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LEGUMIN-LIKE PROTEINS IN GYMNOSPERMS

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Key Word Index—*Ginkgo biloba*; Ginkgoaceae; electrophoresis, IEF, serology; legumin-like proteins, storage proteins.

Abstract—Evidence is given for the production of legumin-like proteins in the seeds of *Ginkgo biloba*. The M_r of the heterogeneous protein and of its S–S bonded acidic and basic subunits, the occurrence of two subunit dimer types ('normal' and 'small'), and the electrophoretic characteristics are similar to well known angiosperm legumins. This is the first gymnosperm for which the production of legumin-like proteins has been demonstrated.

INTRODUCTION

In 1827 Braconnot [1] originally termed a crude protein extract from leguminous seeds 'legumin'. What is now called legumin is a well described storage protein, the origin, structure, and function of which has been elaborated during the past 10–15 years. The prototype of legumin has been studied intensively from *Vicia faba* [2, 3] and from other closely related legumes. Reviews of the present stage of knowledge have been given by Wright [4], and Casey and Domoney [5]. In addition to the *Vicia* related leguminous plant taxa, legumin-like proteins have been detected outside the Fabaceae in other families, e.g. Amaranthaceae [6], Brassicaceae [7, 8], Caryophyllaceae [9], Euphorbiaceae [10, 11], Pedaliaceae [12] and Ranunculaceae [13, 14]. Also, the glutelins from some Poaceae species are considered to be homologous proteins [15–18]. The widespread occurrence of legumin-like proteins has been additionally demonstrated by serological cross-reactivity [19], physicochemical characteristics [20], and DNA nucleotide and protein amino acid sequences [21].

These legumin-like proteins are produced and stored in the endosperm, or in the storage tissue of the cotyledons if the endosperm is reduced at the expense of an enlarged embryo. The tissues concerned are either diploid (embryo) or triploid (endosperm) which develop subsequent to pollination and 'double' fertilization.

In contrast to these angiosperm storage tissues, the storage seed tissue of the gymnosperms ('primary endosperm') is haploid and homologous with the megaprothallium of heterosporic ferns. It develops independently, and before fertilization, of the egg cell.

The occurrence of legumin-like proteins in gymnosperms has yet to be clearly shown. Blagrove *et al.* [22] reported that macrozin, the storage protein in seeds of *Macrozamia communis*, is related neither to legumin nor vicilin.

We demonstrate in this paper that the gymnosperm *Ginkgo biloba* produces a legumin-like protein in the haploid tissue of the primary endosperm.

RESULTS AND DISCUSSION

Evidence for the legumin-like structure of the major protein in Ginkgo *seeds*

Although the amino acid sequences of the *Ginkgo* seed proteins examined are not known, evidence is given for the homology of these major seed proteins with legumins and legumin-like proteins from angiosperms. This correspondence includes the high M_r (*ca* 400 000) of the Ginkgo legumin-like protein and the characteristic disulphide bonds between α and β subunits. This latter can be demonstrated by SDS gel electrophoresis using non-reducing and reducing conditions (Fig. 1).

Only two small bands at M_r 15 200 and 14 400 are not affected. Their isoelectric bands are mainly located in the basic range, although they are poorly visible (Fig. 3B;*). It can be assumed that they belong to the legumin protein since they appear in the 2-D profile (Fig. 6) as well as in the SDS electrophoresis of immunoprecipitated legumin (not shown).

Another correspondence between the *Ginkgo* and angiosperm legumin-like proteins is the occurrence of at least two subunit dimer types. For *Ginkgo*, 'normal' and 'small' dimers are present, which on 2-D electrophoresis are clearly separated (Fig. 2).

The following M_r data are calculated by comparing the electrophoretic distances of the reduced and non-reduced SDS bands in polyacrylamide gradient gels with marker proteins. It is necessary to take into consideration that these M_r data are only approximate, because of the differing mobility of reduced and non-reduced proteins even where there are no intermolecular –S–S– bonds, and because of the differences between apparent M_r on SDS gels and the M_r calculated from sequence data, which are particularly marked for the acidic subunits of legumin and glycinin [4, 23–25].

The 'normal' 50 000 M_r dimer separates into 28 000 and 20 100 M_r subunits. As a result of an identical staining intensity of both subunits, the well known 1:1 subunit composition is likely. The 'small' 31 000 dimer separates into 20 100 and 13 200 M_r subunits. For this 'small' dimer,

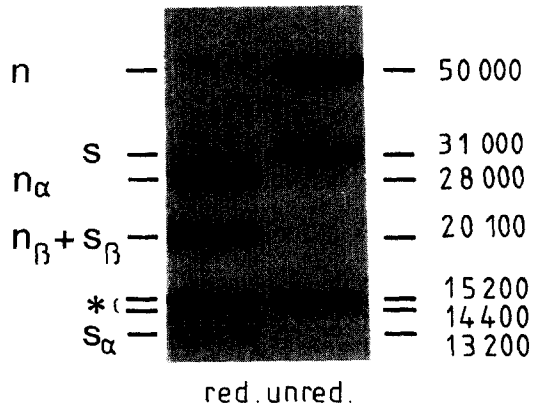
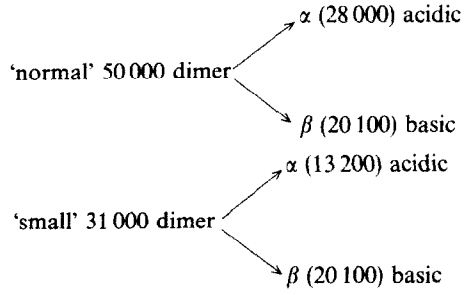


Fig. 1. SDS electrophoresis of the *Ginkgo* legumin-like protein under reducing and non-reducing conditions. The bands for the 'normal' (n) and 'small' (s) legumin-like protein and their α- and β-subunits are indicated; the M_rs are shown on the right side. Two polypeptides M_r 15200 and 14400 are unaffected by reducing conditions.

the 13200 M_r subunits are only slightly stained in contrast to the 20100 M_r banding, which does not appear to fit the 1:1 model for the legumin-like protein. However, the smaller subunit of the 'small' dimer of *Glycine max* legumin is also much less stained than the larger subunit, although both are reported to be present in *ca* equal molar ratio [26].

The acidic and basic character of the subunits and their M_r range is a further similarity between the legumin-like proteins of both *Ginkgo* and angiospermous plants. This can be deduced from the 2-D isoelectric focusing gel of Fig. 3. Both 'small' and 'normal' dimers consist of acidic and basic subunits and are assembled in the following way:



The β 20100 subunits from the normal and small dimers are identical in isoelectric focusing band patterns.

It is interesting, that for the 'small' dimer the basic subunit is the larger one. This 'twisted' phenomenon, however, is also known from the angiosperms, e.g. *G. max* [26, 27].

Beside the differentiation into 'normal' and 'small' dimers and into α and β subunits additional heterogeneity occurs, which is also known for all investigated angiospermous legumin-like proteins. In several electrophoresis experiments at least two adjacent bands each for the 'normal' and 'small' *Ginkgo* dimers have been detected (not shown). In addition heterogeneity in the isoelectric banding pattern has been observed, especially in the case of the acidic subunits (Fig. 3).

The legumin-like protein heterogeneity is represented by a uniformly broad banding in gel electrophoresis of the non-denaturated legumin-like protein fraction, purified by AcA 34 Ultrogel gel filtration (Fig. 4 B). This heterogeneity can be explained by the occurrence of differently charged legumins. These legumins can be separated by using DEAE Sephadex A50 ion exchange chromato-

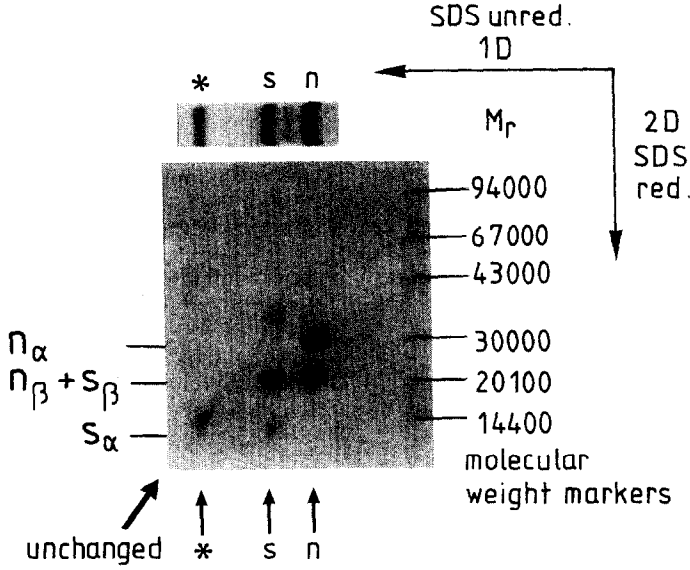


Fig. 2. 2-D SDS electrophoresis of the *Ginkgo* legumin-like protein. The 'normal' dimers (n) separate into nα- and nβ-components, the 'small' dimers (s) into sβ- and sα-components. Unchanged in the second dimension are the –S–S-unbonded polypeptides of 15200 and 14000 M_r and the main component of the H protein (34000 M_r).

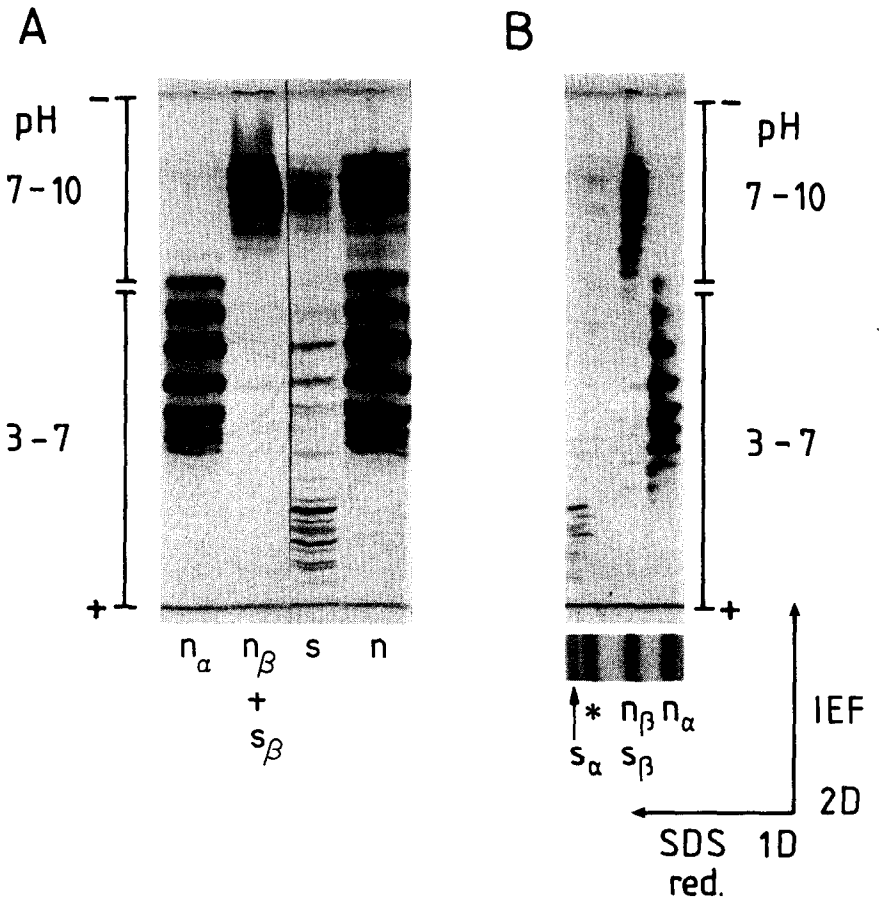


Fig. 3. Isoelectric focusing patterns of legumin-like proteins and their subunits. A: Focusing patterns of various bands cut out from the SDS- PAGE gel (reduced/non-reduced) (i.e. n = 50 000, s = 31 000, nβ + sβ = 20 100, and nα = 28 000, from right to left; see also Fig. 1). B: 2-D focusing patterns from the polypeptides of SDS gel electrophoresis, reducing conditions, showing the extended IEF pattern of the basic subunits (nβ, sβ). The asterisk indicates the polypeptides which are not -S–S- bonded. The proteins used for 3 A and 3 B originate from different fractions of the DEAE anion exchange profile (Fig. 4A); 3 A corresponds to I and 3 B corresponds to II in Fig. 4 A

graphy (Abb. 4, I, II, III). The protein fraction I not absorbed by the DEAE material shows the least electrophoretic mobility. For the fraction eluted at higher sodium chloride molarities the electrophoretic distances increase because of increased net negative charge. It should be kept in mind, that all DEAE fractions originate from a narrow peak in the AcA 34 Ultrogel filtration profile represented by a protein of uniform M_r. The different legumins are serologically identical using polyclonal antisera against the AcA 34 Ultrogel legumin fraction. Similar heterogeneity has been reported for (e.g.) the *Pisum* legumin [28].

Occurrence of a second seed protein

Interestingly, an additional protein ('H protein') was observed to co-elute with the *G. biloba* legumin-like proteins on gel filtration. However, in ion exchange experiments the elution of H protein is delayed with regard to the legumin-like proteins and it forms a shoulder in the elution profile (Fig. 4 AIV).

No cryoprecipitation has been observed for the H protein in contrast with the legumin-like protein, which heavily precipitates at 4° under conditions of low ionic buffer strength.

The H protein is serologically different from the legumin-like proteins; no cross-reactivity (i.e. no precipitation of the purified protein with legumin antiserum) has been observed. Analysis of the specific immunoprecipitation line (Fig. 5) demonstrated the occurrence of 33 800 (H_1) and 14 400 (H_2) M_r non-disulphide linked components. In other experiments, a 50 000 component (H_0) has been detected (Fig. 6). Since the M_rs of these non-S–S- bonded polypeptides correspond with the M_rs of the pea vicilin [4] including proteolytic cleaved fragments, the H protein could be a vicilin homologous protein. This, however, remains to be proved.

Serological cross-reactivity of the G. biloba *legumin-like antisera*

For systematic purposes, *G. biloba*-antisera were produced against the legumin-like protein peak (which also contains H protein) from the Ultrogel AcA 34 gel filtration experiment. Serological cross-reactions between these antisera and antigenic materials from other taxa

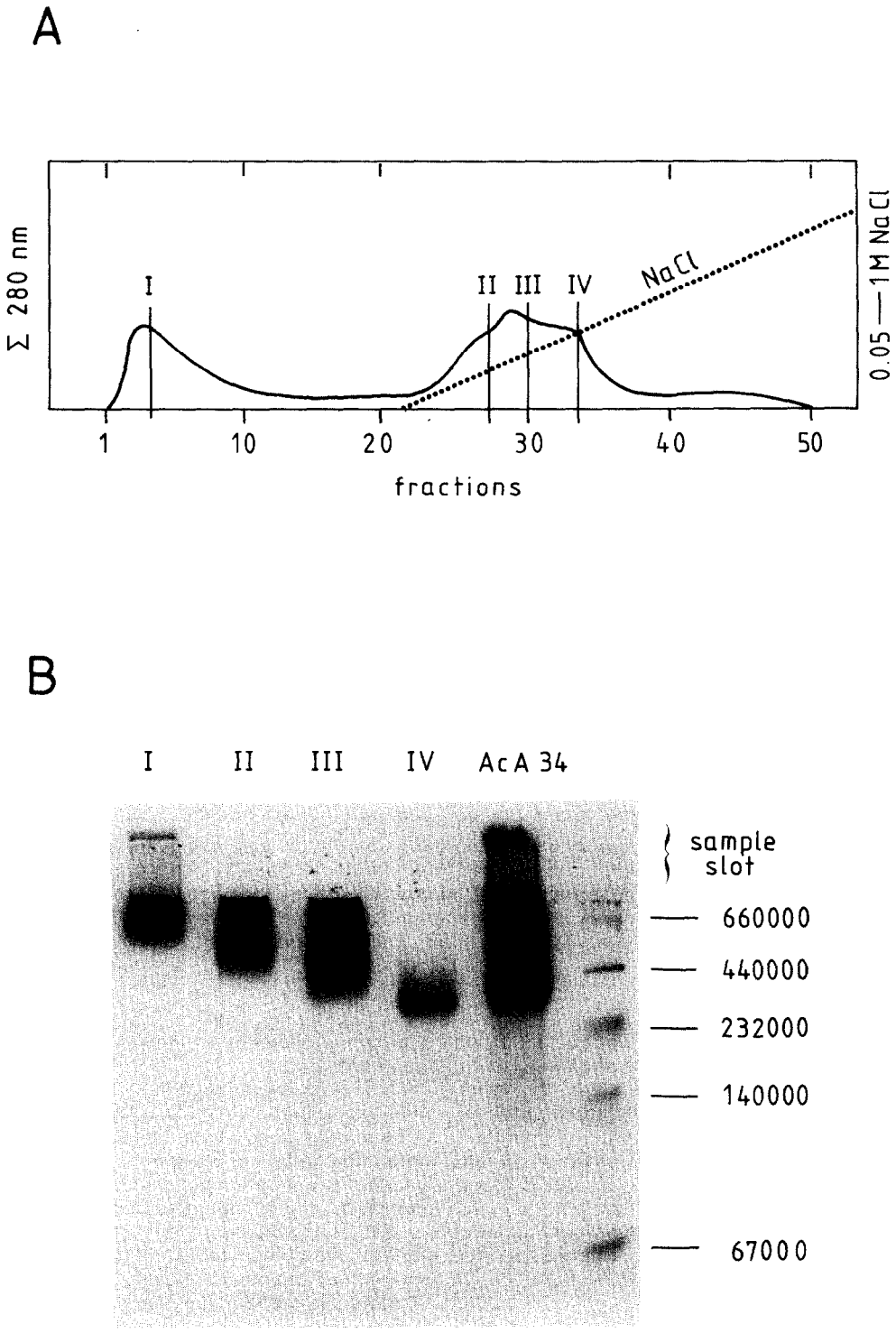


Fig. 4 A. Elution profile on DEAE Sephadex A 50 anion exchange chromatography of the *G. biloba* legumin-like protein (containing the H protein) isolated from AcA Ultrogel filtration. I = unadsorbed peak. B. Non-denaturing gradient PAGE of fractions I, II, III, IV from Fig. 4 A and for the complete AcA Ultrogel filtration fraction.

were performed. No legumin cross-reactions have been observed between the *G. biloba*-antiserum and seed extracts from conifers (*Picea*, *Pinus*) or between *G. biloba*-antisera and legumin extracts from the Ranunculacean genus *Thalictrum*. In both cases, however, a faint precipitation band has to be attributed to another protein. Preliminary western blotting experiments indicated that the H_0 protein is involved. Compared to the often observed cross-reactivity of legumins within angiosperm families and subclasses [13, 19], the missing cross-reac-

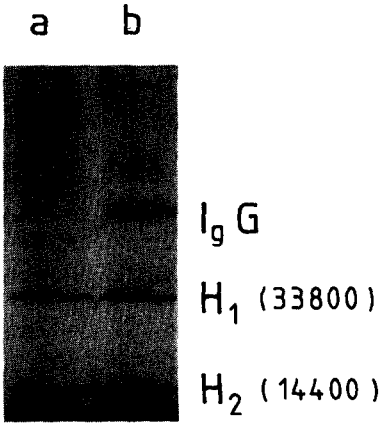


Fig. 5. SDS-polyacrylamide gradient gel electrophoresis of immunoprecipitated H protein, using non-reducing conditions (a) and reducing conditions (b).

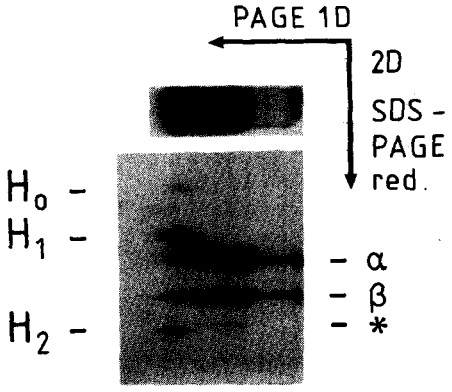


Fig. 6. 2-D electrophoresis of a fraction from gel filtration containing *Ginkgo* legumin-like proteins plus H protein. In the first dimension, non-denaturing conditions are used, in the second dimension SDS gel electrophoresis (reducing conditions) was performed. The different electrophoretically separated legumins are visible, forming a broad protein zone for the 28 000 (α)- and for the 20 100 (β) subunits. Below the β subunits a faint spot for the -S–S–unbonded polypeptides (*) in the 15 000 M_r range is visible. The H proteins separate in SDS electrophoresis into the two main H_1 and H_2 subunits; probably the H_0 subunit additionally belongs to the H protein.

tivity between *G. biloba* and the conifers demonstrates a wide phylogenetic gap between both plant groups. Although morphological links have been demonstrated [29, 30], both plant groups are taxonomically separated by their inclusion into different classes [31, 32] and are phylogenetically believed to be derived from the progymnosperms [32] as the common ancestors.

CONCLUSIONS

Although no amino acid sequence studies have been performed, there is no doubt that the major storage proteins in *G. biloba* seeds are legumin-like proteins. Compared with the legumins known from the Fabaceae and homologous proteins from other dicots and monocots, they have corresponding M_r, charge, subunit properties including disulphide bonds, and similar molecular heterogeneity. If this is accepted, the statement of Borroto and Dure III [21] that 'at least one gene for legumin existed at the beginning of angiosperm evolution' should be stretched back at least to the beginning of gymnosperm evolution. However, the production of legumin-like proteins in all seed plants including gymnosperms is not surprising, as Templeman *et al.* [33] demonstrated legumin-like proteins even in spores of the fern *Mattheucia struthiopteris*. Therefore, these proteins probably represent phylogenetically old and universal storage proteins of haploid spores and haploid megasporic tissue of gymnosperm seeds as well as of diploid/triploid angiosperm seeds. In gymnosperms the production and accumulation of this protein is obviously independent of the fertilization process.

G. biloba is the only gymnosperm for which the production of legumin-like protein has been demonstrated. In preliminary experiments, we failed to demonstrate -S–S–bridges for the major storage proteins of some conifers, and Blagrove *et al.* [22] could not show typical legumin-like proteins in *Macrozamia* (Cycadaceae).

In the course of evolution, considerable changes in the molecular structure of the legumin proteins have taken place. Lack of serological cross-reactivity between *G. biloba* and conifers as well as between *G. biloba* and angiosperms document such molecular evolution. No evidence is available for phylogenetical connections with other storage proteins or for its evolutionary origin from non-storage proteins in evolution [5], although the observed oxaloacetate decarboxylase activity of a *Cucurbita* legumin-like protein [34] indicates one possibility.

EXPERIMENTAL

Protein isolation. Seeds of *Ginkgo biloba* L. were obtained from trees in Berlin (leg. G. Jensen) and Bologna (leg. Dr. G. Cristofolini) respectively. The storage proteins were extracted as previously described [14]. Testas were removed from dry seeds. Seeds were milled and extracted at *ca* 15° with a Tris–glycine-buffer (0.01 M Tris; 0.08 M glycine), pH 8.2 + 2% NaCl. After 20–30 min the extract was centrifuged at *ca* 27 000 *g* for 20 min at 10°. The supernatant which contained dissolved storage proteins was defatted with freon. Legumin was purified by Ultrogel AcA 34 gel filtration, followed by DEAE Sephadex A 50 ion exchange chromatography (Fig. 4A) as described by Jensen [14].

Polyacrylamide gradient gel electrophoresis. Electrophoresis was carried out in 5–20% polyacrylamide gradient gels and a Tris–glycine pH 8.2 buffer system; 300 V, 18 mA, 18 hrs. M_rs were estimated using the standard proteins thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), and bovine serum albumin (67 000).

SDS-polyacrylamide gradient gel electrophoresis. (a) Electrophoresis was carried out in 7.5–20% polyacrylamide gradient SDS gels using a Tris–borate pH 7.9 buffer system; starting conditions 480 V, 40 mA, 4 hr. 0.06 MTris–HCl buffer pH 6.8 containing 3% SDS and samples were mixed in a 1:1 ratio. For reducing conditions 0.5% DTE (1,4-dithioerythritol) was added to the buffer. The mixture was boiled in a water bath at 100° for 5 min. M_rs were estimated using the standard proteins phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

(b) SDS-polyacrylamide gradient gel electrophoresis of immunoprecipitate. The immunoprecipitate from a double immunoprecipitation experiment in Bacto-Agar was cut out. After washing in 1% NaCl soln and double dist. H_2O, the bands were incubated for 1.5 hr in a 3% SDS buffer containing 0.5% DTE for reducing conditions at room temp. The bands were placed on

a horizontal SDS polyacrylamide gradient gel and removed after 1 hr running time. For conditions of SDS-electrophoresis see (a).

Two-dimensional electrophoresis. (a) SDS-PAGE under non-reducing vs reducing conditions: after the first dimension electrophoresis under non-reducing conditions, the gelstrip was incubated in a 0.06 M Tris–HCl buffer, pH 6.8, containing 3% SDS and 0.5% DTE, for 1 hr at room temp. Then the gelstrip was placed on a horizontal SDS gradient gel and after 1 hr electrophoresis removed from the gel. The complete electrophoresis period was 4 hr at 500 V/40 mA.

(b) PAG electrophoresis versus SDS-PAGE under reducing conditions: The first dimension gel electrophoresis was performed in gradient polyacrylamide gel (5–20% acrylamide) using a Tris–glycine pH 8.2 buffer system. For the second dimension conditions see (a).

(c) SDS-PAGE under reducing and non-reducing conditions versus isoelectric focusing. After SDS-PAGE the gelstrip was incubated in deionized 8 M urea containing 0.03 M DTE and applied to the anodic side of the IEF gel for 50 min. For the IEF gel (4.7% T, 3% C, 10% glycerine), 7.4 M urea and 2.4% ampholine (pH 3–10/pH 5–8 = 4:1) were used, following ref. [35]. Focusing was carried out at a constant power 7 W for 110 min (max. set to 2000 V, 40 mA) and then reset to max. 2000 V, 10 mA and 40 W for *ca* 60 min. The focusing was complete when 2000 V was reached. The pH gradient in the gel was estimated by comparison with marker proteins LKB 1860 202/1860 203.

Serology. For the production of antisera, *G. biloba* seed extracts were purified by Ultrogel AcA 34 gel filtration. The fractions of the legumin-like protein peak containing H protein were injected into rabbits weekly (*ca* 5 mg; 2 ml). Samples of blood were removed monthly, starting 8 days after the last injection.

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