They reported that it contains 30% of lectin in its total protein.

Figure 2.2 shows the elution profiles of protein and agglutinating activity on the affinity chromatogram (the final step of purification). Agglutinating activity was

Table 2.1 Purification of the trunk bark lectin of *Sophora japonica*

Fraction	Vol. (ml)	Act. (titer)	Conc. (mg/ml)	Total Act. (titer × ml)	Total Protein (mg)	Specific Activity
1	6.0	2560	3.45	15360	19.4	790
2	15.0	960	0.92	14400	13.8	1040
3	14.5	626	0.37	9090	5.3	1710

Fraction 1, 2 and 3 are crude juice, solution obtained by ethanol precipitation and by affinity chromatography, respectively. Volume (Vol.), hemagglutinating activity (Act.) and protein concentration (Conc.) are experimental data. Total activity (Total Act.), protein content (Total Protein) and Specific Activity are calculated data.

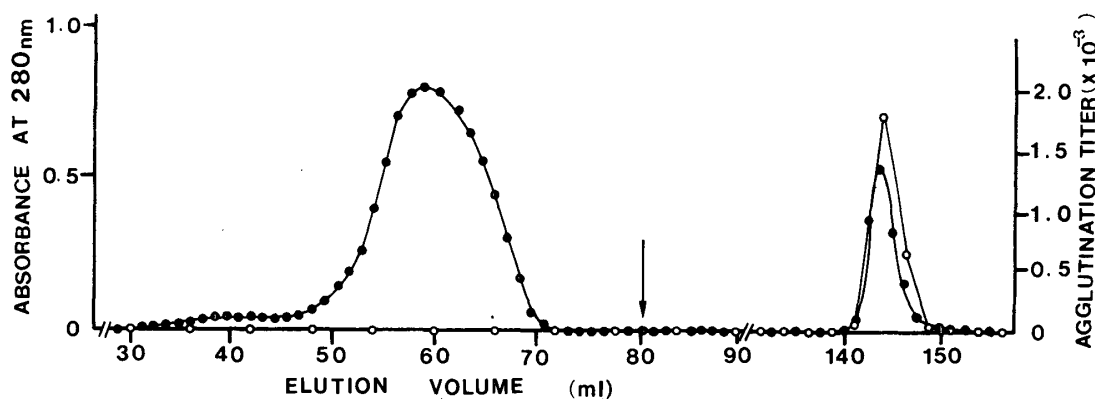


Fig. 2.2 Elution profile of *Sophora japonica* bark lectin on acid-treated Sepharose 6B. At the point indicated by an arrow, 0.2 M galactose was added to the elution solvent (20 mM TBS; pH 8.7). Closed circles show absorbance at 280 nm ( $A_{289}$ ). Open circles show hemagglutinating activity. No activity and absorbance were found in 0–30 and 90–130 ml fractions, so that they were omitted.

detected only in the fraction of second peak of  $A_{280}$ : after galactose was added. This figure indicates that the bark lectin was able to be purified by the method used for the seed lectin of the same species, essentially<sup>45)</sup>. When the crude juice fraction was directly applied to the affinity column, the agglutination activity was detected in both peaks before and after galactose addition. In other words, the lectin in the crude extracts did not fully adsorb to the bed. When the ethanol precipitated fraction was added, the lectin was completely adsorbed to the bed. Instead of ethanol precipitation, gel filtration (Sephadex G-25) led to the same result (the data are not shown). These findings suggested that the crude juice of a *Sophora japonica* bark contains a factor which partially impedes the adsorption of the lectin to the affinity bed. This factor is probably a low molecular material, because it is removed by a gel filtration (data not shown). The impeding factor is possibly related to the physiological roles in the bark.

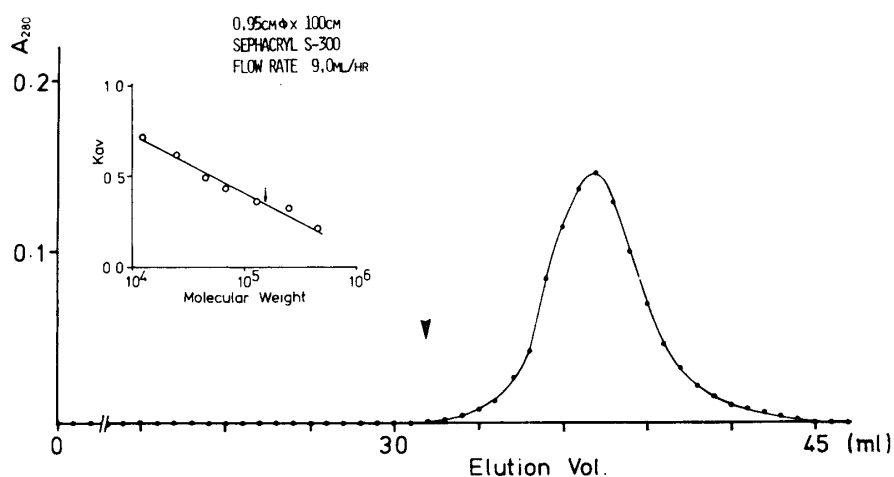


Fig. 2.3 Gel filtration chromatogram of the purified bark lectin of *Sophora japonica* on Sepharcl S-300.

Void volume of this column (0.95 cm  $\times$  100 cm) was found to be 32 ml (arrowhead) by using blue-dextran solution. The calibration of this column was shown. The arrow indicates the molecular weight of the bark lectin.

## 2.2 Characterization of the lectin

The bark lectin was analyzed by gel filtration and polyacrylamide gel electrophoresis (PAGE). The trunk bark lectin showed a single peak on a gel filtration chromatogram, and its molecular weight was  $135000 \pm 5000$  (Fig. 2.3). This result, which was in accordance with ultracentrifugation data<sup>23)</sup>, suggests that this lectin might have a single molecular weight and be similar to that of the seed lectin reported as 132,800<sup>25)</sup>. On the other hand, when analyzed by using native PAGE, the bark lectin showed a different migration pattern from that of the seed lectin both under acidic

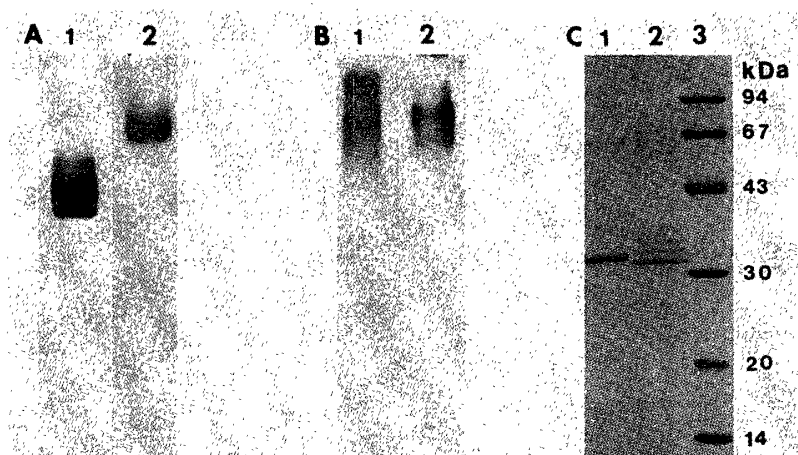


Fig. 2.4 Analysis of bark and seed lectins of *Sophora japonica* by PAGE. A: pH 4.0, native. B: pH 8.9, native. C: SDS-PAGE. Lane 1 is purified seed lectin. Lane 2 is purified trunk bark lectin. Lane 3 is Mr marker.

and basic condition (Fig. 2.4A and B). The bark lectin showed multiple bands under both of the conditions and their patterns were different from those of the seed lectin. These findings suggest that the bark and seed lectins are different in their molecular species. This suggestion was supported by the data of SDS-PAGE and observation of hemagglutination specificity. Where the seed lectin showed a main band of 33 kDa and minor bands of 34.5 and 36 kDa, the bark lectin showed a main band of 32 kDa and minor bands of 33.5 and 35 kDa (Fig. 2.5C). The trunk bark lectin showed a different specificity for blood types from the seed lectin. The seed lectin was specific for types A and B<sup>25,47)</sup>, whereas the trunk lectin equally agglutinated types A, B and O (Table 2.2).

Table 2.2 Hemagglutinating activities of the fractions during purification of the trunk bark lectin of *Sophora japonica*

Fraction	Blood Types		
	A	B	O
1	5120	5120	5120
2	640	640	640
3	640	640	640

Comparing with the seed lectin, the bark lectin of *S. japonica* had a similar molecular weight, different charge variants, different molecular weights of subunits, a broader specificity for the human blood types. These results demonstrate that the bark lectin is different from the seed lectin. The bark lectin showed multiple bands on PAGE under both acidic and basic conditions, while the seed lectin showed three under basic and a single under acidic condition. The peculiar band patterns of the



seed lectin is ascribed to the conformational changes but not to the constructive variation of the subunits<sup>46)</sup>. On the other hand, the reason why the bark lectin shows the multiple band patterns was unknown. Hankins et al.<sup>22)</sup> have just reported five lectins, which are different in their charge, from the lectin of branch bark in *S. japonica*. In the present study, multiple PAGE pattern might be caused by such charge variant show in their report.

### 2.3 Discussion

The trunk bark lectin has one dominant subunit of 32 kDa and two minor subunits of 33.5 kDa and 35 kDa shown by using SDS-PAGE, whereas branch bark lectin was reported to have two major subunits of 30 kDa and 33 kDa and a trace of 30.1 kDa<sup>22)</sup>. Although trunk and branch are apparently similar, being different in their age, they have some anatomical differences. One of the most different characters between their barks is: Branch, or young, living bark consists of cortex, which originates from ground meristem, and phloem, which originates from cambium, while trunk living bark consists of only the latter. Especially, the stem of *Sophora japonica* keeps cortex for a long time, approximately 20–30 mm in diameter. Such anatomical difference between the branch and trunk may cause the difference of their subunits. In this species, different tissues contain different lectins from one another<sup>22,26)</sup>. On the other hand, the trunk bark lectin may vary with geographical population still also remains unsolved. Proetz et al.<sup>25)</sup> discussed that the seed lectin of *Sophora japonica* may vary with geographical population.

It was shown herein that the purification of the trunk bark lectin of *Sophora japonica* is possible by a simple method: ethanol precipitation and affinity chromatography. The affinity bed is also prepared easily. Because the trunk bark and seed lectins are different in their molecular species, the trunk bark lectin may become valuable as another carbohydrate-binding reagent.

### Conclusion

Lectin is the dominant protein in the inner bark of *S. japonica*, and its content in the bark changes during the year, the average amount in summer being about 50% of that in winter.

By immunohistochemistry the lectin was demonstrated to be localized in the ray and axial parenchyma cells. Neither lectin nor other cross reactive materials were observed in the cambium, sieve tubes and companion cells. In xylem another lectin might be present<sup>48)</sup>. The distribution and localization of the lectin changed in relation to the development of the bark tissue. The distribution of lectin in winter was similar throughout the inner bark. In contrast, in summer the innermost region hardly contained any lectin, and the outermost region contained less lectin than the

middle. Lectin localization in tissues and cells differed also depending on tissue age. In new tissue, produced in the current year, lectin was absent in summer, located in the cytoplasmic layer between cell wall and vacuole in autumn, and sequestered in the vacuoles in winter. On the other hand, lectin in old tissue (formed in the previous year) was located mainly within the vacuoles throughout the year, with only very small contents in the cytoplasmic layer in autumn. Within the outermost (oldest) region, in which the lectin content was low in summer, the cells which bordered the outer bark never contained any lectin in summer.

The intracellular localization of the lectin in new tissue in autumn, determined by immuno-gold electron microscopy, was in the lumen of the endoplasmic reticulum (ER) and vesicles and hardly observed in the cytoplasmic matrix. From these findings it is concluded that lectin is synthesized on the ER most vigorously in the new tissue in autumn, and that it is mainly consumed in the outermost bark regions, where dilatation occurs and/or where cork cambium is differentiated.

Further electron microscopical observations indicates that the bark lectin of *S. japonica* is localized in the Golgi apparatus. This result suggested that the lectin is transported through the Golgi apparatus on the way from the ER to the vacuole. On the other hand, it was sometimes observed that ER-like endomembrane contains electron-dense materials by conventional electron microscopy. Moreover, such endomembrane appeared to fuse to the tonoplast. Some of the lectin molecules or subunits may be transported through the Golgi apparatus and the others of them may be transported directly from ER to vacuole in the parenchyma cells of the bark of *S. japonica*<sup>49)</sup>.

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