



Sainfoin lectin : molecular characterization and amino acid sequence  
by Rozenn Nicole Kouchalacos

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF  
PHILOSOPHY in Biochemistry  
Montana State University  
© Copyright by Rozenn Nicole Kouchalacos (1982)

**Abstract:**

Sainfoin lectin, a glycoprotein from the legume *Onobrychis viciifolia*, Scop., has been characterized according to its complete amino acid sequence, carbohydrate binding, metal content and molecular stability. The protein subunit was a single polypeptide chain containing 236 amino acid residues. Nitrogen and carboxyl termini were alanine and threonine, respectively, and the single site of carbohydrate attachment was asparagine 118. Nb isolectin forms were detected. The sequence showed extensive homology with other leguminous lectins. Dissociation constant with D-glucose was 3.52 mM by equilibrium dialysis, and similar values were obtained by affinity electrophoresis. One carbohydrate binding site was determined per protein subunit. Sainfoin lectin contained one Ca<sup>++</sup> and lesser amounts of Mn<sup>++</sup> and Mg<sup>++</sup> per subunit. The protein was normally heat and trypsin resistant. Succinylation of lysine residues and carboxymethylation of the single cysteine resulted in a modified protein that, upon trypsin hydrolysis, yielded manageable peptide fragments.

i

to my husband David

with love

SAINFOIN LECTIN: MOLECULAR CHARACTERIZATION AND AMINO ACID SEQUENCE

by

ROZENN NICOLE KOUCHALAKOS

A thesis submitted in partial fulfillment  
of the requirements for the degree

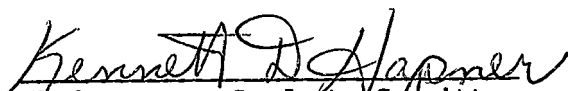
of

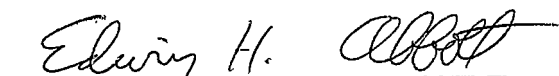
DOCTOR OF PHILOSOPHY


in

Biochemistry

Approved:

  
Chairperson, Graduate Committee

  
Head, Major Department

  
Graduate Dean

MONTANA STATE UNIVERSITY  
Bozeman, Montana

August, 1982

## ACKNOWLEDGMENT

I would like to express my gratitude to Dr. K. D. Hapner for his constant help throughout this study. His assistance and advice were most valuable, and his friendly moral support was appreciated.

I wish to acknowledge a fruitful collaboration with Dr. R. A. Bradshaw who provided funds, expertise and technical assistance for the sequence analyses.

I am thankful to Dr. J. E. Robbins for his interest in my research project and numerous helpful discussions.

During the course of this study, I received two travel grants from the Dean of the Graduate School of Montana State University and financial support from Montana Agricultural Experiment Station and Montana State University Department of Chemistry.

## TABLE OF CONTENTS

	<u>Page</u>
LIST OF TABLES . . . . .	x
LIST OF FIGURES. . . . .	xi
ABSTRACT . . . . .	xiv
CHAPTER ONE. Introduction . . . . .	1
CHAPTER TWO. Goals and Objectives . . . . .	16
CHAPTER THREE. Experimental Procedure . . . . .	18
Materials . . . . .	18
Purification of Sainfoin Lectin (SL) . . . . .	19
Gel Filtration of SL. . . . .	20
Affinity Chromatography of SL at Various pH Values. . . . .	21
Anion Exchange Chromatography of SL . . . . .	21
Immuno Double Diffusion Agar Gels . . . . .	22
Metal Analysis. . . . .	22
Equilibrium Dialysis. . . . .	23
Agglutination Assay . . . . .	24
Standard Electrophoretic Procedures . . . . .	24
Synthesis of Allyl Alpha-Glycosides . . . . .	25
Synthesis of Glycosyl Copolymers. . . . .	26
Affinity Electrophoresis. . . . .	27
Performic Acid Oxidation. . . . .	28
Carboxymethylation. . . . .	29
Reaction with 5,5'-Dithiobis-(2-nitrobenzoic Acid) . . . . .	29
Succinylation . . . . .	30
Trinitrobenzene Sulfonic Acid (TNBS) Assay. . . . .	30
Cyanylation . . . . .	31
Synthesis of 2-Nitro-5-thio[ <sup>14</sup> C]cyano Benzoic Acid (N <sup>14</sup> CB) . . . . .	31
Cleavage at Cysteine. . . . .	32
Gel Filtration of SL Cysteine Cleavage Product. . . . .	32

(continued)

## TABLE OF CONTENTS (continued)

	<u>Page</u>
Gel Filtration of Succinylated SL Cleaved at Cysteine . . .	33
Partial Acid Hydrolysis . . . . .	33
Digestion with Trypsin. . . . .	34
Digestion with Thermolysin. . . . .	34
Digestion with Carboxypeptidases. . . . .	35
Fractionation of SL Tryptic Digest at Low pH. . . . .	35
Gel Filtration of the pH 3.5 Soluble SL Tryptic Peptides. . . . .	36
Repurification of T2 and T10 on LH20. . . . .	36
Cation Exchange Chromatography of the pH 3.5 Soluble SL Tryptic Peptides. . . . .	37
Gel Filtration of the pH 3.5 Insoluble SL Tryptic Peptides. . . . .	38
Repurification of T4 and T7 by High Performance Liquid Chromatography (HPLC). . . . .	38
Digestion of T6 with Beta Xylosidase. . . . .	39
Boron Chromatography of T6. . . . .	39
Chromatography of T6 on WGA-Sepharose . . . . .	39
Carbohydrate Analysis . . . . .	39
Gel Filtration of the Thermolytic Peptides. . . . .	40
Cation Exchange Chromatography of the Thermolytic Peptides. . . . .	40
Arginine Detection. . . . .	41
Ninhydrin Analysis. . . . .	41
Amino Acid Analysis . . . . .	42
End Group Analysis. . . . .	42
Sequence Analysis . . . . .	43
 CHAPTER FOUR. Results . . . . .	 46
Section 1. Sainfoin Lectin Stability . . . . .	46
Gel Filtration of SL . . . . .	46
Solubility of SL in Aqueous Solution . . . . .	49
Affinity Chromatography of SL at Various pH Values. . . . .	50
Trypsin Digestion of SL. . . . .	52
Characteristics of Succinylated SL . . . . .	54

(continued)

## TABLE OF CONTENTS (continued)

	<u>Page</u>
Section 2. Studies on Apparent Heterogeneity of Sainfoin Lectin. . . . .	62
Ion Exchange Chromatography of SL. . . . .	62
Analyses of SL Ionic Species . . . . .	65
Affinity Electrophoresis of SL at pH 4.0 . . . . .	69
Affinity Electrophoresis of SL at pH 9.5 . . . . .	72
Section 3. Ligand Interaction Studies. . . . .	75
Metal Analyses . . . . .	75
Estimation of the Dissociation Constant for SL- Glucose Complex by Affinity Electrophoresis . . . . .	77
Equilibrium Dialysis . . . . .	82
Section 4. Primary Structure Determination . . . . .	86
Partial Acid Hydrolysis. . . . .	86
Cleavage at Cysteine . . . . .	90
Trypsin Cleavage at Arginine . . . . .	99
Purification of the pH 3.5 Soluble SL Tryptic Peptides . . . . .	.104
Purification of the pH 3.5 Insoluble SL Tryptic Peptides . . . . .	.111
Analyses of SL Tryptic Peptides. . . . .	.116
Peptide T1. . . . .	.116
Peptide T2. . . . .	.117
Peptide T3. . . . .	.118
Peptide T4. . . . .	.119
Peptide T5. . . . .	.119
Peptide T6. . . . .	.119
Assignment of Carbohydrate Position . . . . .	.119
Other Characteristics of SL Tryptic Glycopeptide . . . . .	.120
Peptide T7. . . . .	.121
Peptide T8. . . . .	.122
Peptide T9. . . . .	.122

(continued)

## TABLE OF CONTENTS (continued)

	<u>Page</u>
Peptide T10 . . . . .	.122
Peptide T11 . . . . .	.123
Peptide T12 . . . . .	.123
Cleavage of SL with Thermolysin. . . . .	.123
Analyses of Arginine-Containing SL Thermolytic Peptides . . . . .	.127
Peptide Th1 . . . . .	.127
Peptide Th2 . . . . .	.128
Peptide Th3 . . . . .	.128
Peptide Th4 . . . . .	.129
Peptide Th5 . . . . .	.129
Peptide Th6 . . . . .	.129
Peptide Th7 . . . . .	.130
Alignment of SL Tryptic Peptides . . . . .	.130
CHAPTER FIVE. Discussion. . . . .	.135
Sequence Strategy--Plan of Attack. . . . .	.135
Production and Purification of Tryptic Peptides . . . . .	.136
Sequence Analyses. . . . .	.137
Alignment of Tryptic Peptides. . . . .	.139
Sainfoin Lectin Amino Acid Sequence. . . . .	.142
Comparison of SL, Concanavalin A, Favin, Lentil Lectin and Soybean Agglutinin. . . . .	.144
Structure Conservation . . . . .	.151
Influence of Circular Permutation and Subunit Structure. . . . .	.151
Secondary Structures. . . . .	.153
Metal Binding Sites . . . . .	.154
Carbohydrate Binding Site . . . . .	.155
Hydrophobic Cavity. . . . .	.156
Monomer-Monomer and Dimer-Dimer Contacts. . . . .	.157

(continued)

## TABLE OF CONTENTS (continued)

	<u>Page</u>
Carbohydrate Attachment Site. . . . .	.159
Structure Conservation, Summation . . . . .	.160
Other Leguminous Lectins . . . . .	.161
CHAPTER 6. Summary and Conclusions. . . . .	.164
REFERENCES . . . . .	.170
APPENDIX. Amino Acid Symbols. . . . .	.177

## LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Behavior of sainfoin lectin on Ultrogel AcA44 . . . . .	48
2. Amino acid composition of sainfoin lectin ionic species . . . . .	67
3. Metal analyses of sainfoin lectin by atomic absorption. . . . .	75
4. $K_d$ values for SL-glucose complexes determined by electrophoresis at pH 4.0 . . . . .	79
5. Amino acid composition of sainfoin lectin . . . . .	.143
6. Identities among ConA, SL, SBA, favin and LL. . . . .	.148

## LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Calibration of Ultrogel AcA44 column. . . . .	47
2. Affinity chromatography of SL on mannose-Sepharose at different pH values. . . . .	51
3. Affinity chromatography of 90% succinylated SL on mannose-Sepharose . . . . .	55
4. Gel filtration of 90% succinylated SL on Ultrogel AcA44 . . . . .	57
5. Affinity chromatography of 100% succinylated SL on mannose-Sepharose . . . . .	59
6. Gel filtration of 100% succinylated SL on Ultrogel AcA44 . . . . .	60
7. Gel filtration of preheated 100% succinylated SL on Ultrogel AcA44. . . . .	60
8. Diethylaminoethylcellulose chromatography of SL . . . . .	63
9. Diethylaminoethylcellulose chromatography of fraction I of Figure 8. . . . .	64
10. Immuno/double diffusion of SL ionic species against antiserum to native SL. . . . .	66
11. Affinity electrophoresis at pH 4.0 of SL. . . . .	70
12. Affinity electrophoresis at pH 9.5 of SL. . . . .	73
13. Plot (L/Lo-L) vs. C derived from the affinity electrophoresis of Figure 11. . . . .	78
14. Determination of the dissociation constant of glucose to SL by equilibrium dialysis . . . . .	83

(continued)

## LIST OF FIGURES (continued)

<u>Figure</u>	<u>Page</u>
15. Sodium dodecylsulfate electrophoresis of SL partial acid hydrolysate. . . . .	87
16. Gel filtration of SL partial acid hydrolysate on Biogel A5m. . . . .	88
17. Sodium dodecylsulfate electrophoresis of SL cleaved at cysteine . . . . .	91
18. Gel filtration of SL cleaved at cysteine on Sephadex G50. . . . .	92
19. Gel filtration of succinylated SL cleaved at cysteine on Sephadex G25 in 0.02 M $\text{NH}_4\text{HCO}_3$ , pH 6.3. . . . .	94
20. Gel filtration of succinylated SL cleaved at cysteine on Sephadex G25 in 3M guanidinium chloride, pH 7.0. . . . .	95
21. Production and purification of sainfoin lectin tryptic peptides. . . . .	.100
22. Solubility of SL tryptic peptides as a function of pH. . . . .	.102
23. Gel filtration of pH 3.5 soluble SL tryptic peptides on Sephadex G25. . . . .	.105
24. Repurification of peptide T10 of Figure 23 on Sephadex LH20 . . . . .	.107
25. Gel filtration of pH 3.5 soluble SL tryptic peptides on Sephadex G25 in 0.01 M acetic acid . . . . .	.108
26. Repurification of peptide T2 from Figure 25 on Sephadex LH20 . . . . .	.109

(continued)

## LIST OF FIGURES (continued)

<u>Figure</u>	<u>Page</u>
27. Cation exchange chromatography of pH 3.5 soluble SL tryptic peptides . . . . .	.110
28. Gel filtration of pH 3.5 insoluble SL tryptic peptides on Sephadex G50. . . . .	.112
29. Repurification of peptide T7 by reversed-phase HPLC. . . . .	.113
30. Repurification of peptide T4 by reversed-phase HPLC. . . . .	.115
31. Production and purification of thermolytic overlaps. . . . .	.125
32. Gel filtration of SL thermolytic peptides on Sephadex G25. . . . .	.126
33. Reconstruction of seven blocks of continuous sequence from results obtained on SL component peptides. . . . .	.131
34. Alignment of SL peptides using favin as a template. . . . .	.134
35. Comparison of the amino acid sequences of ConA, SL, SBA, favin and LL . . . . .	.145
36. Schematic alignment of SL and favin with ConA . . . . .	.147

## ABSTRACT

Sainfoin lectin, a glycoprotein from the legume Onobrychis viciifolia, Scop., has been characterized according to its complete amino acid sequence, carbohydrate binding, metal content and molecular stability. The protein subunit was a single polypeptide chain containing 236 amino acid residues. Nitrogen and carboxyl termini were alanine and threonine, respectively, and the single site of carbohydrate attachment was asparagine 118. No isolectin forms were detected. The sequence showed extensive homology with other leguminous lectins. Dissociation constant with D-glucose was 3.52 mM by equilibrium dialysis, and similar values were obtained by affinity electrophoresis. One carbohydrate binding site was determined per protein subunit. Sainfoin lectin contained one  $\text{Ca}^{++}$  and lesser amounts of  $\text{Mn}^{++}$  and  $\text{Mg}^{++}$  per subunit. The protein was normally heat and trypsin resistant. Succinylation of lysine residues and carboxymethylation of the single cysteine resulted in a modified protein that, upon trypsin hydrolysis, yielded manageable peptide fragments.

## CHAPTER ONE

### INTRODUCTION

Lectins are multivalent proteins and glycoproteins with carbohydrate binding abilities (Goldstein & Hayes, 1978). This family of molecules does not include antibodies against carbohydrate structures, glycosidases or monovalent carbohydrate binding proteins. No lectin has been convincingly described that exhibits enzymatic activity.

Lectins were originally detected in plants. A review on the history of lectin discovery and uses is available by Coulet (1979). Lectins are now thought to be ubiquitous. They are found in plants, microorganisms, and animals, including both vertebrates (Simpson *et al.*, 1978) and invertebrates (Cohen, 1974). Several recent reviews dealing with biochemical properties of purified lectins, their interaction with cell surfaces and possible functions have appeared (Barondes, 1981; Goldstein & Hayes, 1979; Brown & Hunt, 1978; Lis & Sharon, 1977; Liener, 1976).

Lectins bind carbohydrate-containing receptors of cell surfaces. Their multivalency allows cross linking of several cells, resulting in agglutination. Although agglutination *per se* is not well understood (Brown & Hunt, 1978), cell agglutination provides an

easy detection assay for lectin activity. Erythrocytes are generally employed as test cells because they are readily available. The assay is semiquantitative. Erythrocytes are added to a serial dilution of agglutinin and the agglutination pattern is scored visually, usually after one hour. The reciprocal of the highest dilution showing agglutination is referred to as hemagglutination titer.

Lectins bind specific carbohydrate structures, hence the term lectin derived from the Latin legere (to choose). The carbohydrate specificity of a lectin is usually determined by measuring the capacity of monosaccharides or oligosaccharides to inhibit lectin-induced hemagglutination. Specific inhibition by a particular monosaccharide is one basis for lectin classification (Goldstein & Hayes, 1978). There are five families of lectins which show binding specificity: D-mannose (D-glucose), N-acetyl glucosamine, N-acetyl galactosamine, D-galactose and L-fucose. This classification is an oversimplification of lectin binding specificity, but it permits a comparison of the properties of lectins within a group. The specificity of lectin binding is illustrated by the range of erythrocytes agglutinated by a given lectin. Some lectins will agglutinate erythrocytes of a single species exclusively. Some lectins that interact with human erythrocytes show blood group and

blood subgroup specificity (Brown & Hunt, 1978). Specific oligosaccharides interacting with given lectins were compiled by Uhlenbruck (1981). Detailed studies of lectin carbohydrate binding sites and lectin receptors are discussed in the Goldstein & Hayes review (1978).

Lectins are purified by affinity chromatography, that takes advantage of their specific saccharide binding. Most lectins purified to date are derived from plant seeds. The seeds are ground, extracted with a saline buffer and the lectin adsorbed to its specific carbohydrate immobilized on a support such as Sepharose. After washing out all the contaminants the lectin is eluted with free carbohydrate and is thereby recovered in a highly pure state in a single step. Purification schemes for 20 lectins can be found elsewhere (Brown & Hunt, 1978).

The binding of lectins to cell surface receptors induces various biological activities such as cell agglutination, mitogenic stimulation of lymphocytes, inhibition of phagocytosis, toxicity, inhibition of growth of tumor cells, inhibition of fertilization of ovum by sperm, insulin-like effects on fat cells, induction of platelet release reaction, and others (Nicolson, 1974). Because plant lectins are readily available and easily purified they have

become important medical and research tools in immunology and cell biology.

Some plant lectins bind the surface of quiescent lymphocytes triggering growth, proliferation, and blast cell formation. The blast cells synthesize immunoglobulins as if they had been stimulated by antigens. The morphological and biochemical changes occurring in lectin-stimulated lymphocytes provide a useful model for antigen-induced immune reactions that take place *in vivo*. Lectins will also induce the differentiation of lymphocytes into cytotoxic T cells with the ability to lyse certain target cells. These cells can be obtained from nonimmune animals and are of great interest for the study of the mechanisms of cellular toxicity. The different steps involved in lymphocyte activation by lectins, the factors affecting this activation and what is known of the activation mechanism has recently been reviewed by Lis & Sharon (1977).

The agglutinability of many cell types by lectin is used to probe molecular events taking place at the cell surface. One most important use of lectins is the study of changes in agglutinability accompanying neoplastic transformation. Transformed cells are often found to be agglutinated at much lower concentration of lectin than is required for the corresponding normal cell type. Although the correlation between increased agglutinability and transformation

does not always hold, these studies indicate that in some cases transformation is accompanied by changes in the cell surface which result in facilitated agglutination. The understanding of selective agglutinability of transformed cells may lead to increased understanding of the role of the cell surface in uncontrolled growth, metastasis and resistance to immunological attack. The importance of the use of lectins in this field therefore cannot be overemphasized. A review of increased lectin agglutinability of transformed cells in vitro and in vivo and of the state of our knowledge of the mechanism of cell agglutination has appeared recently (Brown & Hunt, 1978). Lectins are also used to probe cell surface changes associated with differentiation and development of cells and tissues (Brown & Hunt, 1978).

Besides their use in investigating fundamental problems, certain lectins are utilized routinely in clinical laboratories. Lectins that are specific for human erythrocytes of a given blood group or blood subgroup are used for blood type screening and secretor diagnosis (Brown & Hunt, 1978). Lectins that are mitogenic for lymphocytes are useful for the detection of congenital and acquired immunologic deficiencies, the monitoring of the effect of various immunosuppressive and immunotherapeutic manipulations and

the detection of genetic abnormalities in chromosome analysis (Lis & Sharon, 1977).

Lectins can be coupled to solid supports such as agarose beads and serve for affinity chromatography of glycoproteins and polysaccharides. Several immobilized lectins are available commercially to this end. The property of certain lectins to bind specific cells is also exploited for specific fractionation of cells (Lis & Sharon, 1977).

In spite of the extensive use of lectins, their physiological roles in the organisms from which they are derived remains hypothetical. Several lines of evidence indicate that lectins may function in recognition processes, and these have been reviewed by Barondes (1981). Lectins were suggested to play a role in the aggregation of slime molds (Barondes, 1981). Slime molds are eukaryotes that change from discrete amoebae to aggregating colonies upon starvation. The development of cell-cell adhesiveness is correlated with the synthesis of lectins detectable on the cell surface, hence the hypothesis that lectins mediate cell cohesion. A thorough review of slime mold lectin properties and of the evidence bearing on their biological role was done by Bartles *et al.* (1982).

Lectins that may be involved in serum glycoprotein clearance have been found at the cell surface of mammalian hepatocytes

(Ashwell & Harford, 1982). The most studied is a galactose binding lectin that binds asialoglycoproteins. When serum glycoproteins are desialylated, thereby exposing their galactose residues, their survival time in the circulation is drastically reduced. The desialylated glycoproteins presumably bind the hepatic galactose-specific receptor, resulting in internalization of ligand-receptor by endocytosis and degradation of the glycoproteins in the lysosomes. Biologically reactive glycoproteins may be cleared from the serum by this mechanism after they serve a particular physiological function (Ashwell & Harford, 1982). A receptor for D-mannose/N-acetylglucosamine and one for L-fucose are also found at the surface of some hepatocytes. They may also play a role in glycoprotein clearance.

A phosphomannosyl receptor is found in liver, spleen, lung, kidney, and testes. It is believed to play a role in the uptake of lysosomal hydrolases and is suggested to direct newly synthesized acid hydrolases to the lysosomes (Ashwell & Harford, 1982). Cells do not transfer proteins from the endoplasmic reticulum (ER) to lysosomes via an exclusively intracellular route. Newly synthesized lysosomal hydrolases are transported from the ER to the Golgi, packaged in secretion granules and secreted. They are then internalized by endocytosis and directed to the lysosomes (Herzog,

1981). It has been suggested that the phosphomannosyl receptor binds the secreted hydrolases, thereby initiating their endocytosis. Furthermore, intracellular phosphomannosyl receptors would guide the hydrolases to the lysosomes (Ashwell & Harford, 1982).

Several vertebrate lectins have been identified that become prominent at a specific stage in the development of individual tissues (Barondes, 1981). Chick embryonic muscle cells show a large increase in lectin levels correlating temporally with fusion of the cells. This suggests the lectin to mediate myoblast fusion. Another chick embryo lectin was speculated to be involved in neuronal maturation and a third one was prominent in embryonic liver and kidney. The three lectins and their potential role in the differentiative process have been reviewed by Barondes (1981). Another important finding is that, although the lectins are developmentally regulated in some tissues, they are especially prominent in others in adulthood. The same lectins may therefore be utilized in different ways (Barondes, 1981).

Plant lectins have also been suggested to be involved in recognition processes (Barondes, 1981). Lectins derived from leguminous plants may play a role in the rhizobium-legume symbiosis. The lectin-recognition hypothesis states that recognition at infection sites involves the binding of specific legume lectins to

unique carbohydrates found on the surface of the appropriate rhizobial symbiont. Evidence supporting the hypothesis has been reviewed by Dazzo (1980) and Barondes (1981). Models of the contribution of lectins to the infection process can be found in Bauer (1981). There is much support and no decisive negative evidence for the role of soybean lectin and clover lectin in binding specific rhizobium to root hairs. A lectin necessary for specific binding to strains nodulating alfalfa has also been described (Paau et al., 1981). The major exception is associated with the tropical legumes (jack bean, peanut, etc.). Tropical legumes undergo infection by a mechanism different from the root hair infection of temperate legumes (Bauer, 1981). The rhizobia intrude into the legume roots through voids created by lateral root emergence. The recognition processes in these legumes are less specific and have broad boundaries. The recognition hypothesis would not hold for those legumes whose lectins can bind indiscriminately to many rhizobia (Dazzo, 1980).

Legume lectins have been postulated by Hankins & Shannon (1978) to be plant enzymes based on their finding of a lectin with alpha galactosidase activity in mung bean. Later screening of a large number of legume species revealed the presence of alpha-galactosidases without hemagglutination activity in certain legumes

having well known lectins (Hankins *et al.*, 1980). Hemagglutination is therefore a transient property of some legume alpha galactosidases, and lectins are not enzymes. These conclusions are supported by the recent purification from soybean of an alpha-galactosidase that has hemagglutinin properties and is different from soybean lectin (Del Campillo & Shannon, 1982).

Several other roles for plant lectins have been suggested for which there is much less experimental evidence. Leguminous lectins were suggested to act as storage proteins in seeds (Millerd, 1975). This hypothesis stemmed from the fact that legume lectins are present in large amount in the seeds and their level decreases dramatically upon germination. There is, however, no experimental data to support this role and the major storage proteins in seeds do not have lectin activity.

Lectins were suggested to protect legume seeds from attack by insect seed predators (Janzen *et al.*, 1976). This hypothesis was based on the sole observation that addition of black bean lectin to the diet of bruchid beetle results in death of the bruchid larvae.

Lectins were speculated to function in transport of carbohydrates, particularly during extension growth of plant cells, a process requiring deposition of sugars for cell wall formation (Kauss & Glaser, 1974). A lectin in potato tubers has a

glycopeptide domain that bears close resemblance to the glycopeptides derived from the plant cell wall, suggesting that the lectin may be a precursor of the insoluble cell wall material (Ashford & Neuberger, 1980). None of these potential lectin involvements in cell wall growth was demonstrated experimentally.

Lectins were postulated to act as receptors for pathogens and to inhibit the growth of microorganisms (Sequeira, 1978). Wheat germ agglutinin (WGA) inhibits fungal growth (Mirelman *et al.*, 1975). The finding that WGA is localized precisely at potential infection sites of germinating wheat (Mishkind *et al.*, 1982) is consistent with a protective function of the lectin. A protective role against fungal infection was also suggested by the experiments of Etzler and colleagues who found a lectin in the cell wall of leaves and stems of the legume Dolichos biflorus. The lectin had biochemical properties indicating a possible precursor relationship to the seed lectin (Talbot & Etzler, 1978). Its level in the plant cell walls increased during fungal infection or upon wounding of the plant, suggesting an antibody-like function (Etzler *et al.*, 1979; Gibson & Etzler, 1979).

The paucity of data supporting possible roles of plant lectins, except for the legume-rhizobium recognition hypothesis, contrasts

with the extensive use of these lectins in cell biology and immunology.

Interest in the determination of structure of lectins, in order to better understand their interaction with cell surfaces, is growing. Goldstein & Hayes (1978) recently reviewed the biochemical characterization of the best known lectins. Although no generalization can be made about the structure of lectins, those derived from leguminous plants show certain common characteristics. There are two types of leguminous lectins, those containing a single type of subunit and those containing two types of subunits. The single chain lectins are usually homologous tetramers. A notorious exception is the lectin from the red kidney bean which is a tetramer of two different kinds of subunits, one mitogenic for lymphocyte, the other one with hemagglutination properties (Miller *et al.*, 1975). The subunit of the single chain lectins has a molecular weight (MW) of approximately 26,000. The amino acid compositions of the lectins show a high content of acidic and hydroxyamino acids and a low content of sulfur-containing amino acids (Goldstein & Hayes, 1978). The two chain lectins have so far all been found in a single tribe of leguminous lectins, VICIEAE. They are composed of a small alpha-subunit, about 6,000 MW, and a large beta-subunit, 14,000 to 20,000 MW. They have the subunit structure alpha-2-beta-2 and

their amino acid composition is similar to that of single chain lectins.

Four leguminous lectins have had their amino acid sequence established: concanavalin A (ConA) from the jack bean (Wang *et al.*, 1975; Cunningham *et al.*, 1975); lentil lectin (LL) (Foriers *et al.*, 1981); favin from the fava bean (Cunningham *et al.*, 1979; Hopp *et al.*, 1982) and soybean agglutinin (SBA) (Hemperly *et al.*, 1981). Only one of them, ConA, had its crystalline tertiary structure established (Becker *et al.*, 1975; Reeke *et al.*, 1975). While ConA and SBA are tetramers of a unique polypeptide chain, LL and favin have the subunit structure alpha-2-beta-2. The four lectins were found homologous in their sequence of amino acids. The two chain lectins resemble SBA when the beta-chain and alpha-chain are aligned as though they were a single polypeptide chain beta-alpha (Hemperly *et al.*, 1981). A circular permutation was observed between ConA and all the other lectins. Favin beta-alpha, LL beta-alpha and SBA are each homologous to a segment of ConA beginning at residue 120. The homology extends to the carboxyl terminus of ConA and continues, without interruption, through its amino-terminal 119 residues (Cunningham *et al.*, 1979; Foriers *et al.*, 1981; Hemperly *et al.*, 1981).

Favin, LL and ConA belong to the D-mannose (D-glucose) binding family; SBA binds N-acetylgalactosamine (Golstein & Hayes, 1978). In spite of their differences in sugar specificity these lectins appear to have a common evolutionary origin.

Sainfoin lectin (SL) is extracted from the seeds of the leguminous forage crop sainfoin (*Onobrychis viciifolia*, Scop.). It is a dimer of two identical or very similar subunits of 26,000 MW (Hapner & Robbins, 1979). Its amino acid composition is typical of leguminous lectins. The lectin exhibits charge heterogeneity by electrophoretic criteria, interpreted as reflecting the possible presence of allelomorphic or isolectin forms (Hapner & Robbins, 1979). Each subunit bears a short carbohydrate chain linked to an asparagine residue (Namen & Hapner, 1979).

A lectin was isolated from sainfoin roots with similar molecular characteristics to the seed lectin (Hapner & Robbins, 1979) indicating that conclusions from studies performed on the seed lectin should apply to the root lectin. Localization of SL on the legume root by fluorescence microscopy was consistent with a putative role in rhizobium recognition (Hapner & Hapner, 1978). Sainfoin lectin belongs to D-mannose (D-glucose) binding family and it agglutinates cat erythrocytes specifically (Hapner & Robbins, 1979). The lectin is mitogenic for mice lymphocytes (Hapner,

unpublished observations). Because of its narrow specificity the lectin may be very useful as a probe for carbohydrate structure and for isolation of glycoproteins and glycolipids.

CHAPTER TWO  
GOALS AND OBJECTIVES

The principal objectives of this research involve the biochemical and structural characterization of sainfoin lectin, a glycoprotein that is isolated from seeds of the leguminous plant Onobrychis viciifolia, Scop.. The sainfoin lectin molecule is known to be a 52,000 molecular weight dimer containing two identical polypeptide chains of 236 amino acid residues each. Specific research objectives are listed below.

1. Determination of the complete primary structure (amino acid sequence) of sainfoin lectin.
  - a. This objective requires a collaborative arrangement with Dr. R. A. Bradshaw, Washington University, St. Louis, Missouri. The actual protein sequencing will be performed in Dr. Bradshaw's laboratory. Protein and peptide preparation will be done at Montana State University, Bozeman, Montana.
  - b. The established sequence will be compared with that of other leguminous lectins which have been studied.

2. Elaboration of successful biochemical methods to produce peptide fragments from purified sainfoin lectin that are suitable for sequence analysis.
3. Determination of carbohydrate binding, metalloprotein and molecular stability characteristics of sainfoin lectin.

## CHAPTER THREE

### EXPERIMENTAL PROCEDURES

#### Materials

Seeds of sainfoin (Onobrychis viciifolia, Scop., variety Eski) were supplied by the Plant and Soil Science Department, Montana State University. Sephadex G-types resins, Sepharose 4B, Sephadex LH20, WGA-Sepharose, and ConA-Sepharose were obtained from Pharmacia. Biogel A5m was purchased from Biorad, DEAE cellulose from Whatman and Ultrogel Aca44 from LKB. DC-X8-11 was from Dionex Chemical. Ultrafiltration membranes and phenylboronate agarose PBA-30 were obtained from Amicon, equilibrium dialysis membranes from Hoeffler. Cat erythrocytes were purchased from Colorado Serum. Biofluor, D-[<sup>14</sup>C(U)]-glucose, iodo[1-<sup>14</sup>C]acetic acid and K<sup>14</sup>CN were from New England Nuclear. Acrylamide, phenantrenequinone, iodoacetic acid, succinic anhydride, guanidine HCl, beta xylosidase, and thermolysin (protease type X) were obtained from Sigma. The iodoacetic acid was recrystallized from petroleum ether before use. Ninhydrin, methylcellosolve, dansylchloride, trinitrobenzene sulfonic acid, and sodium dodecylsulfate were from Pierce. Bisacrylamide, beta mercaptoethanol and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Aldrich. Cyanogen bromide,

pyridine, acetonitrile and HPLC water were obtained from Baker. Pyridine was distilled (1 g ninhydrin/liter) before use. Dithiothreitol was from Calbiochem, 2-nitro-5-thiocyanobenzoic acid from Eastman. Standard dansyl amino acids were purchased from Seikagaku Kogyo Company. Polyamide sheets for thin layer chromatography of the dansyl amino acids were purchased from Gallard Schlesinger Chemical Company. Trypsin treated with N-tosylphenylalanine chloromethyl ketone was obtained from Millipore Company. Carboxypeptidase B was from Worthington. Sequence grade phenylisothiocyanate, heptane, heptafluorobutyric acid, benzene and ethyl acetate were purchased from Beckman. Polybrene was a gift from Abbott Laboratories. Silica thin layer plates with fluorescent indicator were from Analtech. Noble agar was purchased from Difco. All other chemicals were of reagent grade. Distilled water was used throughout.

#### Purification of Sainfoin Lectin

Sainfoin lectin was purified from the seeds of sainfoin (Onobrychis viciifolia, Scop.), variety Eski, by a modification of the method of Hapner & Robbins (1979). Dehulled and finely ground (200 g) seeds were stirred overnight with one liter PBS (0.01 M sodium phosphate, 0.15 M sodium chloride) containing 0.1 M glucose, 0.01 M ascorbic acid, 3 M sodium azide, pH 7.0. The insoluble

material was removed by squeezing through cheesecloth and subsequent centrifugation at 10,000 X g for 20 min. The soluble extract was dialyzed 48 h against 10 liters PBS with two changes of buffer. Precipitate from the dialyzate was removed by centrifugation at 13,000 X g for 30 min.

The supernatant was submitted to affinity chromatography in a batchwise fashion. Mannose-Sepharose beads (50 ml) prepared as previously described (Fornsted & Porath, 1975) were added to the supernatant solution and the mixture was stirred 3 to 6 h. The beads were collected on a Buchner, washed extensively with PBS and packed into a 2.5 X 10 cm column. The lectin was eluted with 0.5 M glucose in PBS. The lectin was dialyzed extensively against PBS and repurified by affinity chromatography on a 2.5 X 5 cm column of fresh mannose-Sepharose.

#### Gel Filtration of SL

Sainfoin lectin (1 mg/ml) was chromatographed on a 1.5 X 115 cm Ultrogel ACA44 column. Elution was performed with three different buffers: PBS, pH 7.0; 0.01 M sodium acetate, 0.15 M sodium chloride, pH 4.5; 0.01 M sodium acetate, 0.15 M sodium chloride, pH 4.0 (with and without 0.1 M glucose). Flow rate was 22 ml/h and absorbance at 280 nm was recorded. Standards used to calibrate the

column were bovine serum albumin (MW 68,000), ovalbumin (MW 43,000), pepsin (MW 35,000) and chymotrypsinogen (MW 25,700).

#### Affinity Chromatography of SL at Various pH Values

Sainfoin lectin (1 mg/ml) was chromatographed on a mannose-Sepharose column 0.9 X 12 cm. Three buffers were used for the elution: PBS, pH 7.0; 0.01 M sodium acetate, 0.15 M sodium chloride, pH 4.5; 0.01 M sodium acetate, 0.15 M sodium chloride, pH 4.0. After washing with 20 ml buffer, elution was performed at 23 ml/h with a linear glucose gradient, 0-0.1 M glucose in 100 ml. The effluent was monitored at 280 nm and 1 ml fractions were collected.

#### Anion Exchange Chromatography of SL

Sainfoin lectin, 32 mg in 4 ml 5 mM sodium phosphate, 0.1 M sucrose, pH 8.3 was chromatographed on a diethylaminoethyl (DEAE) cellulose column 2.6 X 95 cm at 22 ml/h. Elution was performed with 0.01 M sodium phosphate, 0.1 M sucrose, 3 mM sodium azide, pH 7.9.

Fractions (2 ml) were collected and their absorbance at 280 nm was measured on a spectrophotometer. Fractions that eluted at the column void volume were pooled, concentrated to 8 mg/ml by ultrafiltration over a UM 10 membrane, and adjusted to pH 8.5. The concentrate was chromatographed again on the DEAE cellulose column.

in 0.01 M Tris, 0.1 M sucrose, 3 mM sodium azide, pH 8.5, at 14 ml/h; 1 ml fractions were obtained.

#### Immuno Double Diffusion Agar Gels

Rabbit antiserum to sainfoin lectin previously produced (Hapner & Hapner, 1978) was utilized. Double diffusion tests were done on 5 cm 0.8% Noble agar plates in 0.05 M sodium barbital, pH 8.4 according to Ouchterlony (1948). Antiserum (50  $\mu$ l) was placed in the center well and SL fractions (25  $\mu$ l) were placed in the outer wells. The plates were incubated at 22°C for 24 h.

#### Metal Analysis

Sainfoin lectin was extensively dialyzed against 0.01 M glucose in metal free water. It was then prepared for metal analysis by one of two procedures:

1. 10 mg lectin in 10 ml solution was made 1 mM in ethylenediamine tetraacetate (EDTA), adjusted to pH 7.0 and incubated 15 min at 80°C followed by centrifugation. The supernatant was evaporated to dryness, 1 N nitric acid was added and the sample was aspirated into the atomic absorption instrument.
2. 20 mg water-dialyzed SL was lyophilized and dissolved in 5 ml concentrated nitric acid with heating. Heating was

continued at a temperature just below the boiling point of nitric acid until 1 ml nitric acid was left. After cooling, 2.5 ml of 20,000 ppm potassium nitrate was added and the final volume adjusted to 25 ml with metal free water. The solution was aspirated into the atomic absorption instrument.

In both procedures protein-free blanks were prepared and analyzed. The metals measured were  $\text{Ca}^{++}$ ,  $\text{Mn}^{++}$  and  $\text{Mg}^{++}$ .

#### Equilibrium Dialysis

Equilibrium dialysis was performed in 0.5 ml dialysis cells in PBS that contained 3 mM sodium azide, pH 7.0. Dialysis membranes were washed exhaustively with distilled water before use. Solutions of  $^{14}\text{C}$ -glucose (0.1-10 mM, 2.6-8.7  $\mu\text{Ci}$ ) and protein (1.58 mg/ml), 0.5 ml each, were placed in adjacent compartments of the dialysis cells. The unit, containing eight cells, was rotated for 72 h at room temperature to establish equilibrium. Two 50  $\mu\text{l}$  samples were then removed from each compartment and mixed with 4 ml scintillation mixture (Biofluor). The radioactivity in each sample was determined in a Tricarb model 3320 (Packard) liquid scintillation counter.

### Agglutination Assay

Agglutination tests were performed in PBS in Microtiter plates by adding 25  $\mu$ l 2% cat erythrocytes in PBS, pH 7.0 to serial twofold dilutions of lectin (25  $\mu$ l). When inhibition by sugars was examined, carbohydrate was included in the diluent PBS. Agglutination was evaluated visually after 30 min to 1 h at 22°C.

### Standard Electrophoretic Procedures

Sodium dodecyl sulfate (SDS) electrophoresis was performed in 10% polyacrylamide gels using a phosphate buffer system according to Weber and Osborn (1975). The gels were stained with 0.25% Coomassie blue R-250 in destaining solution (methanol:acetic acid:water, 227:46:227). Protein and peptide samples, 10-50  $\mu$ g in sample buffer containing 1% SDS and 1% beta-mercaptoethanol, were heated 5 min on a boiling water bath prior to electrophoresis. Mobilities were calculated relative to bromophenol blue tracking dye.

Disc electrophoresis in 7% polyacrylamide gels was performed at pH 9.5 in Tris, glycine buffer and at pH 3.8 in beta-alanine, acetic acid buffer using procedures and chemicals provided by Canalco. Gels were stained with Coomassie blue R-250 or, alternatively, with 0.04% Coomassie brilliant blue G-250 in perchloric acid:water, 3.5:96.5 according to Reisner *et al.* (1975).

Gels for isoelectric focusing each contained protein sample (10-50  $\mu$ l) in 1.5 ml water, 0.56 ml of 30% (w/v) acrylamide, 1% (w/v) bisacrylamide in water, 0.056 ml of 40% (w/v) Ampholine Carrier ampholytes, pH 3-10 range (LKB), and 0.13 ml potassium persulfate 1% (w/v) in water. After the gels were polymerized they were focused at 1 mA/tube for 3 to 5 h. Tank electrode solutions were 0.2% sulfuric acid on the anodic side and 0.4% ethylenediamine on the cathodic side. After completion of focusing, the ampholytes were allowed to diffuse out of the gels by soaking the gels 30 min in a solution consisting of methanol:water, 30:70 containing 3.45% (w/v) sulfosalicylic acid and 11.5% (w/v) trichloroacetic acid. The gels were then stained in Coomassie blue R-250 and destained in ethanol:acetic acid:water, 250:80:670.

#### Synthesis of Allyl Alpha-Glycosides

Allyl alpha-glucoside and allyl alpha-galactoside were synthesized by the method of Horejsi (1974): 100 g D-glucose or D-galactose was suspended in 200 ml allyl alcohol containing 6 g dry HCl. The mixture was stirred 4 h at 80°C. After cooling, the solution was neutralized with concentrated ammonia and the allyl alcohol was removed by rotary evaporation at 70°C. The resulting brown syrup was extracted eight times with 100 ml dry acetone. The extracts were combined and evaporated. The solid residue was

dissolved in hot ethanol. In the case of allyl alpha-galactoside, it crystallized readily upon cooling in ice. The ethanol solution containing allyl alpha-glucoside was treated with activated vegetable carbon and concentrated. Anhydrous ether was added to cloudiness and the solution was incubated at  $-10^{\circ}\text{C}$  where it crystallized. The product was recrystallized twice from ethanol:ether, 70:30. Both allyl alpha-glycosides showed a single spot of  $R_f$  0.37 on TLC in butanone:ethanol:water, 75:8:3. The alpha nature of the linkage was confirmed by carbon  $^{13}\text{NMR}$  through comparison of the C-1 chemical shift with standard compounds.

#### Synthesis of Glycosyl Copolymers

Glucosyl and galactosyl copolymers were prepared by the method of Horejsi (1977): 500 mg allyl alpha-glycoside, 1 g acrylamide and 20 mg ammonium persulfate were dissolved in 10 ml water. The solution was boiled on an oil bath for 5 to 9 min. It was then diluted with 20 ml water and extensively dialyzed against water. The carbohydrate content of the glycosyl copolymer was determined spectrophotometrically (Dubois, see carbohydrate analysis) with reference to the corresponding free sugar.

### Affinity Electrophoresis

Solutions used for the polymerization of affinity gels at pH 4.5 were as follows:

- A: 60 ml 1 N potassium hydroxide, 21.5 ml acetic acid, 5 ml N,N,N',N'-tetramethyl ethylene diamine, 13.5 ml water, pH 4.5.
- B: 37.5% (w/v) acrylamide, 1% (w/v) bisacrylamide in water.
- C: 0.35% (w/v) ammonium persulfate in water.

The polyacrylamide affinity gels (7%) were made by mixing 1 ml A with 2 ml B and 4 ml C; glucosyl copolymer and solid free glucose were added to the mixture in amounts needed for the desired final concentrations of immobilized and free carbohydrate; the mixture was then adjusted to 10 ml total volume with water. Sample size was 20  $\mu$ l protein applied to each gel. Disc electrophoresis was performed using the beta alanine: acetic acid buffer system of Canalco, at 5 mA/gel, 10°C for 3½ h. Gels were stained with Coomassie blue R-250 and destained in methanol:acetic acid:water, 227:46:227.

Two affinity gels were electrophoresed for each combination of immobilized glucose and free glucose concentrations. Control affinity gels were polymerized with the amount of galactosyl copolymer required to yield an immobilized galactose concentration

equivalent to the immobilized glucose concentration of the experimental affinity gels.

Solutions used for the polymerization of affinity gels at pH 9.0 were as follows:

- D: 60 ml 1 N HCl, 45.25 g Tris base, 0.3 ml N,N,N',N'-tetramethyl ethylene diamine, H<sub>2</sub>O up to 200 ml, pH 9.0.
- E: 35% (w/v) acrylamide, 0.92% (w/v) bisacrylamide in water.
- C: as for pH 4.5 affinity gels.

Polyacrylamide affinity gels (7%) were made by mixing 2 ml D with 2 ml E and 2 ml C. Glucosyl copolymer and free glucose were added to the mixture in an analogous fashion to the pH 4.0 affinity gels. Disc electrophoresis was performed using the Tris:glycine buffer system of Canalco, at 2 mA/gel for 6½ h. Other procedures were as described for affinity electrophoresis at pH 4.0.

#### Performic Acid Oxidation

The single cysteine residue of SL (Hapner & Robbins, 1979) was oxidized to cysteic acid with performic acid by the method of Hirs (1967). Performic acid reagent was prepared by mixing 1 ml 30% hydrogen peroxide with 19 ml 99% formic acid and allowing the mixture to stand 2 h at 25°C. One mg protein was dissolved in 1 ml formic acid and incubated at -5°C. Methanol (0.2 ml) and 0.5 ml performic acid were added to the protein solution. The reaction

mixture was incubated at  $-5^{\circ}\text{C}$  for  $2\frac{1}{2}$  h. It was then diluted with 25 ml water and lyophilized twice. Cysteic acid was measured by amino acid analysis after acid hydrolysis of the modified protein.

#### Carboxymethylation

Sainfoin lectin was carboxymethylated according to Thomas *et al.* (1981). Briefly, the lyophilized protein was dissolved in 6 M guanidinium chloride, 0.1 M Tris base, 1 mM bis(aminoethyl)-glycoether-N,N',N',N'-tetraacetic acid (metal chelator), pH 9.5. It was reduced by addition of tenfold protein molar excess dithiothreitol, in the dark, under a  $\text{N}_2$  atmosphere, for 2-4 h. The protein was then carboxymethylated at pH 8.0 with threefold molar excess (over total sulfhydryl groups) iodol- $^{14}\text{C}$ acetic acid (specific activity  $7.47 \times 10^8$  cpm/mole). The reaction was stopped after 5 min at  $25^{\circ}\text{C}$  with excess beta-mercaptoethanol. Reagents were removed by dialysis in water and the carboxymethylated protein was lyophilized.

#### Reaction with 5,5'-Dithiobis-(2-nitrobenzoic acid)

Native or succinylated (see below) protein at 1 mg/ml in 0.01 M Tris, 1 mM EDTA, 0.1% (w/v) octylglucoside was incubated at pH 2 for 30 min. The pH was then adjusted to 8.0, fivefold protein molar excess DTNB was added, and the solution was incubated at  $50^{\circ}\text{C}$  for 20

min. Extent of the reaction was estimated by measuring the liberation of 2-nitro-5-thiobenzoate using the molar extinction coefficient of  $13,600 \text{ M}^{-1}$  at 412 nm (Ellman, 1959).

#### Succinylation

Sainfoin lectin was succinylated by a procedure derived from Klotz (1967). The protein (5 mg) was dissolved in 5 ml 0.01 M Tris, 0.1 M NaCl, 0.1 M glucose, pH 8.0. Fiftyfold molar excess (over total protein amino groups) solid succinic anhydride was added in five increments at 10 min intervals. The pH was maintained at 8.0 with 0.1 N NaOH. Excess reagent was removed by extensive dialysis against water. When carboxymethylated protein was succinylated, the succinic anhydride was added to the reaction mixture 10 min after the quenching of the carboxymethylation reaction with beta-mercaptoethanol.

#### Trinitrobenzene Sulfonic Acid (TNBS) Assay

Free amino groups were estimated by colorimetric titration with TNBS according to Means & Feeney (1971). To 1 mg protein in 1 ml PBS were added 1 ml 4% (w/v) sodium bicarbonate, pH 8.5 and 1 ml TNBS 1% (w/v) in water (freshly prepared). The reaction mixture was incubated at 40°C for 2 h followed by the addition of 1 ml 10% (w/v) SDS and 0.5 ml 1 M HCl. Absorbance at 335 nm was measured against a

protein-free blank. The number of amino groups was calculated using the molar extinction coefficient  $14,000 \text{ M}^{-1} \text{ cm}^{-1}$ .

#### Cyanylation

Five mg native or succinylated protein in 5 ml 6 M guanidine HCl, 0.01 M Tris, 1 mM EDTA, pH 9.0, was cyanylated at 25°C with fivefold protein molar excess NTCB or  $^{14}\text{C}$ -NTCB (see below for synthesis). The reaction was measured by the liberation of 2-nitro-5-thiobenzoate, using the molar extinction coefficient of Ellman (1959). When the reaction was complete, the protein solution was acidified to pH 2.0 and filtered through a Sephadex G10 column, 1.5 X 43 cm, in 0.02 M sodium chloride. Elution was monitored by the 280 nm absorbance. The desalted protein was concentrated to 2 ml by ultrafiltration over a UM 10 membrane.

#### Synthesis of 2-Nitro-5-thio[ $^{14}\text{C}$ ]cyano Benzoic Acid (NTCB)

The hemipotassium salt of [ $^{14}\text{C}$ ]NTCB was synthesized according to Degani & Patchornik (1971): 250 mg potassium bicarbonate and 70 mg potassium cyanide were dissolved in 5 ml water followed by the addition of 0.25 mCi  $\text{K}^{14}\text{CN}$ . The mixture was stirred and 100 mg DTNB was added. It was incubated 30 min at 25°C. A 3% (w/v) cyanogen bromide solution in water was slowly added to the reaction mixture until no more color was discharged. The pH was then decreased to 5

with acetic acid:water, 50:50. Nitrogen was bubbled through the solution for 4 h. The solution was then acidified with 6 N HCl until white crystals appeared. After cooling in ice, the crystals were collected and dried in a dessicator. The product was recrystallized from 95% ethanol. The yield of synthesis was 47%. The synthesized [ $^{14}\text{C}$ ]-NTCB had a melting point of 237°C, a specific activity of 910,295 cpm/mg, showed a single spot of Rf 0.70 on TLC in butanol:acetic acid:water, 25:6:25, and exhibited two UV absorption maxima at 291 nm and 202 nm which corresponded with the commercial unlabeled reagent.

#### Cleavage at Cysteine

Cyanylated SL was cleaved at the cyanylated cysteine according to Jacobson *et al.* (1973). Five mg derivatized protein in 5 ml 0.01 M sodium borate, 1 mM EDTA, 6 M guanidinium chloride, pH 9.0, was incubated at 37°C for 40 h. The reaction was quenched by acidifying to pH 6 with acetic acid.

#### Gel Filtration of SL Cysteine Cleavage Product

Five mg peptides derived from the cleavage of cyanylated SL were chromatographed on a Sephadex G50 medium column, 1.5 X 117 cm, at 28 ml/h. The elution buffer was 0.01 M sodium phosphate, 0.1%

SDS, pH 7.0. Fractions (1.1 ml) were collected and their absorbance at 230 nm was measured.

#### Gel Filtration of Succinylated SL Cleaved at Cysteine

Succinylated protein cyanylated with  $^{14}\text{C}$ -NTCB and cleaved at cysteine was gel filtered through a Sephadex G25 fine column, 1.5 X 115 cm. The column was eluted with 0.02 M ammonium bicarbonate, pH 6.3 in one case and 3 M guanidinium chloride, 0.01 M sodium phosphate, pH 7.0 in another case. Flow rate was 29 ml/h.

Fractions of 1.8 ml were collected and their absorbance at 230 nm was measured. The fractions collected from the chromatography in 3 M guanidinium chloride were read at 280 nm because of the high UV absorbing background of the solvent at 230 nm. Aliquots of the fractions (0.4 ml) were mixed with 4 ml scintillation mixture (Biofluor) and counted for 10 min in a Tricarb liquid scintillation counter.

#### Partial Acid Hydrolysis

Acid sensitive peptide bonds of SL were cleaved as described by (Fraser et al., 1972). Protein (20 mg) in 4 ml 6 M guanidinium chloride, 10% (v/v) acetic acid, pH 2.5, was incubated at 40°C for 120 h. The digest was then adjusted to pH 4.5 and submitted to gel filtration.

The partial acid hydrolyzate was fractionated on a Biogel A5m column, 1.5 X 110 cm, at 5.4 ml/h. The elution buffer was 6 M guanidinium chloride, 0.1 M sodium acetate, pH 4.5. One ml fractions were collected and their absorbance at 230 and 280 nm was recorded.

#### Digestion with Trypsin

The method used was as follows, unless otherwise indicated: 2.8  $\mu$ moles (73 mg) carboxymethylated succinylated SL in 35 ml 1 mM Tris, 1 mM EDTA, pH 8.0 was incubated at 50°C for 30 min. Trypsin (1 mg/ml) was dissolved in 1 mM HCl, 1 mM calcium chloride and kept at 4°C prior to its use. Trypsin (1%, w/w) was added to the protein substrate and the pH was maintained at 8.0 with 0.1 N NaOH. When base uptake ceased, the solution was acidified to pH 2.0 with HCl and incubated at 50°C for 1 h. The pH of the solution was then readjusted to 8.0 and a second trypsin addition was made. The digestion was continued until base uptake stopped. The tryptic digest was held at 4°C until fractionation at pH 3.5.

#### Digestion with Thermolysin

One  $\mu$ mole carboxymethylated succinylated SL was dissolved in 3 ml 0.12 M ammonium bicarbonate, 1 mM calcium chloride, pH 8.0. Thermolysin (1% w/w) was added to the protein substrate. The

reaction mixture was incubated at 25°C for 1 h and submitted to gel filtration on Sephadex G25 immediately.

#### Digestion with Carboxypeptidases

Digestion with carboxypeptidase B was as follows. An aliquot containing 20 nmoles trypsin-treated SL in 200  $\mu$ l 0.2 M Tris, pH 8.0 was incubated with 10  $\mu$ g carboxypeptidase B (Ambler, 1972) at 37°C for 2 h. The reaction mixture was acidified to pH 2.5 with acetic acid. One ml of 0.2 M sodium citrate, pH 2.2, was added and the solution was centrifuged to remove any precipitate. The supernatant solution was analyzed for its basic amino acid content with the amino acid analyzer. An enzyme blank was prepared and analyzed similarly.

Digestion of SL tryptic peptides with carboxypeptidase Y was performed according to Hayashi (1977).

#### Fractionation of SL Tryptic Digest at Low pH

In order to choose a pH for the fractionation of the tryptic digest, aliquots of tryptic digest were adjusted to different pH values and centrifuged. Absorbance at 280 nm of the supernatants were recorded.

Trypsinized SL was routinely adjusted to pH 3.5 and incubated at 0°C for 30 min. It was then centrifuged and precipitated and

supernatant solutions were separated. The soluble peptides were adjusted to pH 7.0 and gel filtered on Sephadex G25 or chromatographed on a cation exchanger. The insoluble peptides were dissolved in 0.02 M ammonium bicarbonate, pH 7.9, and submitted to gel filtration on Sephadex G50.

#### Gel Filtration of the pH 3.5 Soluble SL Tryptic Peptides

One  $\mu$ mole of pH 3.5 soluble tryptic peptides was concentrated to 4 ml by rotary evaporation and chromatographed on a Sephadex G25 fine column, 2.5 X 120 cm, at 35 ml/h. Elution buffer was 0.02 M ammonium bicarbonate adjusted to pH 6.3 with carbon dioxide or, alternatively, 1 mM acetic acid. Fractions (2.5 ml) were collected and their absorbance at 230 nm was measured. When the peptides were derived from the digestion with trypsin of SL previously carboxymethylated with iodo- $^{14}$ C acetic acid, 100  $\mu$ l aliquots of the gel filtration column were mixed with 5 ml scintillation mixture and counted for 10 min in a Tricarb liquid scintillation counter.

Ultraviolet absorption spectra of the peptides were obtained on a Varian Techtron 635 recording spectrophotometer.

#### Repurification of T2 and T10 on LH20

The fraction pool from Sephadex G25 chromatography, containing the peptide of interest, was concentrated to 1 ml by rotary

evaporation and chromatographed on a Sephadex LH20 column, 0.9 X 95 cm, at 4.5 ml/h. The column was eluted with water. Fractions (1 ml) were collected and their absorbance at 230 nm was recorded. In the case of peptide T2, 10  $\mu$ l aliquots of the fractions were counted 5 min for radioactivity.

Cation Exchange Chromatography of the pH 3.5 Soluble SL Tryptic Peptides

One  $\mu$ mole of pH 3.5 soluble tryptic peptides were lyophilized, dissolved in 1.5 ml 30% (v/v) acetic acid, 0.4% (v/v) pyridine (in water), pH 2.5, and chromatographed on a DC-X8-11 column, 0.9 X 20 cm, at 30 ml/h according to the method of Bradshaw *et al.* (1980). After loading the sample, elution was performed with sample buffer for 5 min. A four chamber buffer gradient was then started which consisted of: a) 200 ml sample buffer; b) 200 ml 27.8% (v/v) acetic acid, 1.61 % pyridine, pH 3.1; c) 200 ml 20% (v/v) acetic acid, 4.03% (v/v) pyridine, pH 3.7; d) 200 ml 14.3% (v/v) acetic acid, 16.1% (v/v) pyridine, pH 5.0. The chromatography was monitored with ninhydrin with an automated peptide analyzer. Three ml fractions were collected.

#### Gel Filtration of the pH 3.5 Insoluble SL Tryptic Peptides

One  $\mu$ mole of pH 3.5 insoluble tryptic peptides were dissolved in 3 ml 0.02 M ammonium bicarbonate, pH 7.9, and chromatographed on a Sephadex G50 superfine column, 2.5 X 120 cm, at 16 ml/h. Elution buffer was 0.02 M ammonium bicarbonate adjusted to pH 6.8 with CO<sub>2</sub>. Three ml fractions were collected and their absorbance at 230 nm was recorded.

#### Repurification of T4 and T7 by High Performance Liquid Chromatography (HPLC)

The Sephadex G50 chromatographic pools containing the peptides to be repurified were concentrated to 2 ml by ultrafiltration over a UM 02 membrane and aliquots (200  $\mu$ l) were chromatographed on a Beckman Ultrasphere ODS HPLC column preequilibrated with 0.01 M ammonium bicarbonate, pH 7.0. Elution was performed at 0.5 ml/min with the following successive buffers: 0.01 M ammonium bicarbonate for 3 min; 20% acetonitrile in 0.01 M ammonium bicarbonate for 3 min; a linear gradient of 20% to 35% acetonitrile in 0.01 M ammonium bicarbonate for 30 min; 35% acetonitrile in 0.01 M ammonium bicarbonate for 5 min. Absorbance at 280 nm was monitored with a Perkin Elmer UV monitor, model LC75. Peaks of 280 nm absorbance were collected manually, lyophilized twice, and analyzed for amino acid content.

#### Digestion of T6 with Beta Xylosidase

T6 (220 nmoles) in 1 ml 0.2 M sodium acetate, 0.1 M sodium chloride, pH 5.5, was incubated with 0.5 units beta xylosidase at 37°C for 4 h. The reaction was quenched by adjusting the pH to 7.2. The digest was chromatographed on a ConA-Sepharose column, 1.5 X 2.5 cm, in 0.02 M Tris, 0.1 M sodium chloride, 1 mM calcium chloride, pH 7.2, at 2 ml/h. The elution was monitored by the absorbance at 230 nm.

#### Boron Chromatography of T6

T6 (200 nmoles) was chromatographed on a phenyl boronate agarose PBA-30 column, 1.5 X 1.5 cm, in 0.05 M ammonium carbonate, pH 9.1, at 2 ml/h. Absorbance at 230 nm was recorded.

#### Chromatography of T6 on WGA-Sepharose

T6 (200 nmoles) was chromatographed on a wheat germ agglutinin (WGA)-Sepharose column, 1.5 X 1.5 cm, in 0.02 M Tris, 0.1 M sodium chloride, 1 mM calcium chloride, 1 mM manganese chloride, pH 7.0, at 2 ml/h. The elution was monitored through the absorbance at 230 nm.

#### Carbohydrate Analysis

Neutral carbohydrates were measured colorimetrically by the method of Dubois *et al.* (1956) as modified by Misaki & Goldstein (1977). One ml of an aqueous solution of glycopeptide was mixed

with 0.5 ml 5% (v/v) phenol in water and 3 ml concentrated sulfuric acid. The solution was incubated at 25°C for 1 h and its absorbance at 490 nm was measured. D-Glucose was used to prepare the standard curves.

Glucosamine was identified on a Beckman 120 C amino acid analyzer after hydrolysis of the glycopeptide.

#### Gel Filtration of the Thermolytic Peptides

The thermolytic digest of SL was chromatographed on a Sephadex G25 fine column, 2.5 X 120 cm, at 29 ml/h. The elution buffer was 0.02 M ammonium bicarbonate, pH 6.4. Fractions (2.8 ml) were collected and their absorbance at 230 nm was recorded. The fractions were pooled appropriately and lyophilized twice.

#### Cation Exchange Chromatography of the Thermolytic Peptides

The thermolytic peptide fractions were dissolved in 1 ml acetic acid:water, 50:50, and chromatographed on the Beckman AA-15 column, 0.9 X 23 cm, of the amino acid analyzer. Gradient elution was performed at 40 ml/h in exactly the same fashion as the one described in "Cation Exchange Chromatography of the pH 3.5 Soluble Tryptic Peptides." At the end of the gradient, the column was eluted with the final buffer for 1.5 h followed by 6 M pyridine for 0.5 h. Aliquots (0.3 ml) of every third fraction (3.5 ml) were

lyophilized and submitted to an arginine fluorescent spot test. Aliquots of the arginine positive fractions were hydrolyzed in base and analyzed with ninhydrin in order to describe completely the peptide elution pattern.

#### Arginine Detection

Arginine was detected by the method of Yamada & Itano (1966). The lyophilized fractions to be analyzed were dissolved in 30  $\mu$ l acetone:water, 50:50, and spotted on 3 MM Whatman paper, 10  $\mu$ l at a time. The paper was dipped in a freshly made mixture of equal volumes of phenantrenequinone 0.02% (w/v) in ethanol and 10% (w/v) sodium hydroxide in 60% ethanol. It was air dried for 30 min and arginine fluorescence was visualized under a UV lamp.

#### Ninhydrin Analysis

Fractions to be analyzed were first submitted to alkaline hydrolysis according to Hirs (1967): the fractions were dried in an oven at 110°C, 0.15 ml 13.5 N NaOH was added, and the samples were autoclaved at 121°C for 20 min. Ninhydrin analysis was performed according to Cocking and Yemm (1954): to each fraction were added 0.25 ml acetic acid, 0.5 ml 0.2 M sodium citrate, pH 5.0, 0.2 ml ninhydrin 5% (w/v) in methylcellosolve, and 1 ml 0.01 M sodium cyanide 2% (v/v) in methylcellosolve. The solutions were heated 15

minutes on a boiling water bath. Absorbances (570 nm) were measured after the samples had cooled.

#### Amino Acid Analysis

Peptides were hydrolyzed with 1 ml 6 N HCl in evacuated glass tubes at 110°C for 18 h according to Spackman *et al.* (1958). Amino acid analyses were performed on a Beckman 120C or a Durrum D-500 analyzer.

#### End Group Analysis

Two methods were used: a) 100 nmoles peptide in 0.2 M sodium bicarbonate, pH 8.4, was added forty-twofold molar excess iodoacetic acid over total amino groups, readjusted to pH 8.4 and incubated at 50°C for 19 h. The alkylated sample was evaporated to dryness, hydrolyzed with 2 ml 6 N HCl and analyzed with the amino acid analyzer. The results were compared to those obtained with another sample of the peptide treated in the same fashion but without addition of alkylating agent.

b) Alternatively, the peptide was analyzed with the dansyl chloride method (Gray, 1972): 20 nmoles peptide was dissolved in 50  $\mu$ l 0.2 M sodium bicarbonate in ammonia free water, 50  $\mu$ l dansyl (dimethyl-amino-naphthalenesulfonyl) chloride, (5 mg/ml in acetone) was added and the solution incubated at 37°C for 3 h. The

solution was evaporated to dryness with a stream of filtered air, 150  $\mu$ l 6 N HCl was added and the sample was hydrolyzed for 16 h at 110°C. It was then dried under vacuum, 25  $\mu$ l 95% (v/v) ethanol was added, followed by centrifugation. Four  $\mu$ l of the supernatant was spotted in the corner of a 5 X 5 cm polyamide sheet. The sheet was developed with formic acid 1.5% (v/v) in water in the first dimension and with toluene:acetic acid, 9:1:, followed by ethylacetate:methanol:acetic acid, 20:1:1, in the second dimension. The fluorescent dansylated amino acid derivatives were revealed under a UV lamp. Amino acid identifications were done by comparison with standard dansyl amino acids.

#### Sequence Analysis

All polypeptide chains were sequenced automatically in a Beckman 890 C Sequencer. The analyses were performed according to Edman & Begg (1967) using a 0.33 M Quadrol buffer degradation program (Thomas et al., 1981) modified from that of Brauer et al. (1975). One degradation cycle is briefly described as follows. The sample in the spinning cup was dissolved in 0.33 M Quadrol [N,N,N',N' tetrakis-(2 hydroxypropyl)-ethylene diamine]; benzene precipitated the peptide and carried off the excess phenylisothiocyanate; ethylacetate washed out the Quadrol and the

breakdown products of phenylisothiocyanate; anhydrous heptafluorobutyric acid cleaved off the amino terminal amino acid as the anilino thiazolinone derivative of the amino acid; butylchloride extracted the cleaved product that was transferred to a fraction collector. About 3 mg polybrene was included with the peptide to diminish its washout from the spinning cup.

The anilinothiazolinones from each cycle were converted to the corresponding phenylthiohydantoins in the following way: the samples in butylchloride were dried at 50°C with a nitrogen stream, 200  $\mu$ l 1 N HCl with 1  $\mu$ l/ml beta mercaptoethanol was added and conversion was done at 80°C for 10 min.

The phenylthiohydantoins were extracted twice with 750  $\mu$ l ethyl acetate. The aqueous phase was saved. The extracts were combined, dried under nitrogen, and dissolved in 50  $\mu$ l ethylacetate. Most residues were identified by a combination of one-dimensional thin layer (Jacobs & Niall, 1975) and gas chromatography (Pisano & Bronzert, 1969). A portion (20-30%) of the ethylacetate phase was spotted in the corner of a 20 X 20 cm silica plate. The plate was developed with methylene chloride:acetic acid, 30:7, dried and visualized under a UV lamp. Derivatives were identified by comparison with standards run in parallel with the unknown on the thin layer plate.

The phenylthiohydantoin of S-[<sup>14</sup>C]-carboxymethylcysteine was identified by either liquid scintillation counting or autoradiography of the thin layer plate, or both. The phenylthiohydantoins of histidine and arginine were identified by spot tests of the aqueous phases (Easley, 1965; Yamada & Itano, 1966).

In the later stages of the work, phenylthiohydantoins were identified by high performance liquid chromatography using a 4.6 mm X 25 cm Altex Ultrasphere ODS 5  $\mu$ m column, 15 mM sodium acetate (pH 5.5) as the aqueous phase, methanol:acetonitrile (17:3) as the mobile phase and detection at 269 nm (Gordon *et al.*, 1982).

## CHAPTER FOUR

### RESULTS

#### Section 1. Sainfoin Lectin Stability

##### Gel Filtration of SL

A column of Ultrogel ACA44, 1.5 X 115 cm, was calibrated with standard proteins (Figure 1). Regression analysis of the plot  $\log MW$  vs. VE yielded a correlation coefficient of  $-0.98$ . Sainfoin lectin was gel filtered on this column at several pH values. In all cases the lectin eluted as a single peak. The elution volume of the peak was correlated to a molecular weight with the plot of Figure 1. Table 1 shows the apparent molecular weight and the recovery of SL when it was gel filtered at pH 7.0, 4.5, 4.0 and 3.0. The molecular weight of the protein decreased from 51,700 at pH 7.0 to 32,400 at pH 4.0. Recovery of the protein decreased when the pH of the elution buffer was decreased. Recovery at pH 4.0 depended upon the presence of interacting carbohydrate. The lectin was completely lost at pH 4.0 in absence of glucose and partially recovered in presence of 0.1 M glucose. However, it was lost at pH 3.0 independent of the presence of glucose.

The elution volume of gel filtered SL depended upon the proportion of monomer and dimer species present under the

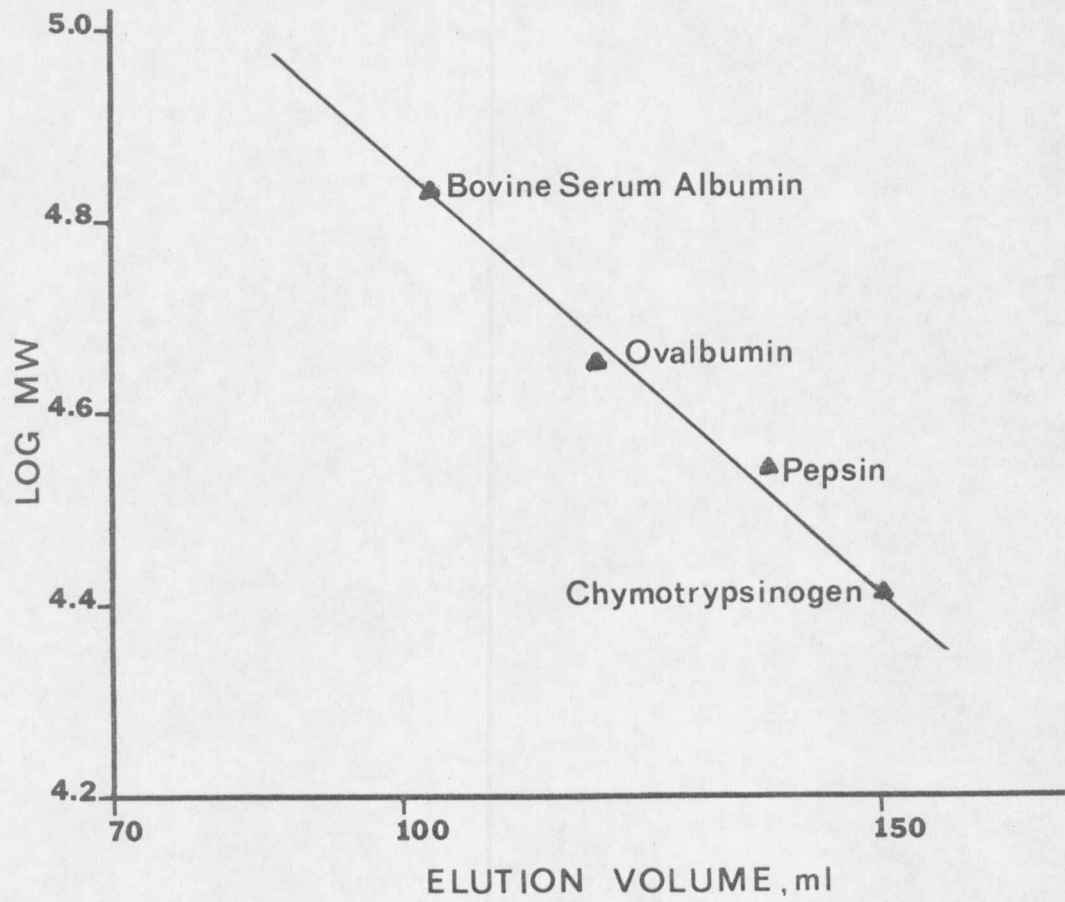


Figure 1. Calibration of Ultrogel AcA44 column, 1.5 X 115 cm

Table 1. Behavior of SL on Ultrogel AcA44

pH	elution volume	apparent MW	recovery
7.0	115 ml	51,678	98%
4.5	135 ml	34,993	76%
4.0	a. $\infty$ in absence of glucose		0%
	b. 139 ml in presence of 0.1 M glucose	32,368	54%
3.0	$\infty$ in presence of glucose		0%

chromatographic conditions. Hapner & Robbins (1979) previously demonstrated SL monomerization at pH 4.0. In the experiments reported here the lectin exhibited an apparent molecular weight of 32,400 at pH 4.0, as opposed to 26,000 for the monomer species. The lectin was presumably incompletely monomerized at pH 4.0. The discrepancy in the results may have been due to the different gel filtration media employed in the two studies. Quaternary changes associated with variations in pH are reported in the literature for concanavalin A (ConA) (McKenzie *et al.*, 1972), wheat germ agglutinin (WGA) (Nagata & Burger, 1974), peanut agglutinin (PNA) (Fish *et al.*, 1979) and mung bean lectin (del Campillo *et al.*, 1981). It is possible that these lectins, including SL, may undergo changes of

quaternary structure in vivo. These changes would be accompanied by alterations in valency that could result in alterations in activity and could be of importance to the physiological role of the lectins. The complete loss of SL during the gel filtration at low pH observed in the experiments reported here was interpreted as denaturation of the protein. The denatured protein presumably precipitated in the Ultrogel matrix or eluted in such a large volume that it could not be detected. Hapner & Robbins (1979) previously obtained evidence for SL denaturation at low pH. The protein cannot be electrophoresed on polyacrylamide gels at pH 2.0 and 2.3; at pH 2.0 it precipitates at the top of the gel. At pH 2.3 it yields a diffuse pattern. Sainfoin lectin is therefore unstable at pH 4.0 and below. In addition, the experiments reported here demonstrated that the lectin could be partially protected from denaturation at pH 4.0 by the addition of glucose. Binding to the carbohydrate site may therefore stabilize the lectin toward low pH denaturation.

#### Solubility of SL in Aqueous Solution

When SL was extensively dialyzed against water or PBS, the maximum concentration of soluble protein achieved at 25°C was about 1 mg/ml. However, in the presence of 0.1 M glucose or sucrose the protein was soluble at concentrations of 10 mg/ml and above.

The increased solubility of SL in the presence of carbohydrate, together with the increased stability of the protein at pH 4.0 in the presence of carbohydrate, supported the possibility of a stabilizing conformational change induced by sugar binding to the carbohydrate site. Such conformational changes are reported in the literature for other lectins and are reflected, among other effects, by altered ultraviolet absorption spectra in the absence and presence of interacting sugars (Matsumoto *et al.*, 1980).

#### Affinity Chromatography of SL at Various pH Values

Sainfoin lectin was chromatographed on mannose-Sepharose at pH 7.0, 4.5 and 4.0. The lectin was eluted from the affinity matrix with a shallow gradient of glucose. Figure 2 shows that SL was completely retained by the mannose-Sepharose at pH 7.0 and 4.5, while only 13% was retained at pH 4.0. The lectin peak eluted with the glucose gradient was much broader in the case of the affinity chromatography at pH 4.5 than at pH 7.0. Total protein recoveries from the affinity chromatographies at pH 7.0, 4.5 and 4.0 were 92%, 71% and 66%, respectively.

The difference of SL behavior on mannose-Sepharose at pH 4.5 and 4.0 was interpreted to be due to protein denaturation at pH 4.0. The monomer species of SL that is the predominant form of the protein at pH 4.5 (see gel filtration of SL above) would bind

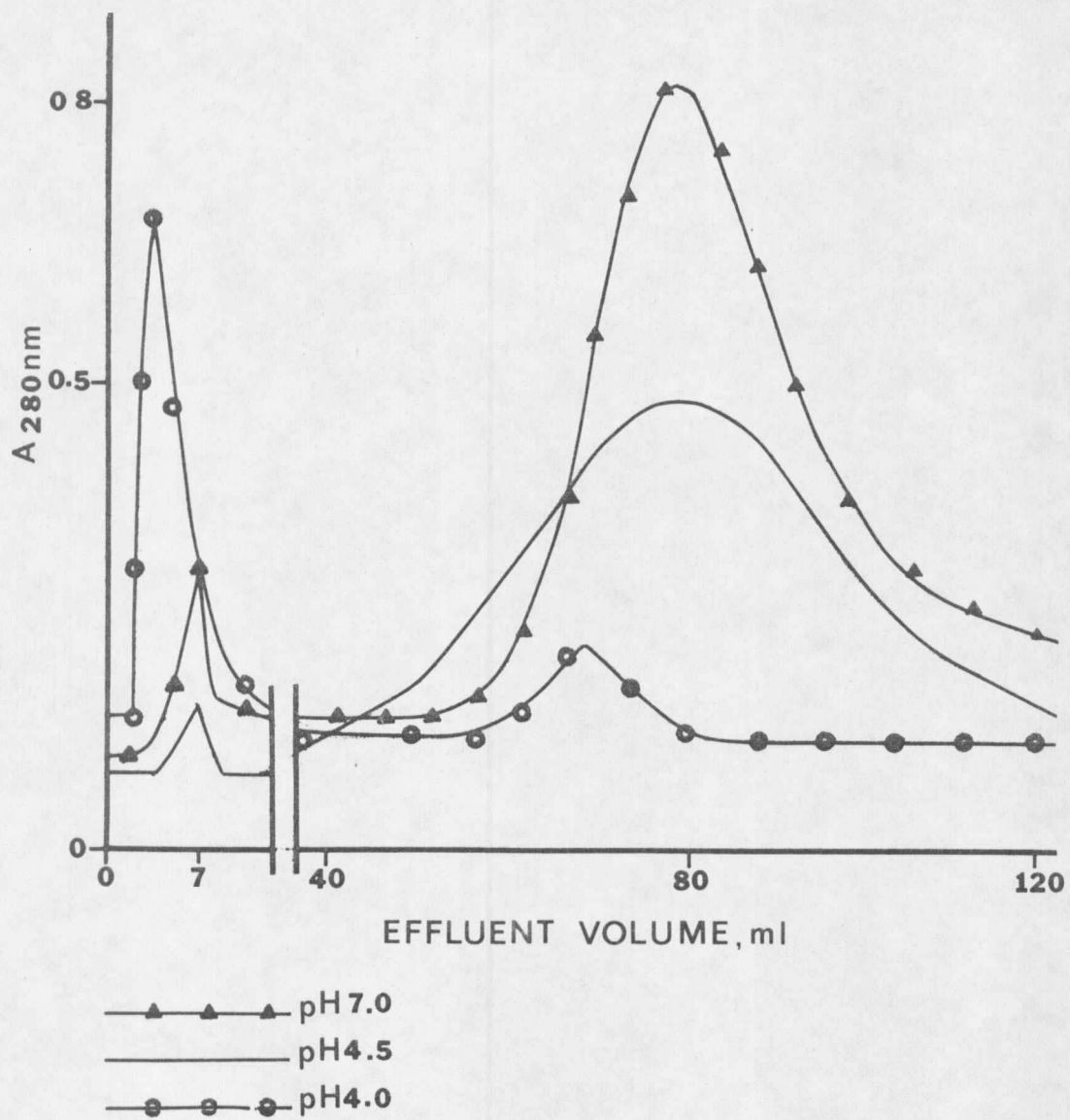


Figure 2. Affinity chromatography of SL on mannose-Sepharose at different pH values

mannose-Sepharose. At pH 4.0, increased denaturation of the monomer would suppress its carbohydrate binding ability. The broadness of the SL peak eluted with the glucose gradient from the affinity matrix at pH 4.5 suggested the presence of different species with similar but non-identical affinities toward the immobilized mannose or the free glucose, or both. These species could be the monomeric and dimeric forms of the protein. The results of affinity electrophoresis presented below support this interpretation.

#### Trypsin Digestion of SL

Sainfoin lectin was resistant to trypsin at pH 8.0, 25°C using a 10% weight ratio of enzyme to protein substrate and a 5 h incubation time. The absence of tryptic cleavage was demonstrated by the absence of base uptake during the incubation with the enzyme and the persistence of intact protein as shown by SDS electrophoresis. Addition of glucose to the reaction mixture did not induce trypsin digestion. Preincubation of SL at pH 2.5, 40°C for 30 min or at pH 11.0, 37°C for 15 min did not make the protein sensitive to trypsin digestion at 40°C.

Sainfoin lectin therefore appeared totally resistant to trypsin in its native conformation in the presence and absence of interacting carbohydrate. Denaturation of the protein at low or high pH did not expose any tryptic sites. It is possible that the

denatured molecules formed a trypsin resistant aggregate or that they renatured quickly when the pH was adjusted to 8.0. The extreme trypsin resistance of SL lead to the hypothesis that the protein could be a trypsin inhibitor. Sainfoin lectin did not affect hydrolysis of the synthetic substrate p-toluenesulfonyl-L-arginine methyl ester by trypsin, indicating that the trypsin active site was not blocked. Trypsin resistance of SL was therefore due to the unavailability of susceptible peptide bonds rather than possible inhibition of the trypsin activity.

Partial digestion of SL with 1% trypsin was observed at pH 8.0, 40°C after the protein was preincubated at pH 11.0, 60°C for 20 min. Digestion of the trypsin treated protein with carboxypeptidase B liberated 4 out of 11 lysine residues and 4.6 out of 12 arginine per monomer lectin. Approximately 36% of lysine and arginine bonds were cleaved by trypsin. When SDS electrophoresis was performed on the tryptic digest, only intact lectin was visible on the gels after the staining-destaining procedure, indicating the absence of large tryptic peptides. It was found that alkylation with iodoacetic acid or disulfide interchange with DTNB of the single cysteine in SL induced at least partial trypsin sensitivity. Complete digestion resulted after incubation at 50°C, pH 2.0 for 1 h prior to a second

trypsin addition. Details of the conditions for complete trypsin digestion are described in Experimental Procedures.

The results presented here demonstrated that partial trypsin digestion of SL did not result in preferential cleavage of a few peptide bonds. Instead a population of denatured molecules was completely digested while the others remained intact. The absence of trypsin sensitive peptide bond suggested that SL did not have a protease sensitive site analogous to the natural cleavage site of ConA, Asn118-Ser119 (Wang *et al.*, 1971). The above results indicated that derivatization of SL cysteine sensitized the protein toward trypsin digestion and further incubation at low pH and high temperature completely denatured the protein. Derivatization of SL was done in the presence of metal chelator and strong denaturant. It is probable that sequestration of metals was at least partially responsible for SL denaturation.

#### Characteristics of Succinylated SL

Sainfoin lectin was succinylated by the method described in the Experimental Procedures. Reaction of an aliquot of the succinylated protein with the amino group reagent TNBS revealed the presence of one underivatized amino group per lectin monomer. The lectin was therefore 90% succinylated. Figure 3 shows the behavior of succinylated SL on mannose-Sepharose at pH 7.0. One-half of the

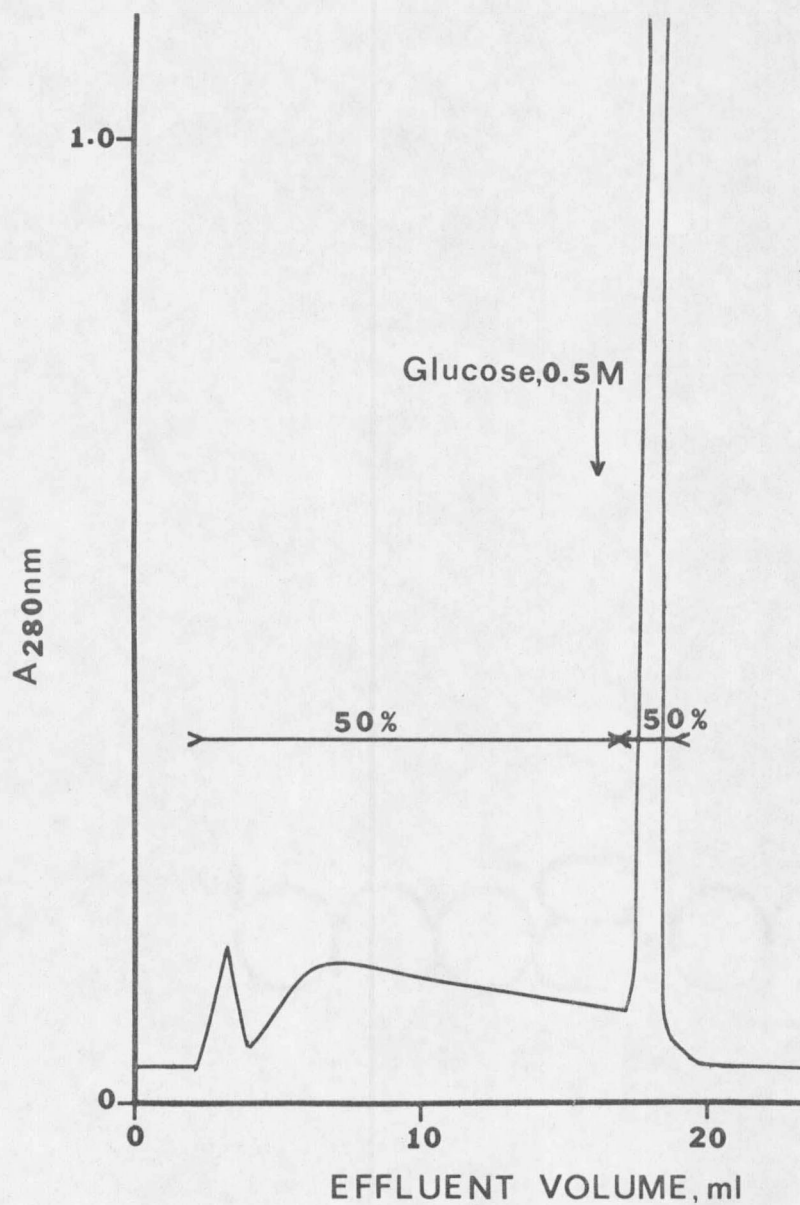


Figure 3. Affinity chromatography of 90% succinylated SL on mannose-Sepharose

protein was only retarded by the matrix and eluted with carbohydrate free PBS. The other 50% was retained and eluted with 0.1 M glucose, suggesting that 90% succinylated SL retained some binding activity. Figure 4 shows the elution profile of the modified protein on the previously calibrated Ultrogel AcA44 column (Figure 1). Two species eluted, of apparent molecular weights 59,000 and 48,000, possibly corresponding to succinylated dimer and succinylated monomer. The succinylated protein was incubated with 1% trypsin at 25°C, pH 8.0 for 2 h. No decrease in intact lectin was apparent by SDS electrophoresis and no tryptic fragments were observed.

The experiments reported above suggest that 11 out of the 12 free amino groups of SL could be succinylated without prior denaturation of the protein. The unmodified amino group could have been any one of the 11 lysine residues or the protein alpha-amino group. This specific residue would be protected from acylation by its local environment in the protein native conformation. Alternatively, the 10% unmodified amino groups could be randomly distributed. The succinylated protein contained two species of 59,000 and 48,000 MW. These MW values differed from those of SL dimer and monomer. Succinylation may have induced a conformational change in the protein resulting in a change in the Stokes radius of the molecule and its apparent molecular weight. It is conceivable

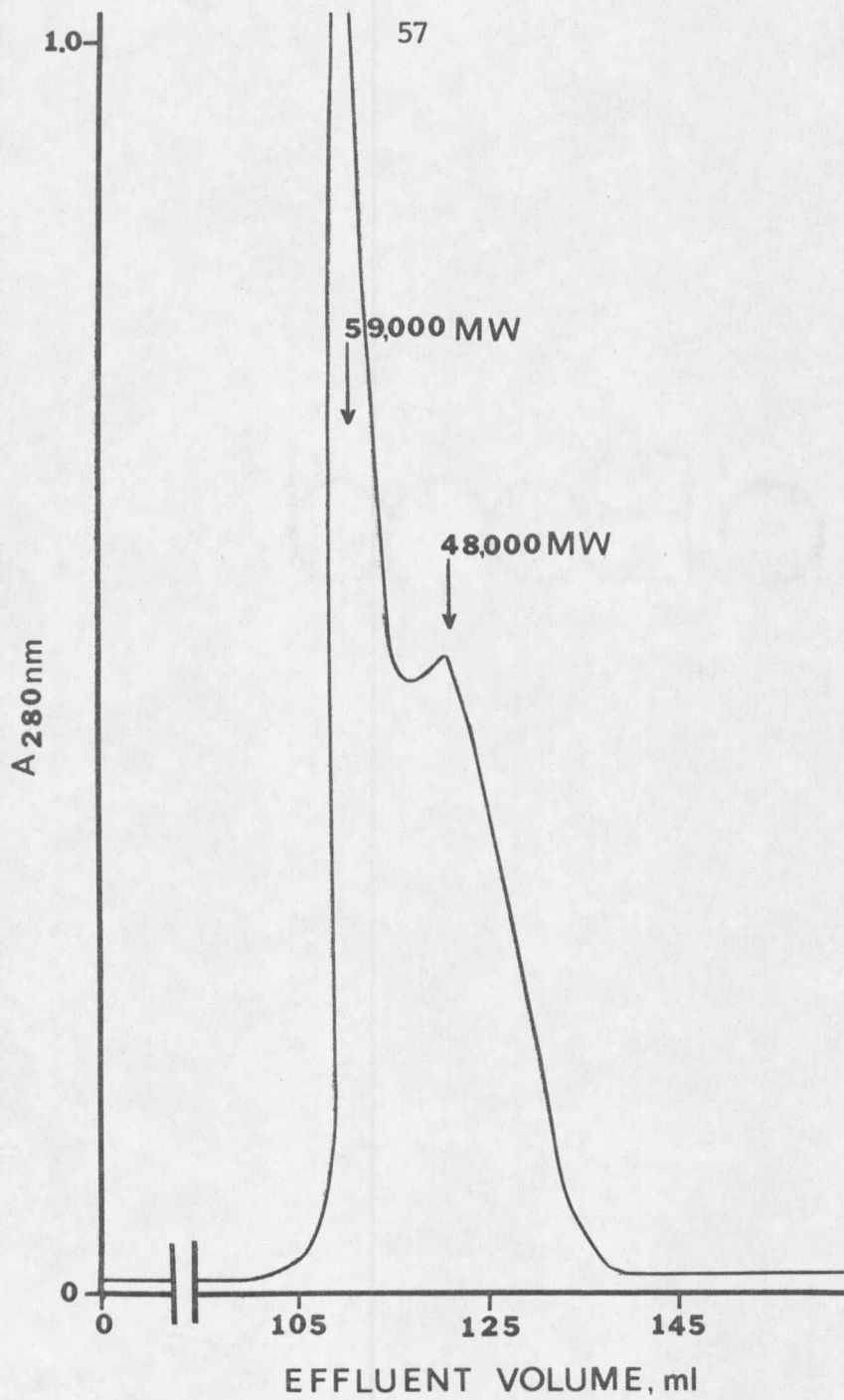


Figure 4. Gel filtration of 90% succinylated SL on Ultrogel AcA44

that the two species were succinylated dimer and succinylated monomer. Predominance of the largest species suggest that 90% succinylated lectin was mostly in the dimer state. The modified protein retained its trypsin resistance. No arginine sites were therefore exposed as a result of succinylation.

The 90% acylated SL was completely succinylated after incubation of the protein at pH 11.0 for 30 min. No free amino group could be detected with TNBS after this additional reaction. Eighty three percent of the completely succinylated SL eluted from mannose Sepharose in absence of free interacting carbohydrate (Figure 5), suggesting that the protein had lost its binding activity. Figure 6 shows the behavior of the completely modified lectin on Ultrogel AcA44. Two species eluted with apparent MW values of 63,100 and 49,000 probably corresponding to 100% succinylated dimer and monomer, respectively. When the modified protein was heated at 60°C for 30 min prior to gel filtration, it eluted mostly as a single species of 39,000 MW (Figure 7). The twice succinylated protein was still trypsin resistant at 25°C. Conditions for the successful trypsin digestion of the succinylated protein were the same as for the native protein, *i.e.*, derivatization of the unique cysteine and denaturation by low pH and heat.

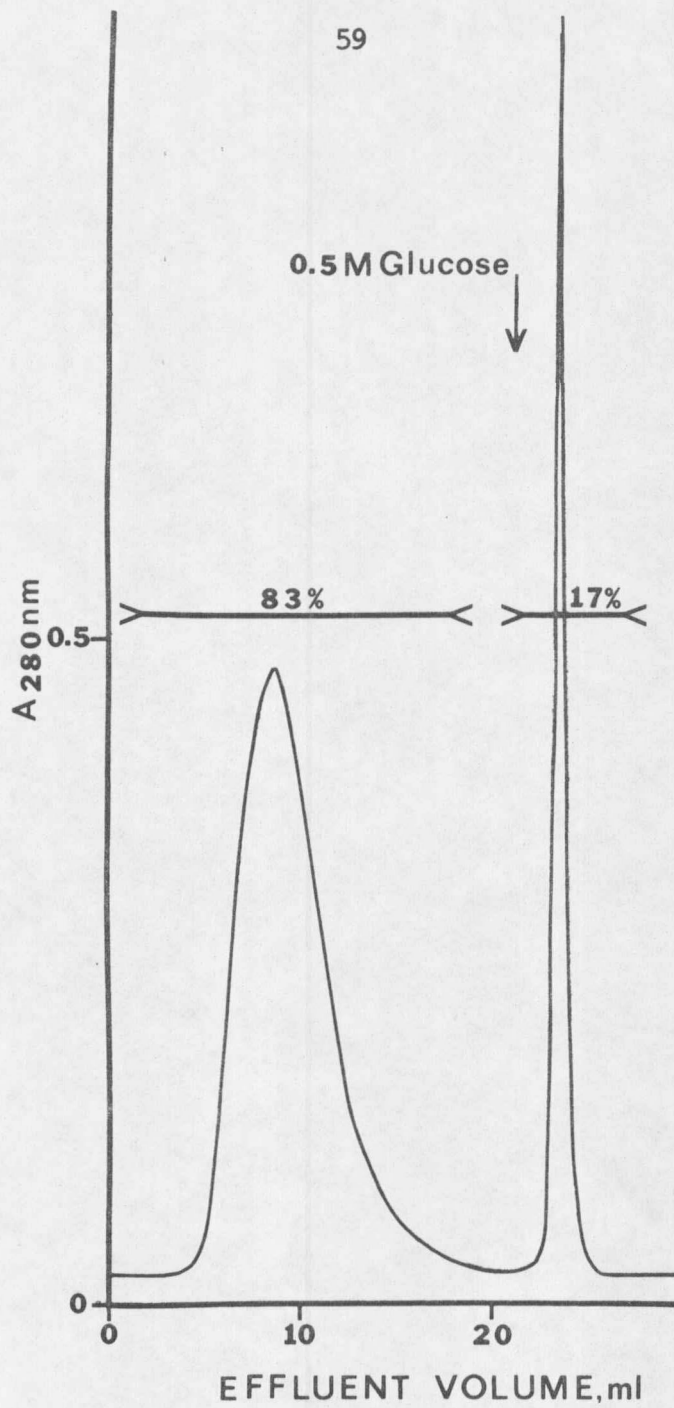


Figure 5. Affinity chromatography of 100% succinylated S1 on mannose-Sepharose

60

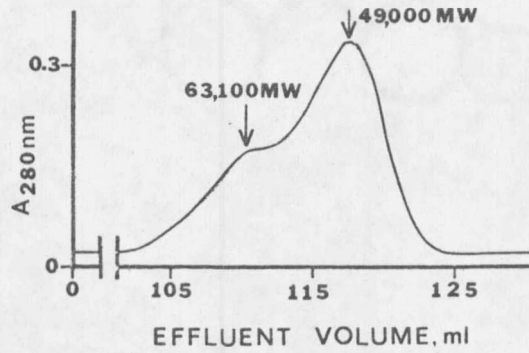


Figure 6. Gel filtration of 100% succinylated SL on Ultrogel AcA44

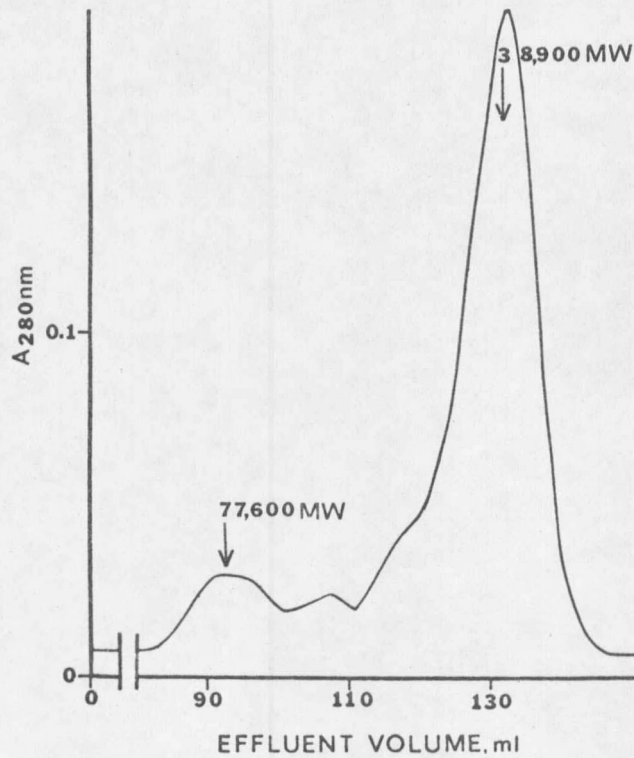


Figure 7. Gel filtration of preheated 100% succinylated SL on Ultrogel AcA44

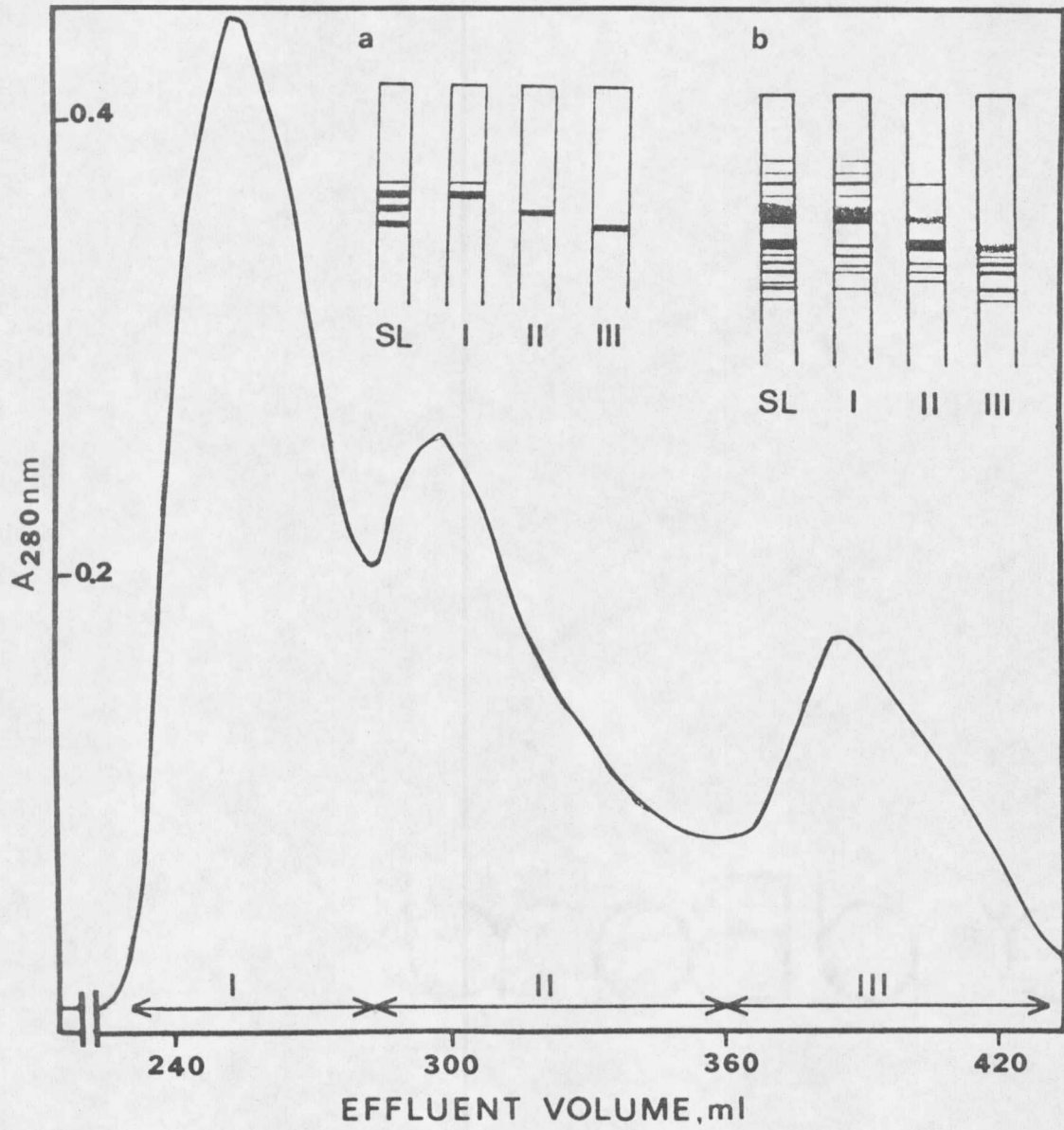
Denaturation of 90% succinylated SL allowed acylation of the previously underivatized amino groups. The 100% succinylated protein contained a large predominance of the 49,000 MW species that was transformed into a 39,000 MW species by heating. It can be speculated that totally succinylated SL was mostly in the monomeric conformation and heating converted it completely to that form. In view of the facts that 90% succinylated SL was a dimer that exhibited an average of one free amino group per monomer, it can be speculated that a single amino group was responsible for the dimer maintenance. The dimer maintenance could be due to formation of a specific salt bridge. An analogous situation stabilizes the tetrameric structure of ConA. In ConA, two lysines of one dimer form salt links with a glutamic acid residue of the other dimer (Reeke *et al.*, 1975); consequently, acylation of ConA with maleic anhydride results in dimerization of the molecule (Gunther *et al.*, 1973). There is, however, no additional evidence to support this idea with regard to SL. In the case of SL, exhaustive succinylation also induced the loss of carbohydrate binding activity indicating extensive conformational changes in the saccharide binding region. Persistent trypsin resistance of succinylated SL, however, emphasized the extreme stability of the molecule with regard to tryptic cleavage.

## Section 2. Studies on Apparent Heterogeneity of Sainfoin Lectin

### Ion Exchange Chromatography of SL

Figure 8 shows the behavior of SL on DEAE cellulose at pH 7.9. The protein was resolved into three fractions. Aliquots of these fractions were submitted to polyacrylamide gel electrophoresis (PAGE) at pH 9.5. Insert Figure 8a shows the results of the electrophoresis. The first fraction to emerge from the DEAE cellulose column (fraction I) contained the two most basic species of native SL. The other two fractions each showed a single electrophoretic band. The DEAE cellulose fractions were also analyzed by isoelectric focusing as shown in the insert Figure 8b. The individual fractions exhibited multiple banding patterns that corresponded with a range or group of isofocusing bands on unpurified protein. The fraction I peak was asymmetrical (Figure 8) and was rechromatographed on the same column at pH 8.5. The elution pattern is shown in Figure 9. The peak was further resolved into two components.

The above results show that SL could be fractionated into four ionic species—IA, IB, II and III—by anion exchange chromatography. Hapner & Robbins (1979) previously reported that SL was homogeneous according to SDS electrophoresis, sedimentation velocity and PAGE at pH 4.0. In contrast, the protein was heterogeneous on PAGE at pH



Insert a. Polyacrylamide gel electrophoresis pH 9.5 of SL and fractions I, II & III

Insert b. Polyacrylamide gel isoelectric focusing of SL and fractions I, II & III

Figure 8. Diethylaminoethylcellulose chromatography of SL

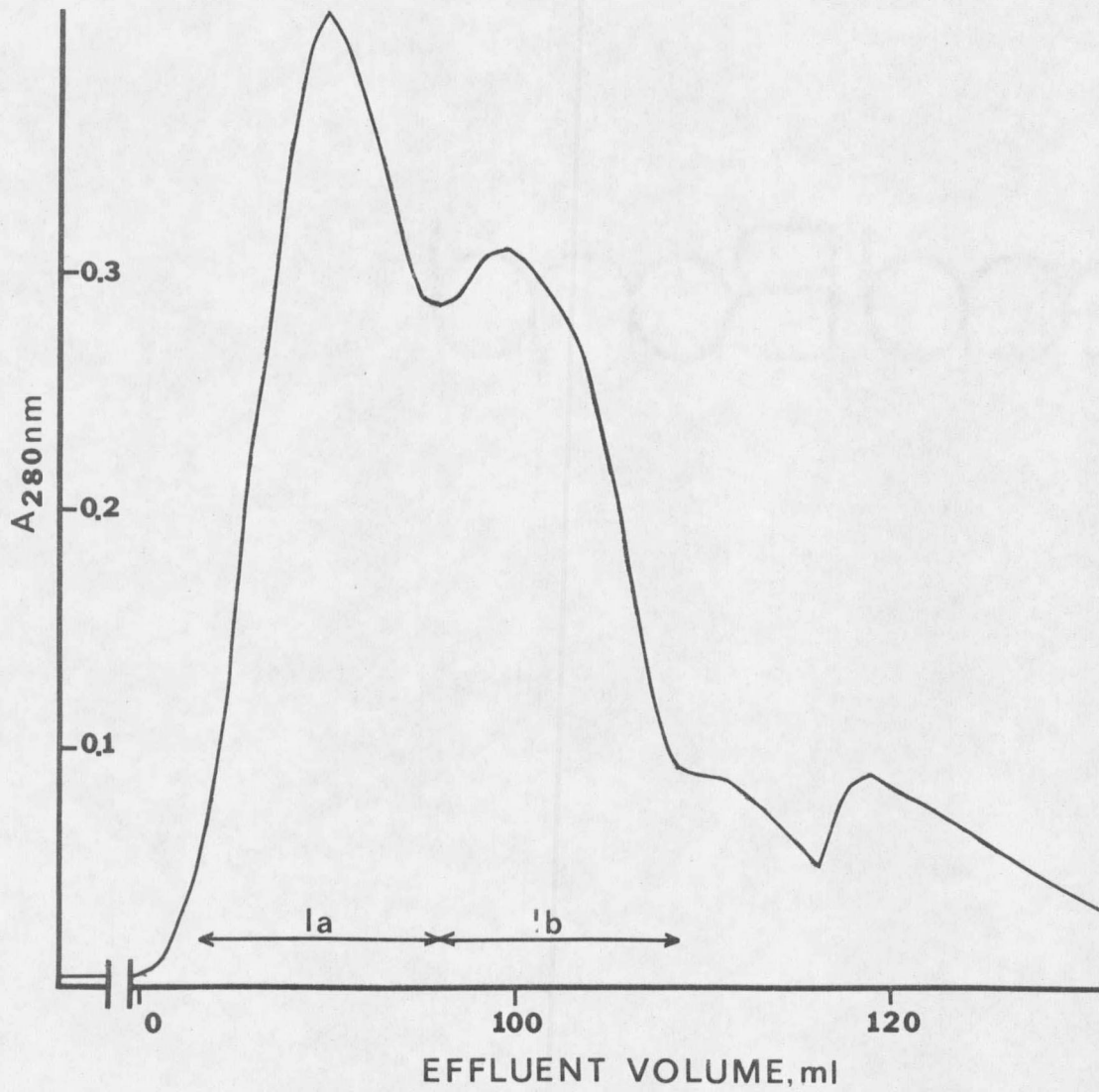
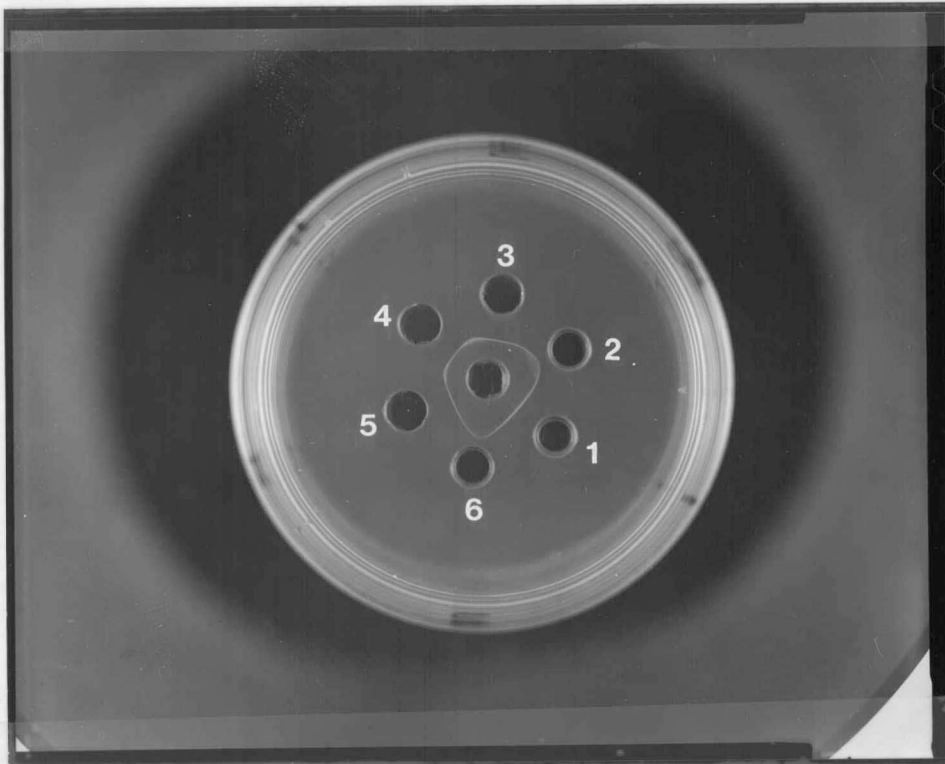


Figure 9. Diethylaminoethylcellulose chromatography of fraction I of Figure 8

9.5 and isoelectric focusing. The authors speculated that the heterogeneity observed under certain conditions may have reflected the presence of isolectins or allelomorphs of the protein. Results from this work confirm SL heterogeneity through isolation of several molecular species. The basis of the heterogeneity was not as speculated above, but rather was derived from charge differences resulting from microheterogeneity among the protein amide groups (see below).

#### Analyses of SL Ionic Species

The four DEAE fractions all showed hemagglutination activity towards cat erythrocytes when tested at a concentration of 200 µg/ml. The four species were therefore lectins that expressed biological activity. Three out of four species exhibited identical immunodiffusion patterns against antiserum to native SL as shown in Figure 10. Species IA showed a slightly different immunoprecipitation pattern. Amino acid compositions of the lectin species are presented in Table 2. The compositions were extremely similar with only minor differences in lysine content. These results must be interpreted in light of the fact that subsequent work showed unfractionated SL to have a unique amino acid sequence (see Section 4 of Results). The similar amino acid compositions of SL ionic species showed them not to be true isolectins, *i.e.*, different gene



- |                      |                      |
|----------------------|----------------------|
| 1. unfractionated SL | 4. ionic species II  |
| 2. ionic species Ia  | 5. ionic species III |
| 3. ionic species Ib  | 6. conA              |

Figure 10. Immuno/double diffusion of SL ionic species against antiserum to native SL

Table 2. Amino acid composition of SL ionic species

	Ia	Ib	II	III
Lys	12.6	11.4	11.4	10.6
His	3.7	3.4	3.3	3.3
Arg	11.9	12.1	12.2	12.0
Asx	35.9	33.4	34.3	34.8
Thr	20.4	20.3	19.8	19.4
Ser	23.4	23.9	24.6	24.7
Glx	19.0	18.6	18.9	19.0
Pro	10.3	10.4	9.8	10.7
Gly	16.3	16.8	16.4	16.3
Ala	12.0	12.6	12.3	12.3
Val	14.8	17.3	16.9	17.2
Ile	12.8	12.8	12.6	12.8
Leu	20.5	19.5	20.0	19.8
Tyr	7.1	7.2	7.2	7.1
Phe	16.2	16.0	16.0	15.8

products. The charge differences allowing fractionation of the species were the result of differences in amide content of the proteins that would be undetectable by amino acid analysis as well as by immunoprecipitation and PAGE at pH 4.0. Charge

microheterogeneity was indicated by the presence of multiple isoelectric focusing bands. The correspondence between isoelectric focusing bands of purified fractions and grouped bands in unpurified protein further indicated that the DEAE column resolved groups or classes of charged species, rather than individual molecular forms. Sequence analysis would not detect amide heterogeneity due to partial conversion of the amide to acid form normally observed during Edman degradation (Pisano & Bronzert, 1969). For the reasons stated above and the improbability of isolectins differing only in aspartic-asparagine and glutamic-glutamine content, SL is concluded to be a single protein that shows charge heterogeneity due to variations in amide content. This variation may be naturally present or it might be an artifact of isolation.

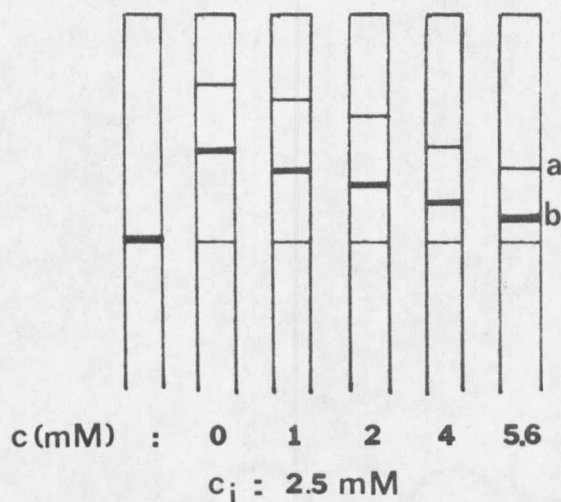
True isolectins have been observed for several plant lectins (Goldstein & Hayes, 1978). Examples of such multiple lectin forms are found in WGA, *Bandeiraea simplicifolia* I and phytohemagglutinin. The presence of multiple forms, as seen here, is not always related to different gene products. Soybean contains four ionic species separable on DEAE cellulose that have very similar amino acid compositions (Lis *et al.*, 1966). Lentil and pea each contain two ionic species that have almost identical amino acid compositions (Goldstein & Hayes, 1978). Peanut lectin can be resolved into eight

ionic species by isoelectric focusing (Miller, 1982); however, no amino acid composition data were shown. It is possible that the type of heterogeneity shown by SL, *i.e.*, amide differences, is common among plant lectins.

#### Affinity Electrophoresis of SL at pH 4.0

Apparent heterogeneous forms of SL differing in carbohydrate binding strength was observed by this technique. Sainfoin lectin was submitted to affinity electrophoresis according to the following. Allyl alpha-glucoside was synthesized and copolymerized with acrylamide to form water soluble copolymers. These copolymers were added to the usual polymerization mixture resulting in glucose entrapment in the polyacrylamide gels. Sainfoin lectin was electrophoresed on these gels using the standard discontinuous buffer system of pH 4.0. A pH value of 4.0 was chosen because SL shows one electrophoretic band at this pH (Hapner & Robbins, 1979). Figure 11 shows the results of a typical affinity electrophoresis run. The first gel on the left was a control gel in which galactose was the immobilized sugar. The other gels contained immobilized glucose and various amounts of added free glucose. Three bands were visible in the affinity gels of SL. One of them showed a migration unaffected by the presence of immobilized glucose. The other two bands had retarded mobilities in the affinity gels that became less

retarded with added concentrations of free glucose. Binding constants toward free and immobilized glucose were calculated from these data and are fully presented in Section 3 of the Results.



Note: The gel at the far left contains immobilized galactose. The other gels contain immobilized glucose in concentration  $c_i$  and free glucose in concentration  $c$ .

Figure 11. Affinity electrophoresis at pH 4.0 of SL

Affinity electrophoresis is a new technique in which proteins can be separated on the combined basis of their specific affinity to immobilized ligands and their ionic charge character (Horejsi, 1981). The presence of immobilized ligand decreases the mobility of the interacting protein. The mobility is restored by the addition

of free ligand to the affinity gels. These results showed that SL was resolved into three components, and since the lectin shows only one band on PAGE at pH 4.0 (Hapner & Robbins, 1979), the components therefore differed by their carbohydrate binding affinity.

Entrapment of a non-interacting sugar (galactose) in the control gel did not affect SL mobility, indicating that the alteration of protein mobility in the glucose containing gels was not due to a non-specific phenomenon. The SL species whose migration was independent of the presence of glucose was presumably denatured SL. The other two components resolved on the affinity gels possibly corresponded to SL monomer and dimer species that differed in their interaction with the immobilized glucose. The  $K_D$  values for free glucose calculated for the two species were very similar, whereas the dissociation binding constants for immobilized glucose were different (see Section 3 of Results). It is conceivable that the dimer would interact more strongly with the entrapped glucose because of its greater valency and would be more retarded on the affinity gels. In agreement with this hypothesis was the observation that the fastest component was in larger amount than the slowest one, as would be expected from the predominance of monomers at pH 4.0 (see Section 1 of Results). Alternatively, dimer and monomer may differ in their access to the immobilized sugar for

steric reasons, or the sugar may have been chemically immobilized in different conformations resulting in different binding constants.

In any case, there is no other evidence that suggests SL may have different carbohydrate binding strengths and the apparently contrary results here must be ascribed to experimental conditions.

Similarly, the apparent conflict with the gel filtration experiments at pH 4.0 which showed little carbohydrate binding (Section I of Results) is likely due to an elevated pH in the affinity electrophoresis gel.

#### Affinity Electrophoresis of SL at pH 9.5

Figure 12 illustrates the behavior of SL on affinity electrophoresis at pH 9.5. The control gel on the left with immobilized galactose exhibited the usual four bands patterns observed in native SL (Hapner & Robbins, 1979). Only one band was present on the affinity gels in the absence of free glucose. When glucose was added the single band progressively diffused into the four-banded pattern characteristic of SL.

Because of the limited migration of the protein in the presence of little or no free glucose, one possible interpretation was that each gel in fact exhibited several bands, but it was not possible to distinguish them. If this hypothesis was correct, the  $K_D$  for



















































































































































































































