LEGHEMOGLOBINS: IMMUNOCHEMISTRY AND PHYLOGENETIC RELATIONSHIPS

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1. Introduction

Comparative immunological analyses of proteins from different phylogenetic groups of animals and plants have been useful in assessing taxonomic relationships and measuring genetic homologies among species, especially in the absence of amino acid sequence data [1,2]. Among some isofunctional, homologous globular proteins antigenic cross-reactivity is seen if the amino acid sequences differ by less than 40% [3,4], the antigenic effects of substitutions being approximately equal and additive.

Previously [5] we have shown that the 5 leghemoglobins isolated from the root nodules of the soybean plant (*Glycine max*. c.v. Lincoln) completely cross-react immunologically with each other and with the main leghemoglobin from serradella (*Ornithopus sativus* Brot.). The major leghemoglobin from snake bean (*Vigna sinensis* L.) only partially cross-reacts with antisera to the soybean leghemoglobin while no cross-reaction at all is seen between the lupin (*Lupin luteus*) and soybean leghemoglobins. These studies have now been extended to leghemoglobins from clover (*Trifolium subterraneum* c.v. Woogenellup) and broad bean (*Vicia faba* c.v. Triple White) using a radioimmunoassay procedure with antisera to soybean and lupin leghemoglobins.

A comparison of immunological cross-reactivities between leghemoglobins from plants of different phylogeny has made possible the construction of a simple phylogenetic tree for the Fabaceae family of the order Leguminale. Further comparison of this tree with phylogenetic relationships based on conventional taxonomic criteria shows that immunological cross-reaction is a good indicator of species differences.

2. Materials and methods

Soybean leghemoglobin was fractionated into 5 components as described [6]. Leghemoglobins from snake bean, lupin and serradella plant root nodules were extracted by established procedures [7]. Leghemoglobins from clover and broad bean were isolated from the nodules of plants grown in sand and vermiculite (1:1, v/v) in a glass house according to the procedure [8]. Antiserum to lupin leghemoglobin was prepared in rabbits. Radio-iodination of the leghemoglobins and the radioimmunoassay experiments were carried out by procedures described [5].

3. Results and discussion

The abilities of leghemoglobins isolated from serradella, snake bean, lupin and broad bean to inhibit the binding of ^{125}I-labeled soybean leghemoglobin *a* to its homologous antibodies are summarized in fig.1a. As noted [5], serradella leghemoglobin completely inhibited the soybean homologous reaction but with several-hundred-fold attenuation of binding affinity, while the snake bean protein shows evidence of incom-

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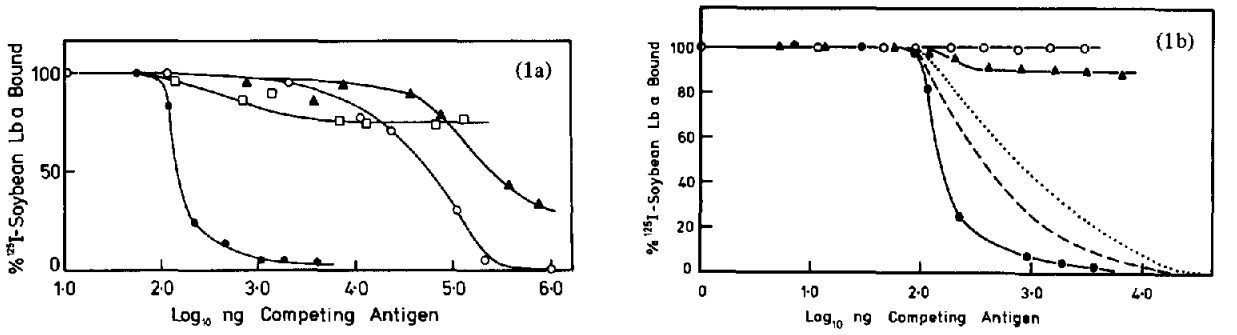

Fig.1. Radioimmunoassay inhibition curves for unlabeled leghemoglobins inhibiting the binding of ^{125}I-labeled soybean leghemoglobin *a* binding to anti-(soybean leghemoglobin *a*) serum. The unlabeled leghemoglobins are:
(1a) Soybean *a* (•); serradella (○); snake bean (▲); broad bean (□).
(1b) Soybean *a* (•); clover (▲); lupin (○).
For comparisons soybean c_1 (---) and soybean c_2 (. . . .) leghemoglobins are shown in (1b).

plete cross-reactivity and even weaker affinity than serradella. Broad bean leghemoglobin is able to inhibit only 25% of the homologous soybean leghemoglobin *a* reaction while clover leghemoglobin inhibits 14% of the reaction (fig.1b). This suggests that only one or two antigenic determinants found on either broad bean or clover leghemoglobins are recognized by antibodies specific to soybean leghemoglobin *a*. The contribution of each determinant to the overall immune response of a protein varies and it is therefore not possible to say exactly how many determinants are common; it is unlikely, however, that there are more than two loci in common between the soybean protein and the broad bean or clover proteins. Lupin leghemoglobin I does not cross-react with soybean leghemoglobin *a* (fig.1b) indicating that soybean and lupin do not belong to closely related tribes of the Fabaceae family.

Radioimmunoassay, using ^{125}I-labeled lupin leghemoglobin I and anti-(lupin leghemoglobin) serum substantiates the distant phylogenetic relationship between lupin and soybean (fig.2a). Soybean, serradella, snake bean and broad bean leghemoglobins do not cross-react with lupin leghemoglobin (fig.2b). Clover leghemoglobin, however, cross-reacts with lupin leghemoglobin (fig.2a) and so provides an

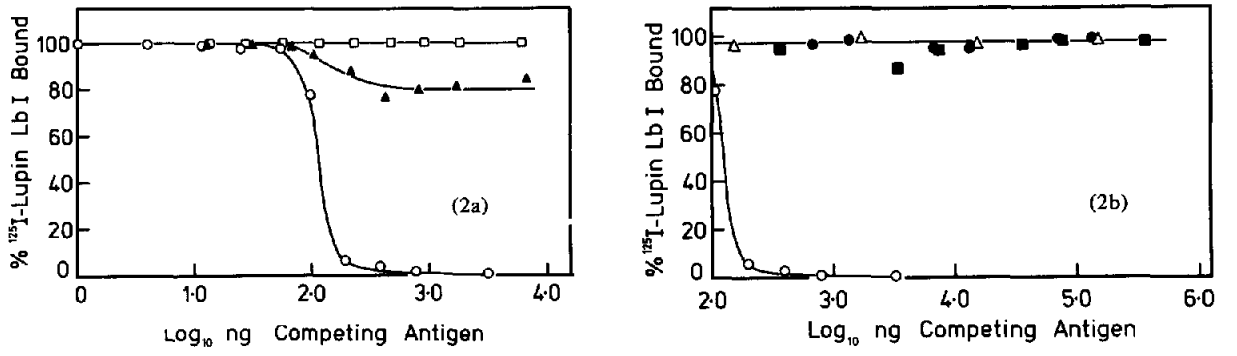

Fig.2. Radioimmunoassay inhibition curves for unlabeled leghemoglobins inhibiting the binding of ^{125}I-labeled lupin leghemoglobin to anti-(lupin leghemoglobin) serum. The symbols for the unlabeled leghemoglobins are:
(2a) Lupin (○); clover (▲); soybean *a* (□).
(2b) lupin (○); broad bean (△); serradella (•); snake bean (■).

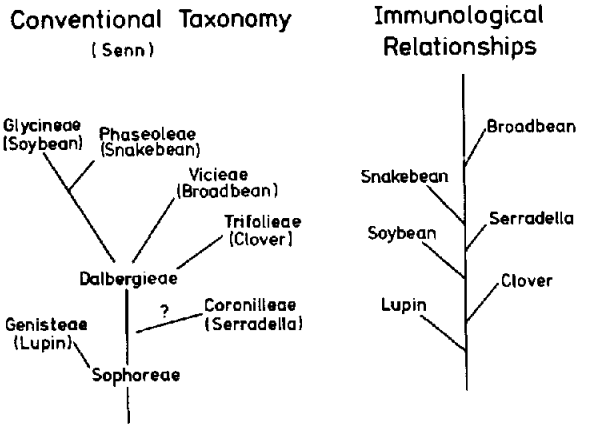

Fig.3. A simple phylogenetic tree for some tribes of the Fabaceae family based on radioimmunoassay cross-reactivities (right-hand side) compared with the phylogenetic tree established from conventional taxonomy [9] (left-hand side).

important immunological bridge between the distantly related lupin and soybean proteins, since it cross-reacts with both; albeit weakly (20% with the former and 14% with the latter).

From the immunological data presented, it is possible to draw up several phylogenetic trees based on cross-reactivity. The simplest of these trees is shown in fig.3 and is in good agreement with the phylogenetic tree based on conventional taxonomic criteria.

The close relationship between the soybean and snake bean observed immunologically with the leghemoglobins is in agreement with the findings of others [9,10] based on conventional methods. In fact the genera *glycine* (soybean) and *phaseolus* (snake bean) are sometimes classified as belonging to the one tribe, Phaseoleae (II), rather than to two different tribes Glycineae and Phaseoleae). Further, this close relationship has been established by double diffusion studies using heterogeneous seed protein extracts [11]. Other immunodiffusion studies with heterogeneous seed protein extracts [12] have substantiated the distant relationship of the genus *lupinus* to *glycine, phaseolus, vicia* and *trifolium*. The position of serradella in the tree based on cross-reactivity data appears not to be consistent with the position by conventional taxonomy. However, very little information concerning the classification of the Coronilleae tribe (Serradella) has appeared in the literature, so that at present, it is felt that the immunological placement is probably sound.

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