PLANT PROTEIN CHEMOTAXONOMY

I. DISC ELECTROPHORESIS OF *LASTHENIA* SEED ALBUMINS AND GLOBULINS

II. PARTIAL CHARACTERIZATION AND SEQUENCE STUDIES OF *SAMBUCUS* FERREDOXIN

by

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ABSTRACT

Proteins indirectly reflect, via messenger-RNA, the information coded in DNA, and are thus considered to be tertiary semantides. Since proteins are amenable to comparative analyses, they can provide a chemical basis for a molecular phylogeny.

This dissertation reports the application of two approaches to the study of plant relationships using protein characters. The first was an electrophoretic comparison of seed storage proteins from all twenty taxa of the genus *Lasthenia*. Albumin and globulin fractions were extracted from dormant achenes. Each protein sample was fractionated by disc electrophoresis in basic 7% polyacrylamide gels. Mean R_p values, coefficients of variation, and 95% confidence intervals were calculated for both types of protein bands. Similarity coefficients, calculated from the distribution of homologous bands, were used to produce dendrograms. Affinities among the taxa differ from the conventional taxonomy of the genus.

The second approach involved the purification and characterization of an iron-sulphur protein from a higher plant and comparison with ferredoxin from other species. Ferredoxin was isolated from leaves of *Sambucus racemosa* L. by the following procedure: 1) homogenization in buffered 50% acetone-water, 2) ion-exchange chromatography on several columns of DEAE-cellulose, and 3) finally purified in good yield by gel filtration. The UV and visible spectrum showed maxima at 277, 331, 423, and 466 nm. The protein sustained an initial photoreduction rate of 86 µmoles NADP per mg chlorophyll per hour.

The amino acid composition was found to be Lys_5, His_2, Arg_1, Asx_{11}, Thr_5, Ser_7, Glx_{17}, Pro_6, Gly_7, Ala_{6-7}, Cys_4, Val_8, Ile_5, Leu_7, Tyr_3, Phe_2, and Trp_1. The molecule has a molecular weight of 10,700 and contains 2 atoms of iron. The amino-terminal sequence is Ala-Thr and the carboxyl-terminal sequence is Leu-Thr-Ala. These properties are discussed in relation to those of other angiosperm ferredoxins. Experiments were performed to investigate the feasibility of sequencing this ferredoxin.

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ABBREVIATIONS

AECys	Aminoethylcysteine
AEI	3-(2-aminoethyl)indole
CMCys	Carboxymethylcysteine
Dansyl	1-dimethylaminonaphthalene-5-sulphonyl
DEAE	Dimethylaminoethyl
NADP	Nicotinamide adenine dinucleotide phosphate
PITC	Phenylisothiocyanate
PTC	Phenylthiocarbamyl
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
TPCK	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone
Tris	Tris(hydroxymethyl)amino methane

See väitekiri on pühendatud minu vanematele, Lainele ja Heinole, kelle armastuse ja õhutuse kaasabi on võimaldanud minu õppinguid lõppule viia.

This thesis is dedicated to my parents, Laine and Heino. Their love and encouragement have made my studies possible.

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P A R T I

DISC ELECTROPHORESIS OF *LASTHENIA* SEED ALBUMINS AND GLOBULINS

CHAPTER I

INTRODUCTION

Lasthenia (Compositae: tribe Helenieae) is a small genus occurring in western North America with a single species native to Chile (1). In North America the genus shows most diversity in California, where it contributes to the floral displays of early spring in a conspicuous manner. It also ranges from northern Vancouver Island to northern Baja California, the Channel Islands, Guadalupe Island, and inland to central Arizona. The sixteen species (which are in six sections, Table I) are ecologically and biochemically diverse, occupy a wide range of habitats, and generally show striking interspecific differences in many morphological and chromosomal characteristics (1). The systematics of *Lasthenia* has been the subject of a recent monograph (1). More recently, the nature of their flavonoid constituents has been investigated (3, 4, 5, 6). Members of the genus produce four anthochlor pigments (two aurones and two chalcones), two luteolin glycosides, eight quercetin glycosides, one kaempferol glycoside, six patuletin glycosides, and one patuletin bisulphate compound (4). In general, however, each species exhibits a distinctive array of flavonoids, and within some species there are as many as three "flavonoid races" which differ in their flavonoid constituents (6).

Proteins, being tertiary semantophoretic molecules, inherently possess a greater potential to reflect more accurately evolutionary history than do episemantic molecules such as

TABLE I

THE GOLDFIELD GENUS *LASTHENIA*[a]

Section	Taxon
Baeria	*L. chrysostoma* (Fisch. & Mey.) Greene
	L. macrantha (Gray) Greene
	subsp. *macrantha*
	subsp. *bakeri* (J. T. Howell) Ornduff
	subsp. *prisca* Ornduff
Burrielia	*L. debilis* (Greene ex Gray) Ornduff
	L. leptalea (Gray) Ornduff
	L. microglossa (DC.) Greene
Platycarpha	*L. platycarpha* (Gray) Greene
Lasthenia	*L. kunthii* (Less.) Hook & Arn.
	L. glaberrima DC.
Hologymne	*L. chrysantha* (Greene ex Gray) Greene
	L. glabrata Lindl.
	subsp. *glabrata*
	subsp. *coulteri* (Gray) Ornduff
	L. ferrisiae Ornduff
Ptilomeris	*L. fremontii* (Torr. ex Gray) Greene
	L. burkei (Greene) Greene
	L. conjugens Greene
	L. coronaria (Nutt.) Ornduff
	L. minor (DC.) Ornduff
	subsp. *minor*
	subsp. *maritima* (Gray) Ornduff

a: According to Ornduff (1, 2).

flavonoids (7). The advantages of using protein data, especially those derived from electrophoretic techniques, in studying plant systematics have been discussed (8, 9). In studies of *Agropyron* and *Hordeum*, disc electrophoresis in polyacrylamide gels of the storage proteins from seeds has shown that the band patterns obtained are: (a) genetically determined and not modified by environmental factors; (b) different enough that species and (in some cases) infraspecific or racial populations may be distinguished; and (c) sufficiently conservative as to accurately reflect the specific relationships within a genus (9). Studies with legume seed proteins have also shown that they are not formed by a random mechanism, and are indeed tertiary semantides (10).

Boulter, Thurman, and Derbyshire pointed out that during the past half century, the proteins of seeds have been classified on various bases, including functional, solubility, and structural or cytological criteria (10). A generally accepted division of seed proteins is that of albumins, globulins, prolamins, and glutelins. As proposed by Osborne, albumins are soluble in water and dilute salt solutions; globulins are soluble in salt solutions but are not, or only slightly, soluble in water; prolamins are soluble in 70-80% (w/v) ethanol but are not soluble in absolute ethanol nor in water; and glutelins are soluble in dilute acids and alkalis but not in water, salt solutions nor aqueous alcohol (11). It is now recognized that enzymes are found in the albumin fraction and that the globulin fraction contains the storage protein (10).

The albumin fractions of seeds from seventeen species of the Leguminoseae have been fractionated by polyacrylamide gel disc electrophoresis and shown to be of taxonomic usefulness (12). A similar but more extensive study of the legume seed globulins has shown that certain tribes possess distinctive protein band patterns (10). In the genus *Crotalaria*, the seed globulin patterns of 24 species have shown reasonable correlation with the existing sectional taxonomy of the genus (13).

As part of a continuing chemosystematic survey of *Lasthenia*, an electrophoretic study of the albumin and globulin seed fractions of *Lasthenia* was undertaken to investigate the taxonomic usefulness of these seed protein characters in the genus.

CHAPTER II

EXPERIMENTAL PROCEDURE

1. MATERIALS

Seed material was obtained from Dr. R. Ornduff of the Botany Department, University of California at Berkeley. For each taxon, dormant achenes from several plants (see Table II for collection numbers) were used.

N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide, acrylamide, and riboflavin were obtained from Eastman Kodak Company of Rochester, New York. All other chemicals were from local suppliers.

2. METHODS

Achenes were separated from chaff and cleaned. Proteins were extracted using modifications of the method of Fox, Thurman, and Boulter (12). Achenes (1 part w) were ground with 5% (w/v) K_2SO_4 (10 parts v) in a mortar and stirred for 20 min. The slurry was centrifuged at 1,000 x *g* for 10 min at 4 C and the supernatant was dialyzed against 4 litres of distilled water for 24 hr. The dialyzate was centrifuged at 1,500 x *g* for 10 min at 4 C and the supernatant was lyophilized. The freeze-dried protein was referred to as the albumin fraction. The precipitate was resuspended in 12 ml of 5% K_2SO_4, dialyzed for 24 hr and centrifuged at 1,500 x *g* . The pellet was suspended in distilled water and lyophilized (globulin fraction). All work was done at 4 C.

TABLE II

COLLECTIONS OF *LASTHENIA* USED FOR DISC ELECTROPHORESIS

Taxon	Collection
L. chrysostoma	Near Dozier, Solano Co., 4941[a]
L. macrantha subsp. *macrantha*	Point Reyes, Marin Co., 4140C
L. macrantha subsp. *bakeri*	Garcia R., Mendocino Co., 4709
L. macrantha subsp. *prisca*	Rogue R., Curry Co., Oregon, Chambers 2466
L. debilis	Elderwood, Tulare Co., 4788
L. leptalea	Atascadero, San Luis Obispo Co., 4976
L. microglossa	Mt. Diablo, Contra Costa Co., 4807
L. platycarpha	Byron, Contra Costa Co., 4718
L. kunthii	Coline, Chile, Schlegel 3974
L. glaberrima	Valley Home, Stanislaus Co., 4726
L. chrysantha	Hanford, Kings Co., 4784
L. glabrata subsp. *glabrata*	Garrapata Cr., Monterey Co., 4983
L. glabrata subsp. *coulteri*	Sorrento Slough, San Diego Co., Witham 200
L. ferrisiae	Soda Lake, San Luis Obispo Co., Twisselmann 4382
L. fremontii	Soda Lake, San Luis Obispo Co., Twisselmann 4450
L. burkei	East Windsor, Sonoma Co., 6067
L. conjugens	Imola State Hosp., Napa Co., 4129
L. coronaria	U. C. Riverside, Vasek, *s. n.*
L. minor subsp. *minor*	Maricopa, Kern Co., Twisselmann 4377
L. minor subsp. *maritima*	South Farallon Id., San Francisco Co., 6130

a: Unless otherwise indicated, all collections are those of Ornduff. Vouchers are at University of California, Berkeley. Localities, except where noted, are in California.

Protein concentration was determined by the method of Waddell (14). For each protein fraction, 8 replicate samples (200 µg/0.1 ml of 10% (w/v) sucrose) were separated by disc electrophoresis on 7% polyacrylamide gels (pH 9.0) which were 6 cm long and prepared according to Davis (15). Gels were run at 4 ma per tube for 50 min at 4 C and then stained in 1% amido black in 7% acetic acid for 1 hr. Protein band mobility (R_p) was expressed as a fraction of the mobility of the bromophenol blue front-tracking dye. Distances were measured from the middle of each band.

The mean R_p value, coefficient of variation, and 95% confidence interval of bands appearing in half or more of the replicate gels were calculated with the aid of an IBM 1130 computer. The position of each mean with its confidence interval was proportionally plotted on 1-m long paper by a Calcomp 565 digital incremental drum plotter, with the taxa listed side by side. On this large scale it was easier to determine band homology. Bands were considered homologous if their confidence intervals overlapped more than 50%. In order to compare protein bands and obtain an index of similarity for each pair of taxa, the similarity coefficient (SI) of Jaccard (Sneath) was used: $SI = IA/(IA + IBC)$, where IA = number of homologous bands present in both taxa; and IBC = number of bands present in only each one of the two taxa (16). The similarity coefficients were used to cluster the taxa by the commonly used unweighted pair group method with average linkage (16).

CHAPTER III

RESULTS

The average coefficients of variation for all albumin and globulin bands which appeared in this study were 0.028 and 0.044 respectively. While both values are within 5% experimental error, the seed globulins may be less suitable for use as electrophoretic characters than the seed albumins.

Homology of bands for the albumin (61 bands) and globulin (20 bands) fractions was determined from computer calculated means R_p values and by comparison of confidence intervals on machine plotted 3 dimensional graphs. Homologous band groupings for both seed protein fractions are shown in Table III. *Lasthenia microglossa* possessed the greatest number of albumin bands (13) while the least number of albumin bands (4) appeared in *Lasthenia glabrata* subsp. *glabrata*. Albumin band 8, the most common (R_p 0.135-0.145) occurred in 11 of the taxa. *Lasthenia minor* subsp. *maritima* showed the most globulin bands (5) while both *Lasthenia fremontii* and *Lasthenia microglossa* showed the fewest (1). The most common globulin band (R_p 0.3-0.314) occurred in only six of the taxa. Each species has a unique protein band profile. Dendrograms, indicating the degree of phenetic similarity among the taxa, were produced for the albumin characters, the globulin characters, and for both protein characters combined (81 bands in total). These are represented in Figures 1, 2, and 3 respectively.

TABLE III. MEAN R_p VALUES OF HOMOLOGOUS BANDS OF ALBUMINS AND GLOBULINS FROM LASTHENIA ACHENES

TAXA	*L. CHRYSOSTOMA* (1)	*L. MACRANTHA* SUBSP. *MACRANTHA* (2)	*L. MACRANTHA* SUBSP. *BAKERI* (3)	*L. MACRANTHA* SUBSP. *PRISCA* (4)	*L. DEBILIS* (5)	*L. LEPTALEA* (6)	*L. MICROGLOSSA* (7)	*L. PLATYCARPHA* (8)	*L. KUNTHII* (9)	*L. GLABERRIMA* (10)	*L. CHRYSANTHA* (11)	*L. GLABRATA* SUBSP. *GLABRATA* (12)	*L. GLABRATA* SUBSP. *COULTERI* (13)	*L. FERRISIAE* (14)	*L. FREMONTII* (15)	*L. BURKEI* (16)	*L. CONJUGENS* (17)	*L. CORONARIA* (18)	*L. MINOR* SUBSP. *MINOR* (19)	*L. MINOR* SUBSP. *MARITIMA* (20)
ALBUMIN BAND NO. 1				0.064				0.067	0.068											0.063
2	0.078	0.077	0.082										0.077		0.079					
3					0.097		0.093			0.090	0.093					0.091	0.086			
4												0.102						0.105		
5									0.117											0.118
6				0.124																
7										0.132	0.129			0.133						
8	0.140	0.135	0.142			0.138	0.139	0.145	0.137				0.141			0.145	0.145		0.143	
9															0.154			0.151		
10					0.161															
11				0.175																
12	0.194								0.192						0.188					
13			0.204			0.204														

TABLE III. MEAN R_p VALUES OF HOMOLOGOUS BANDS OF ALBUMINS AND GLOBULINS FROM LASTHENIA ACHENES (CONT'D)

TAXA		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)
ALBUMIN BAND NO.	14										0.212	0.213									
	15					0.220												0.219			0.219
	16							0.233													
	17				0.241																
	18	0.256	0.253	0.259								0.258			0.257						
	19				0.269		0.268	0.266		0.263								0.267			0.265
	20																0.272				
	21		0.280						0.278		0.276	0.280	0.280						0.282		
	22													0.286							
	23	0.293																			
	24						0.301													0.300	
	25				0.313	0.314								0.312							
	26						0.336	0.336													
	27			0.368																	
	28	0.377									0.375										
	29		0.384																		
	30				0.415					0.411		0.410				0.411	0.407				0.414
	31	0.419						0.422			0.418								0.425		
	32		0.439	0.440			0.436						0.443								
	33					0.452				0.454		0.456									
	34	0.463													0.462		0.460		0.465		
	35				0.482		0.479		0.474												0.477
	36					0.501						0.495	0.495								
	37		0.510											0.504	0.512			0.507	0.508		0.502
	38						0.525										0.528	0.525		0.520	
	39							0.539	0.543					0.542							
	40	0.551			0.558					0.564	0.555								0.555		
	41							0.573												0.573	
	42			0.590																	
	43															0.597					
	44							0.623			0.614							0.617		0.617	
	45						0.639		0.645					0.638					0.629		0.634
	46																			0.659	
	47				0.678																

TABLE III. MEAN R_p VALUES OF HOMOLOGOUS BANDS OF ALBUMINS AND GLOBULINS FROM LASTHENIA ACHENES (CONT'D)

TAXA		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)
ALBUMIN BAND NO.	48									0.683		0.687									
	49	0.696				0.697		0.693													
	50																0.700	0.702			
	51		0.706	0.708			0.713		0.713						0.712						0.710
	52																			0.733	
	53				0.741																
	54									0.752	0.755										
	55							0.767													
	56					0.783															
	57																			0.794	
	58							0.838						0.836							0.830
	59									0.887	0.889	0.890									
	60							0.932									0.929	0.929			
	61				0.956																
GLOBULIN BAND NO.	1												0.057								
	2	0.080												0.069	0.076			0.076			
	3										0.087									0.090	
	4					0.105						0.098									
	5																				0.112
	6		0.143	0.143	0.149										0.136						
	7																				0.162
	8		0.179	0.185			0.185														
	9				0.197			0.202		0.192											
	10					0.210			0.208			0.213		0.212						0.210	
	11										0.219								0.217		
	12	0.225											0.226		0.225		0.226				
	13		0.231	0.236																	0.231
	14				0.248							0.248								0.251	
	15						0.269			0.267											
	16								0.280										0.285		
	17										0.296					0.296					0.293
	18	0.300				0.310						0.314					0.309	0.304		0.301	
	19													0.425							
	20													0.473							

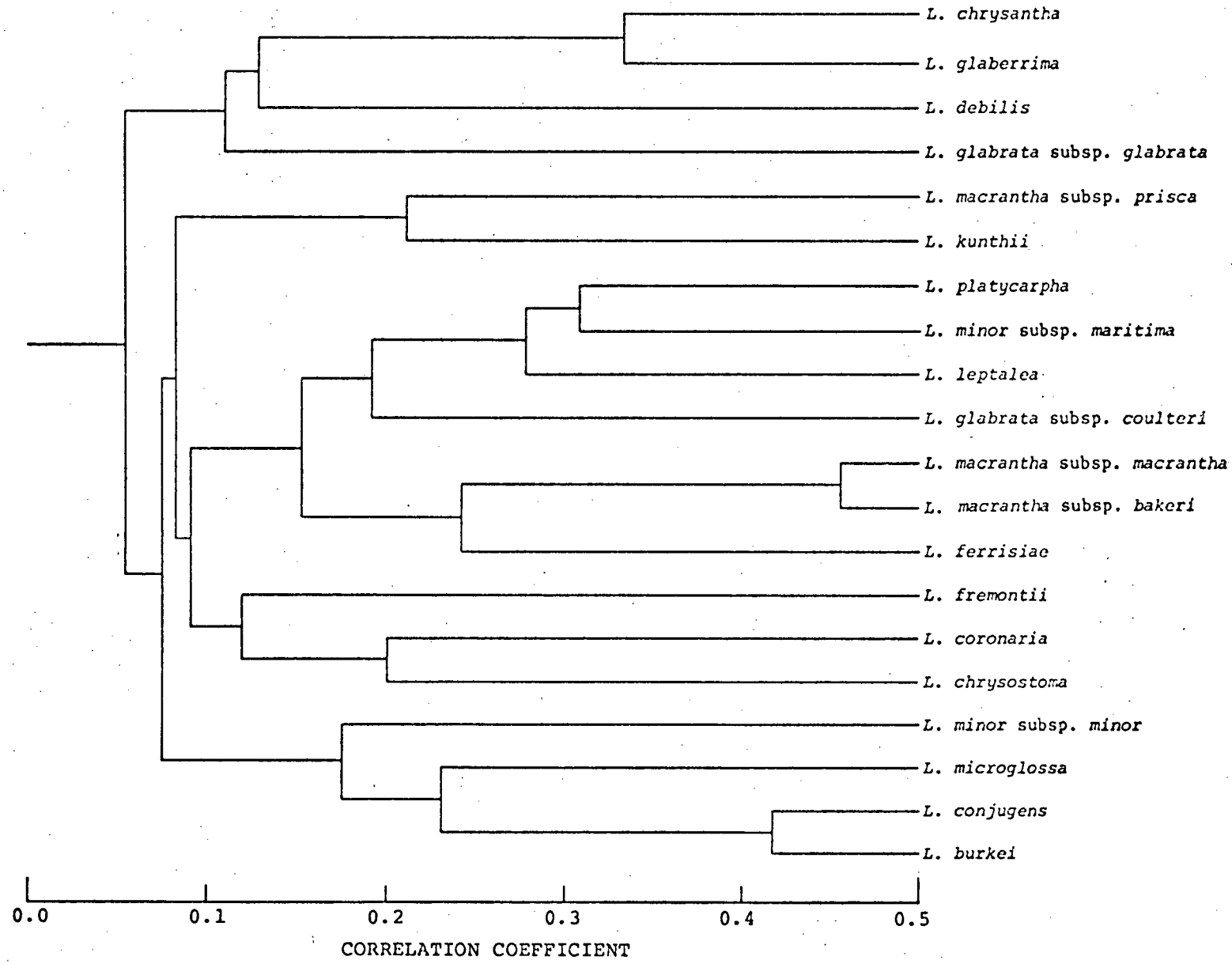

Fig. 1 Dendrogram for unweighted pair-grouping of *Lasthenia* taxa based on albumin data.

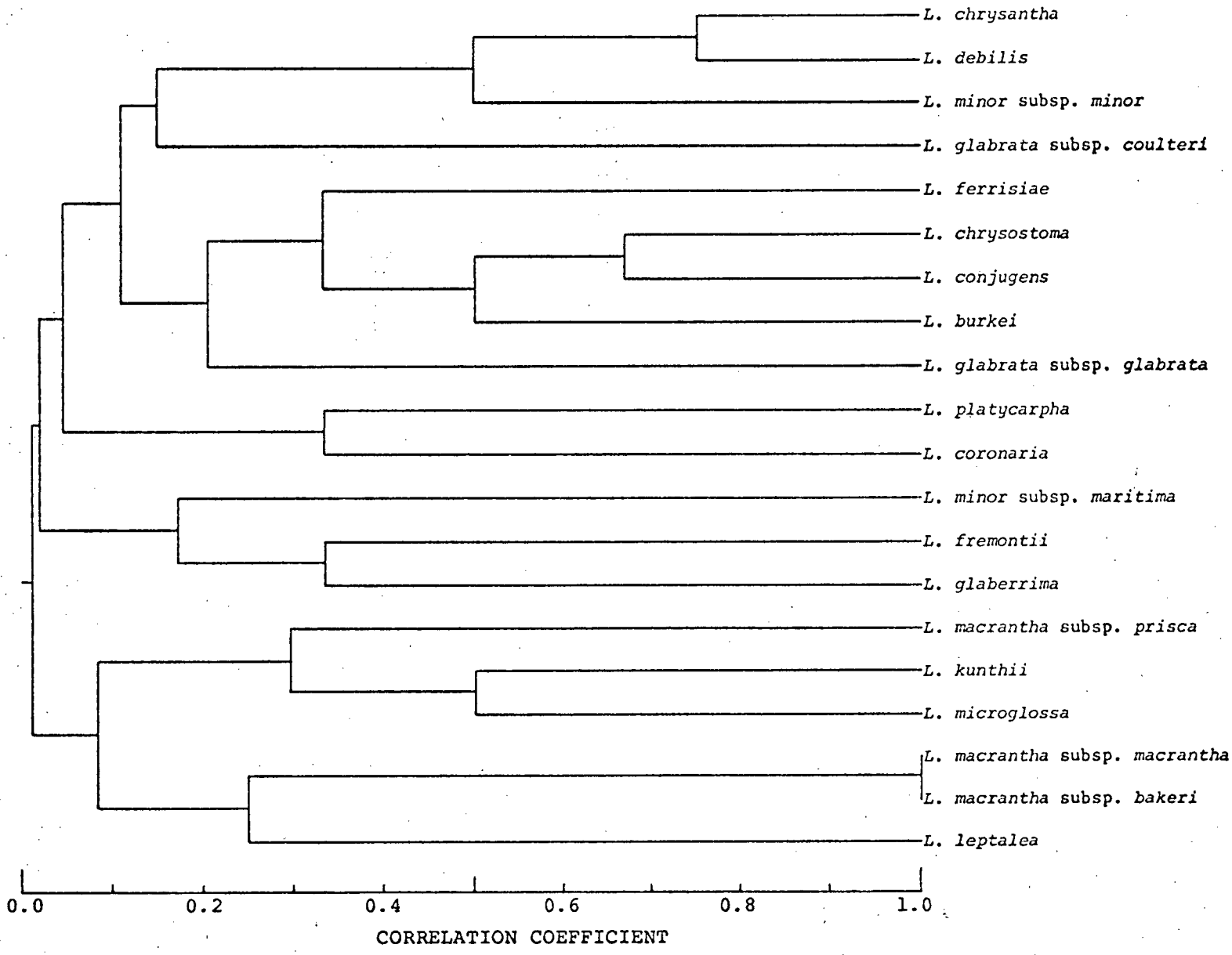

Fig. 2 Dendrogram for unweighted pair-grouping of *Lasthenia* taxa based on globulin data.

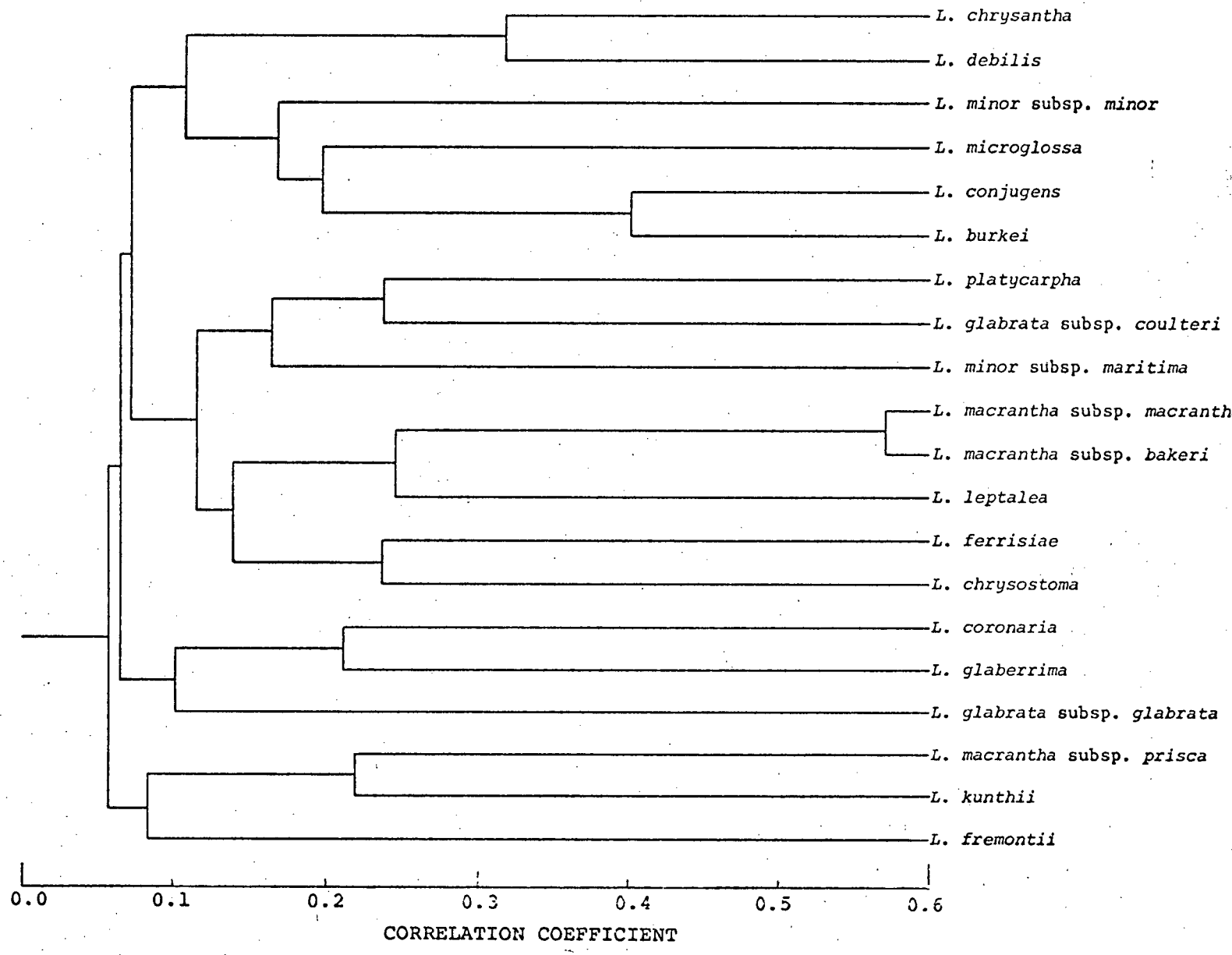

Fig. 3 Dendrogram for unweighted pair-grouping of *Lasthenia* taxa based on combined albumin and globulin data.

CHAPTER IV

DISCUSSION

Lasthenia has been divided into six sections (Table I) on the basis of morphology, cytology, and hybridization studies (1). The flavonoid chemistry of the genus supports this sectional treatment (4). In the present study, each protein fraction resulted in a very different clustering of the taxa. For example, in the albumin dendrogram (Fig. 1), 15 taxa clustered into six groups at an arbitrary correlation coefficient of 0.2 or higher. Only four of these 15 taxa (*Lasthenia macrantha* subsp. *prisca* and *Lasthenia kunthii*, *Lasthenia macrantha* subsp. *macrantha* and *Lasthenia macrantha* subsp. *bakeri*) are clustered together at a comparable level in the globulin dendrogram (Fig. 2). Other such comparisons indicate equal disagreement between the two protein classifications. This indicates the distinctiveness and independence of the two seed protein fractions, and this must be considered when either of them is used as an evolutionary indicator. The sectional groupings of the taxa were not clearly reflected in any of the dendrograms. In the albumin dendrogram, only two of the six clusters mentioned above contained species belonging to the same section, and in each of these a third species belonging to another section also was included. In the globulin dendrogram, all four taxa of section Hologymne clustered together in a 9-member group at a low correlation coefficient of 0.118. Due to the influence of the dissimilarity of their albumins, this section was further dispersed

in the other two dendrograms.

Section Baeria contains two species, *Lasthenia chrysostoma* and *Lasthenia macrantha*. The latter has been subdivided into three subspecies on morphological and cytological grounds (1, 2). *Lasthenia macrantha* subsp. *macrantha* (n = 24) has branching stems which are cespitose and decumbent, usually has a tap root, and leaves that are 2-15 mm wide. *Lasthenia macrantha* subsp. *bakeri* (n = 24) has simple erect stems arising from thickened fibrous roots, and narrow leaves 1-2 mm wide. *Lasthenia macrantha* subsp. *prisca* (n = 16) has smaller leaves (2). Although all three subspecies share a similar flavonoid chemistry, subsp. *bakeri* and subsp. *prisca* have more in common with subsp. *macrantha* than they do with one another. *Lasthenia macrantha* subsp. *bakeri* is unique in the species in synthesizing four quercetin glycosides (4). In this study, *Lasthenia macrantha* subsp. *macrantha* and subsp. *bakeri* were the only two taxa to share an identical protein banding profile. Due to their three homologous globulin bands, they clustered at correlation coefficient 1.0 in the globulin dendrogram. By sharing five albumin bands, these two taxa were also most similar (albumin correlation coefficient 0.455). However, out of a total of 28 different albumin and globulin bands occurring in the species *Lasthenia macrantha*, subspecies *prisca* possessed only one band (globulin band 6) in common with subsp. *macrantha* and subsp. *bakeri* (Table III). *Lasthenia macrantha* subsp. *prisca* was removed from the above two taxa in all three

dendrograms, and was most closely related to *Lasthenia kunthii*.

In contrast, other studies have shown that species, which have been subdivided on morphological, cytological, or other grounds, contain seed proteins which exhibit uniform band patterns within the species. For example, in the genus *Suaeda* (Chenopodiaceae), three varieties of *Suaeda maritima*, differentiated by morphological and ecological criteria, showed almost identical (92.2% similarity) seed protein profiles (17).

The classification of *Lasthenia* (Table I) has been described as taxonomically conservative, stressing the phenetic unity of the genus and minimizing the differences which exist within it (1). The striking singularity of the seed protein profile exhibited by *Lasthenia macrantha* subsp. *prisca*, when compared with those exhibited by the other two subspecies, *bakeri* and *macrantha*, suggest that more consideration may need to be given to the biochemical diversity existing within the genus.

Lasthenia leptalea (section Burrielia) is closely related to members of section Baeria (1). The flavonoid chemistry of *Lasthenia leptalea* is identical to that of the three subspecies of *Lasthenia macrantha* in section Baeria (4). The globulin seed proteins of *Lasthenia leptalea* are closest to those of *Lasthenia macrantha* subsp. *macrantha* and subsp. *bakeri* at a correlation coefficient of 0.25. However, *Lasthenia leptalea* shows the greatest similarity in albumin proteins to *Lasthenia platycarpha* and *Lasthenia kunthii*,

and clusters with *Lasthenia macrantha* at 0.154. Taking both types of protein into consideration, *Lasthenia leptalea* first clustered with *Lasthenia macrantha* subsp. *macrantha* and subsp. *bakeri* at a correlation coefficient of 0.244, supporting the suggestion that this species is related to those in section Baeria.

A close relationship between sections Lasthenia and Hologymne is suggested by their occasional inclusion in a distinct genus, *Lasthenia* sensu stricto. Supportive evidence for this is the occurrence of the Hologymne flavonol pattern in *Lasthenia glaberrima* (4). In the albumin dendrogram, *Lasthenia glaberrima* is most closely allied to *Lasthenia chrysantha* (Hologymne), clustering at a relatively high value of 0.333, and it is also similar to *Lasthenia glabrata* subsp. *glabrata* (Hologymne). With globulins, no such close relationship was found. In the combined dendrogram, *Lasthenia glaberrima* is next closest to *Lasthenia glabrata* subsp. *glabrata*, although it clusters with it at a very low value of 0.099.

Three closely related taxa, *Lasthenia fremontii*, *Lasthenia burkei*, and *Lasthenia conjugens* (section Ptilomeris) have been the focus of several recent studies (5, 6, 18). On cytological and morphological grounds, one proposed evolutionary scheme places *Lasthenia burkei* intermediate to the other two species in the evolutionary pathway: (*Lasthenia fremontii* → *Lasthenia burkei* → *Lasthenia conjugens* or *Lasthenia conjugens* → *Lasthenia burkei* → *Lasthenia fremontii*). On the other hand, some interspecific hybrid progenies between

Lasthenia fremontii and *Lasthenia conjugens* are morphologically indistinguishable from *Lasthenia burkei*, suggesting the possibility that this species is of hybrid origin. Flavonoid studies do not offer evidence in support of either of these alternative schemes, though they also do not negate either of them (4). *Lasthenia fremontii* has only 1 globulin band, while *Lasthenia burkei* and *Lasthenia conjugens* have two bands each. *Lasthenia fremontii* has only five albumin bands while *Lasthenia burkei* has eight albumin bands and *Lasthenia conjugens* has nine. This progression of increasing band complexity in both protein fractions may support the first evolutionary scheme, *Lasthenia fremontii* → *Lasthenia burkei* → *Lasthenia conjugens*. *Lasthenia burkei* and *Lasthenia conjugens* cluster together at a relatively high level (0.5) in the globulin dendrogram and are the second most similar taxon pair in the albumin dendrogram (correlation coefficient .04417). This close similarity is confirmed in the combined dendrogram. In all three dendrograms, *Lasthenia fremontii* has little in common withtthe other two members of this closely knit phylad. Protein banding patterns of interspecific hybrids may further elucidate relationships in this group.

Disc electrophoresis of crude seed-protein extracts has been extremely useful in elucidating evolutionary relationships in many genera. In *Gossypium*, comparison of banding patternsshas provided additional information regarding the genetic origin of natural allotetraploids (19). Similar studies clearly support the amphidiploid origin of *Triticum*

aestivum from the hybridization of the tetraploid *Triticum dicoccum* and the wild diploid species *Aegilops squarrosa* (20). Seed protein profiles fully corroborate morphological, cytological, and flavonoid data in delineating the internal relationships of a phylogenetic reticulum in *Phlox* containing twelve taxa (21). The seed proteins of seven *Coffea* taxa showed a correlation with genetic and taxonomic information (22).

In the present study, disc electrophoresis of albumin and globulin seed fractions has provided some additional information about the genus *Lasthenia*, although the number of clear relationships that arose are few. Dendrograms based on unweighted pair-group clustering of similarity coefficients do not reflect the sectional taxonomy of the genus. Although the present study would suggest that nearly every species of *Lasthenia* has a unique array of seed proteins, further sampling of additional populations may reveal the existence of intraspecific variation. At present, however, most of the interspecific affinities that would be deduced on the basis of seed protein similarities are not in concordance with the affinities that have been suggested on the basis of evidence from morphological, cytological, and other biochemical studies.

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P A R T I I

PARTIAL CHARACTERIZATION AND SEQUENCE STUDIES OF *SAMBUCUS* FERREDOXIN

CHAPTER I

INTRODUCTION

The ferredoxins are low molecular weight proteins which function in a variety of metabolic systems as electron carriers. They constitute one group of a larger class of electron carriers and enzymes, the iron-sulphur proteins.

Previously called non-haeme iron proteins, the iron-sulphur proteins are defined as those in which iron is bound via sulphur-containing ligands. They occur widely, in bacteria, plants, and animals. The function of iron-sulphur proteins in carbon and sulphur metabolism, nitrogen fixation, photosynthesis, respiration, and hormone synthesis is considered central (1). Although these proteins were recognized but a decade ago, interest in them has increased dramatically during the past several years. One result has been the proliferation of trivial names for the members of this unique class of proteins. Only recently has a systematic nomenclature been developed (Figure 1). The chemical, physical, and biological properties of iron-sulphur proteins have been reviewed (1, 4, 5, 6, 7), and a comprehensive treatise of this class of proteins is now available (3). It is not surprising that a substantial portion of this excellent two-volume work is dedicated to ferredoxins because, of the three groups of non-conjugated iron-sulphur proteins (see Figure 1), the ferredoxins are most widely distributed and numerous.

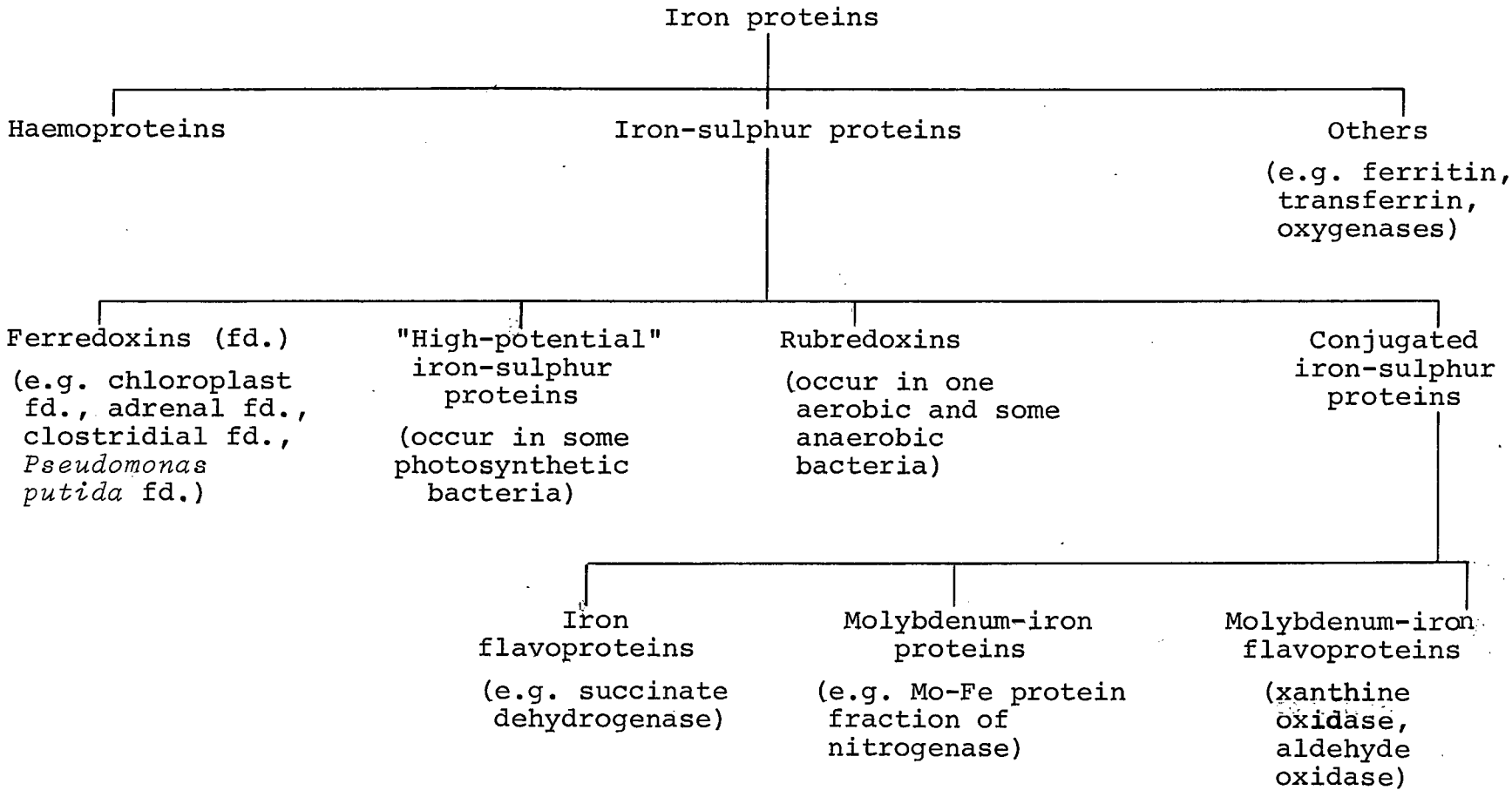

Fig. 1 Recommended classification of iron-sulphur proteins. Modified from refs. 2,3.

Ferredoxins have been found in a wide range of organisms, from the fermentative, aerobic, and photosynthetic bacteria, through the blue-green algae, protozoa, and green algae to all higher plants and animals investigated thus far (8, 9). They are relatively small proteins, consisting of a single polypeptide chain, with molecular weights between 6,000 and 13,000. They contain two to eight atoms of iron and equal amounts of inorganic (acid-labile) sulphur. Ferredoxins are the most electronegative proteins known. They transfer one or two electrons at redox potentials (-300 to -500 mV at pH 7) close to that of hydrogen gas. Due to their high content of acidic amino acids, ferredoxins show a great affinity for diethylaminoethyl (DEAE)-cellulose, and this property is utilized in their purification. Ferredoxins are distinguished from the other groups of iron-sulphur proteins (see Figure 1) by having a characteristic electron paramagnetic resonance (EPR) signal at g=1.94 for the reduced protein (2, 10). The orientation and mechanism of their iron-sulphur active centres have been the focus of an ever-increasing number of biophysical, spectroscopic, and X-ray structural studies (reviewed in refs. 3, 11). More general properties of ferredoxins have also been reviewed recently (3, 4, 5, 8, 12, 13).

The metabolic roles of ferredoxin electron carriers are as diverse as the organisms in which they have been found. For example, ferredoxins, containing eight atoms of iron and sulphur per molecule, have been isolated from twelve species of *Clostridium* (9). They function in a host of key oxidation-reduction reactions, some of which involve pyruvate, xanthine,

sulphite, butyrate, formate, and nicotinic acid metabolism; and nitrogen fixation (14). Another anaerobic, nonphotosynthetic bacterium, *Desulfovibrio gigas*, contains a $4Fe\text{-}4S^{2-}$ ferredoxin which is required for the reduction of sulphite by molecular hydrogen. In the aerobic *Pseudomonas putida*, a $2Fe\text{-}2S^{2-}$ ferredoxin (previously called putidaredoxin) acts as a redox protein in a camphor hydroxylating system. The green photosynthetic bacterium *Chlorobium* has an $8Fe\text{-}8S^{2-}$ ferredoxin which catalyzes the carboxylation of acetyl CoA and succinyl CoA to pyruvate and α-ketoglutarate respectively (9). Bovine and porcine adrenal ferredoxin (formerly referred to as adrenodoxin) functions as a component of a steriod 11β-hydroxylating system. A ferredoxin also containing two iron atoms and two sulphides has a similar function in porcine testes (9). In algal and higher plant chloroplasts, the photoreduction of the $2Fe\text{-}2S^{2-}$ ferredoxin is the terminal photochemical event (15). Ferredoxin plays a key role in photosynthesis. Besides being connected to NADP reduction, the photoreduction of ferredoxin is coupled to oxygen evolution and to noncyclic and cyclic photophosphorylation. Also it appears that reduced chloroplast ferredoxin plays a regulatory function in carbon assimilation by activating fructose diphophatase (15). In view of the multiplicity of physiological roles already known for ferredoxins, it will not be considered unusual if the continued study of this group unveils still a greater variety of functions.

Structurally and biosynthetically, bacterial ferredoxins are the simplest electron carriers known (16). They have been studied from about 35 different species (8,9). Examination of

the amino acid compositions of four clostridial and micrococcal ferredoxins shows that they contain only 14 different amino acids, nine of which are common to all four organisms. These nine - glycine, valine, alanine, proline, glutamic acid, serine, cysteine, and isoleucine - are identical with those amino acids which are readily synthesized in laboratory conditions simulating the supposedly primitive Earth environment. Significantly, the first six amino acids were those found in the Murchison meteorite which fell in Australia in 1969 (10). These and other considerations suggest that ferredoxins may have been one of the first proteins formed during prebiological evolution (16,17).

Generally, bacterial ferredoxins are composed of 54-56 amino acid residues and have a molecular weight of about 6,000 (9). The amino acid sequences of ferredoxins from eight anaerobic fermentative bacteria are known and these sequences are extremely homologous (9, 18). The differences which occur between them can be attributed to deletions, insertions, and point mutations during the evolution of the bacteria (19). Ferredoxins have been studied from about 20 algal and plant species (9). They all have a molecular weight double that of bacterial ferredoxins, about 11,000, and contain 96-100 amino acid residues. The amino acid sequences of ferredoxins from five plant and algal species are known and these are strikingly similar. More interestingly though, the algal and plant sequences are homologous to the bacterial ferredoxin sequences when the amino-terminal regions of these various ferredoxins are aligned. All present-day ferredoxins may have evolved from a common archetype by gradual lengthening of the genome (9, 18, 20, 21).

Two intermediate ferredoxins support this hypothesis. Ferredoxin from the green photosynthetic bacterium *Chlorobium* consists of 60 amino acid residues and has a molecular weight of 6,900. *Chromatium*, a purple photosynthetic bacterium, has a ferredoxin containing 81 residues with a molecular weight of 10,000. The amino acid sequences of both ferredoxins are known and also show homology when aligned with other ferredoxins (9, 18). Comparison of the structural and functional characteristics of all ferredoxins which have been studied suggests the pattern of evolutionary development shown in Figure 2.

Amino acid sequence data are of great value in the study of evolution (22, 23). Changes in specific amino acids in the sequences of homologous proteins can be used by the application of techniques such as calculation of mean base difference per codon to trace the development of these proteins from "primitive" types (19, 24). Amino acid sequence differences in cytochrome *c*, haemoglobin, and fibrinopeptides have been successfully applied in constructing phylogenetic trees which agree well with those phylogenies deduced by more classical studies of organisms (19). Recently, a family tree has been constructed from amino acid sequences of 21 higher plant cytochromes *c* (25). The phylogeny of higher plants is also being investigated by the study of the amino acid sequences of plastocyanin (26, 27). However, the use of ferredoxin in studying plant evolution is considered more advantageous (17). Biologically, ferredoxins are found in the widest range of organisms - fermentative obligate anaerobes, facultative anaerobes, and aerobes. Technically, ferredoxins are obtained in higher yields from plant material than are

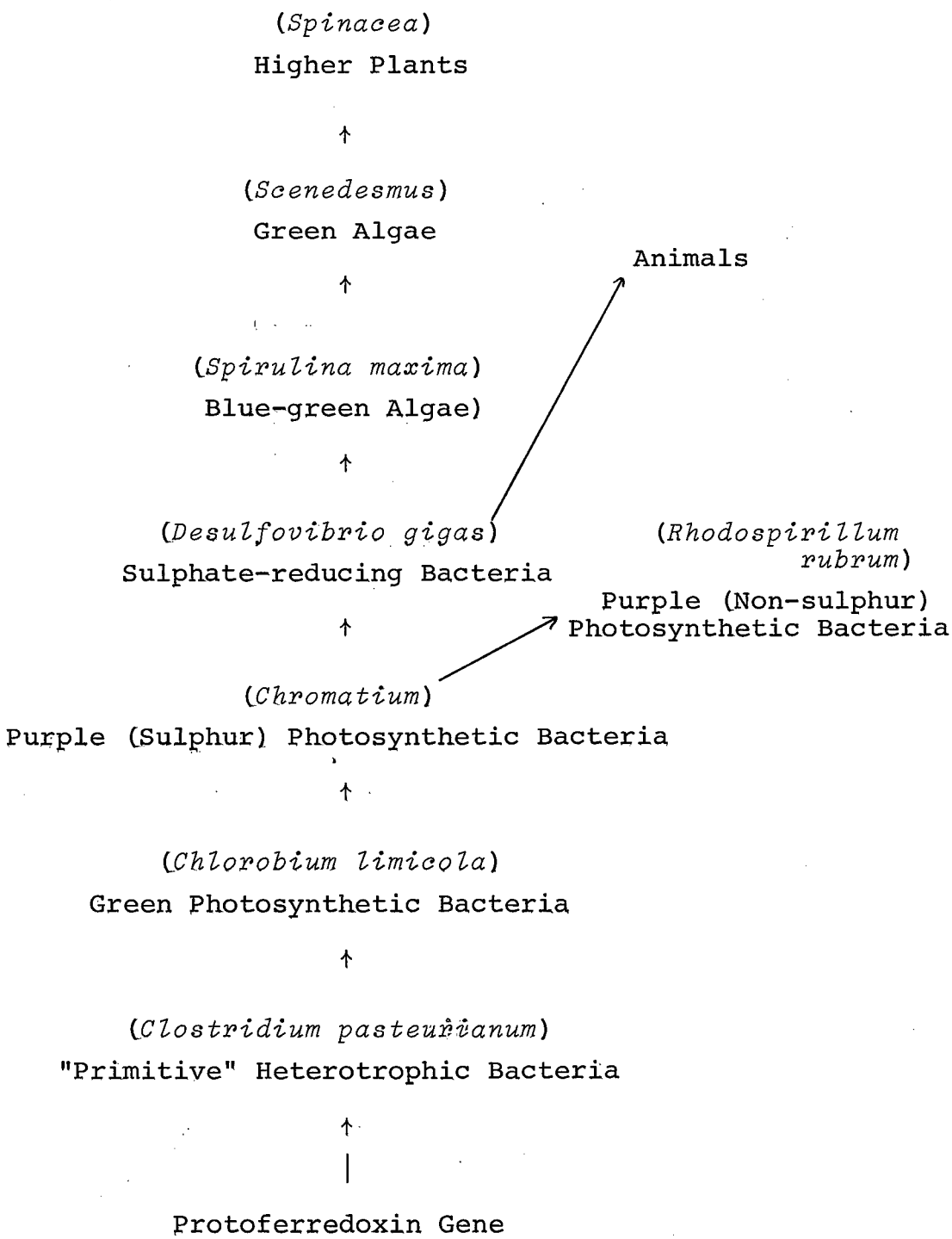

Fig. 2 Evolutionary development pattern of ferredoxin based on characteristics of the respective protein from each organism. Examples in parentheses are present-day species representative of the groups shown. Modified from refs. 9, 18, and 17.

cytochrome *c* and plastocyanin, thus facilitating the sequence determination. The phylogenetic tree of ferredoxins calculated from the amino acid sequences known to date is shown in Figure 3. Many more ferredoxin sequences must be obtained before the data can be used to solve plant systematic problems. It is hoped that such information will not only help in our understanding of the evolution of plant families, but also aid in clarifying relationships within families.

Ferredoxins from only four angiosperms have been sequenced thus far, as indicated in Figure 3. (The amino acid sequences of three of these ferredoxins are presented in the Appendix.) However, ferredoxins have been purified and characterized from these additional angiosperms: *Zea* and *Cyperus* (Graminales), *Amaranthus* (Chenopodiales), *Gossypium* (Malvales), and *Petroselinum* (Umbellales) (9).

Chemosystematic and phytoserological studies of the Caprifoliaceae (Rubiales) have indicated possible trends among the constitutive genera from primitive to advanced status in the family (28, 29). With respect to flavonoid characters, the relationship of *Sambucus* to *Lonicera* and *Viburnum*, and to the rest of the family as a whole is of special interest (28). The future availability of protein sequence information from these genera will hopefully add much to our understanding of the taxonomic relationships within the Caprifoliaceae. Toward this goal, the technical feasibility of performing amino acid sequence studies with ferredoxins isolated from these plants must first be investigated. The wide distribution of *Sambucus* and the ready availability of plentiful leaf material from

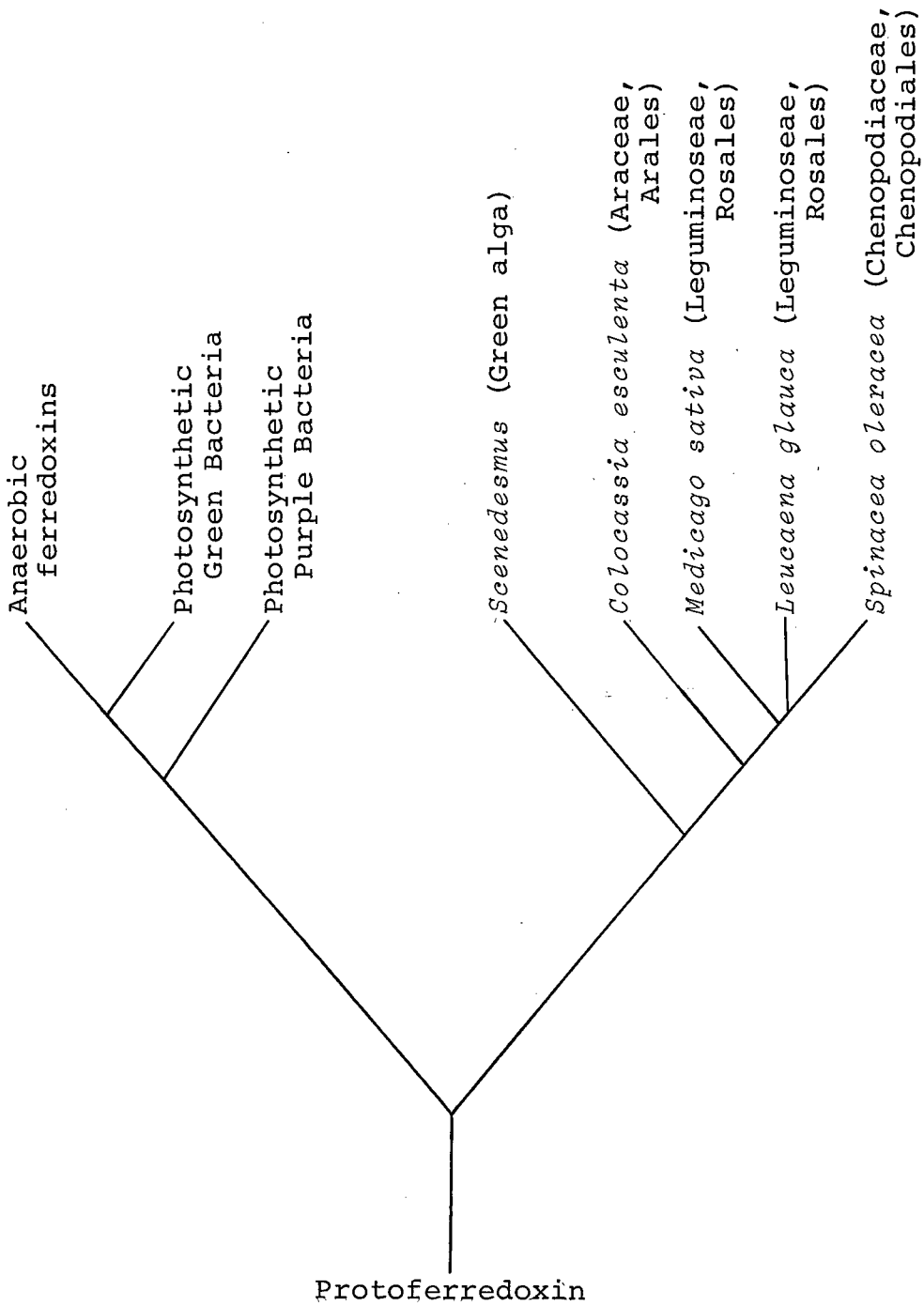

Fig. 3 The phylogenetic tree of ferredoxins. Modified from refs 9.

this member of the Caprifoliaceae suggest it as an initial candidate. The choice of *Sambucus* as the first source of ferredoxin for continuing protein sequence studies in higher plants is also appropriate because both cytochrome *c* (25) and plastocyanin (26) have already been sequenced from this genus. It will eventually prove interesting to see what evolutionary relationships these three protein families yield for a single genus.

The research described in this thesis was undertaken to determine whether ferredoxin could be isolated from leaves of *Sambucus racemosa* L. (redberry elder), and thus add to the growing amount of evidence showing the ubiquity of this iron-sulphur protein. The investigation of various methods for the extraction and purification of ferredoxin from leaf material therefore formed an integral part of the work. The development of an efficient mode of preparation of the protein was considered an important goal. The subsequent determination of homogeneity, biological activity, and chemical and physical characteristics could indicate the degree of similarity between *Sambucus* ferredoxin and other plant ferredoxins. Also, it was hoped that an investigation of the practicability of various strategies for the determination of the sequence of *Sambucus* ferredoxin would aid in the future elucidation of its primary structure.

CHAPTER II

EXPERIMENTAL PROCEDURE

1. MATERIALS

A. Plant Material

Leaves of *Sambucus racemosa* L. ssp. *pubens* (Michx.) House var. *arborescens* Gray were collected from the University of British Columbia Endowment Lands. Only green leaves with no obvious signs of infection were used. A voucher specimen has been deposited in the University of British Columbia Herbarium (Herbarium Sheet Accession Number 144617).

B. Chemicals and Solvents

Diethylaminoethyl(DEAE)-cellulose and tris(hydroxymethyl)amino methane (Tris) were obtained from Nutritional Biochemicals Corporation, of Cleveland, Ohio. Whatman DE 23 cellulose powder was obtained from W. and R. Balston (Modified Cellulose) Limited, Maidstone, England. Sephadex G-25 Fine (20-80 μ dry particle diameter) and Sephadex G-75 (40-120 μ) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Nicotinamide adenine dinucleotide phosphate (NADP), thermolysin (protease type X), N,N-dimethyl-*p*-phenylenediamine (grade III purity), and dansyl amino acids were all purchased from Sigma Chemical Company, St. Louis, Missouri. N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide, riboflavin, and 2-amino-2-methyl-1,3-propanediol were obtained from Eastman Kodak Company of Rochester, New York.

Ammonium persulphate and concentrated hydrochloric acid were "Baker Analyzed" reagent grade and were obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey. Ninhydrin, 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl-Cl), *p*-toluenesulphonic acid (PTSA), and dimethylformamide (silylation grade) were obtained from Pierce Chemical Company, Rockford, Illinois. Sodium dodecyl sulphate (SDS), phenylisothiocyanate (PITC) in 1 ml ampules, N-ethylmorpholine, and trifluoroacetic acid (TFA) were all "Sequenal" grade, from Pierce Chemical Company. Dr. C. O. Parkes donated 3-(2-aminoethyl)indole (AEI) after it was converted to the free base from the monohydrochloride form, which was also purchased from Pierce. Potassium hydrogen phthalate, 1,10-phenanthroline, and ferrous ammonium sulphate were certified ACS grade, Fisher Scientific Company, Fair Lawn, New Jersey. Cyanogum 41 and pyridine (analytical reagent grade) were obtained from British Drug Houses (Canada) Limited, Toronto, Ontario. Fluorescamine [4-phenylspiro(furan-2[3H],1'-phthalan)-3,3'dione] was donated by Dr. C. O. Parkes and was obtained from Hoffmann-La Roche (Canada) Incorporated, Montreal, Quebec. The protein molecular weight standards used in SDS electrophoresis were also donated by Dr. Parkes, and were obtained from the same suppliers as noted by Eng and Parkes (30). Ethyleneimine was purchased from Koch-Light Laboratories Limited, Colnbrook, England. Trypsin-L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Trypsin-TPCK) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. For dansylation, Pyrex culture tubes without rims, 6 x 50 mm, (9820) were

purchased from Corning Glass Works, Corning, New York. Polyamide sheets were obtained from Cheng Chin Trading Company, Tapei, Taiwan. Nitrogen was L grade (20 ppm oxygen) and was obtained from Canadian Liquid Air Limited, Vancouver, British Columbia. All other chemicals were reagent grade.

2. METHODS

A. Preparation of Column Chromatographic Media

(1) DEAE-Cellulose

DEAE-cellulose was precycled and equilibrated before use, according to standard procedures (31). The dry cellulose powder (60 g) was stirred into 1 liter of 0.5 M HCl and left to stand for 1 hr with occasional stirring. The swollen gel was filtered and washed with deionized water in a large Buchner funnel using a water aspirator. When the pH pf the filtrate reached 4.0, the moist bed of cellulose was stirred for 2 hr in 1 liter of 0.5 M NaOH, filtered and washed as before. The 0.5 M NaOH treatment was repeated. The neutral exchanger was then stirred into 1.5 liters of deionized water in a 4 liter Buchner flask. The pH of the slurry was adjusted to 3.0 with 1 M HCl. The DEAE-cellulose was degassed by stirring it magnetically under water pump vacuum until boiling occurred. It was titrated to pH 7.5 with saturated Tris solution. Fine particles were removed by suspending the stirred slurry in a 2 liter graduated cylinder, letting it settle for 1 hr,

and siphoning off the supernatant. This was repeated 5 times. Each time, the volume of the slurry was made up to 2 liters with deionized water. The exchanger was then equilibrated by: (a) suspending it in the appropriate buffer, (b) titrating it to pH 7.5 with the appropriate buffer component, and (c) filtering it in a Buchner funnel. Steps a, b, and c were repeated until the pH of the filtrate remained for at least 2 successive washes. The DEAE-cellulose was finally degassed and stored in the cold room (4 C). Columns were poured in the cold room immediately prior to use, and were stabilized by running equilibration buffer through them.

(2) Sephadex Gels

Sephadex gels were boiled for 1 hr in excess buffer, left to stand overnight, and degassed. Columns were poured, stabilized, equilibrated, stored, and their void volumes determined according to standard methods (32).

B. Preparation of Ferredoxin

(1) Preparation of Buffers

Buffers were made with distilled water and titrated to the correct pH. Nitrogen was bubbled through them for 5 min. After degassing (water pump), and adding β-mercaptoethanol if necessary, the pH was checked. Buffers were stored

in the cold room at 4 C. Throughout the extraction procedure all solutions were adjusted to pH 7.5.

(2) Initial Trials

Initially the procedure of Keresztes-Nagy and Margoliash (33) was chosen for the purification of ferredoxin from *Sambucus* leaves because it did not involve the use of organic solvents, large-scale centrifugation, dialysis, or precipitation. But after several trials, the method proved unsuccessful. After homogenization and filtration through cheesecloth, the aqueous extract did not pass through a large bed of DEAE-cellulose because a lipid-like residue coated the top of the resin bed and restricted the flow. It seemed possible to overcome this problem by using the procedure of Petering and Palmer (34). The aqueous cheesecloth filtrate was treated with DEAE-cellulose by batch technique, but the separation and subsequent washing of the resin was laborious, due to the unavailability of a 6 liter centrifuge. Also, when the washed resin was poured into a column for elution of adsorbed ferredoxin, the flow rate was prohibitively slow. Since wax-like material from the aqueous leaf extract was the apparent cause of poor chromatographic performance, a new procedure using acetone was employed. The method of Crawford and Jensen (35), with some modifications, proved successful.

(3) Purification of *Sambucus* Ferredoxin

The entire preparation was carried out in the cold room (4 C). At anytime at which it was necessary to interrupt the preparation, the solution containing ferredoxin was flushed with and stored under nitrogen. All solutions, except 50% acetone-water, contained 1 mM β-mercaptoethanol.

a. Homogenization

Fresh *Sambucus* leaves were collected, immediately taken into the cold room and the petioles removed. Leaflets (400 g) were placed loosely in a 5 liter Waring Blendor and homogenized with 800 ml of 50% acetone-water (-20 C) containing 1.8 g Tris. A fine slurry was obtained, usually within 3 min, by varying the speed of the blendor using a voltage regulator. Homogenization was continued for 2 min at high speed. The homogenate was squeezed through 4-ply cheesecloth in a large Nalgene funnel and the filtrate was collected in a 1 liter flask. The pH of the cheesecloth filtrate was adjusted to 7.5 with cold 1 M NaOH or 1 M HCl if necessary. The filtrate was poured into six 250 ml Nalgene centrifuge bottles and centrifuged at 20,000 x *g* for 10 min at -15 C.

b. DEAE-Cellulose Adsorption

The dark green supernatant was decanted into a 1 liter flask. It was then siphoned through a 6 x 2 cm bed of DEAE-cellulose, equilibrated with 2 mM Tris-HCl, in 50% acetone-

water, pH 7.5, in a 200 ml coarse sintered disc funnel. The flow rate was about 20 ml/min. The bed was washed with 10 mM Tris-HCl, pH 7.5 until the effluent was clear (ca. 2 liters). The top of the bed was exposed and buffer (0.1 M Tris-HCl, 0.8 M NaCl, pH 7.5) was carefully pipetted on. The adsorbed protein was slowly eluted in about 100 ml of red-brown solution.

c. DEAE-Cellulose Chromatography

The ferredoxin-containing solutions, from two 400 g lots of leaves, were combined in a Mariotte bottle, and diluted with 4 parts of 1 mM β-mercaptoethanol. The solution was readsorbed on a 2.5 x 45 cm DEAE-cellulose column, equilibrated with 0.1 M Tris-HCl, 0.2 M NaCl, pH 7.5 buffer. The column was washed with 1.5 liters of this buffer. The flow rate was 4 ml/min. A diffuse yellow red band containing the ferredoxin separated from the dark brown zone retained at the top of the column. The column was further developed with 500 ml of 0.1 M Tris-HCl, 0.3 M NaCl, pH 7.5. Twenty ml fractions were collected and scanned from 210 to 450 nm using a Unicam SP1800 recording spectrophotometer. Ferredoxin eluted as a distinct red-brown solution, showing a peak at 330 nm, usually contained in the 1630-1720 ml fraction. The effluent prior to and following the ferredoxin band was light yellow.

d. Sephadex G-75 Chromatography

The fractions containing ferredoxin were pooled and

diluted 1:3 with 1 mM β-mercaptoethanol. The solution was concentrated by readsorption on a 3 x 1 cm bed of Whatman DE 23 cellulose, equilibrated with 10 mM Tris-HCl, pH 7.5, in a 45 ml coarse sintered disc funnel and by slow elution with 0.1 M Tris-HCl, 0.8 M NaCl, pH 7.5 buffer. The concentrated ferredoxin solution (ca. 20 ml) was siphoned through a 3-way valve on to a 4.7 x 100 cm Sephadex G-75 column and eluted with 10 mM Tris-HCl, 0.2 M NaCl, pH 7.5. The flow rate was 60 ml/hr with a hydrostatic pressure of 30 cm. The sharp red band of pure ferredoxin separated from two slower moving light green bands, and had an elution volume (V_e) of 1160 ml.

e. Desalting and Lyophilization

The pure ferredoxin solution was concentrated as in step (d) above, except there was no need for prior dilution. The concentrated ferredoxin solution (ca. 20 ml) was put on a 4 x 20 cm column of Sephadex G-25 Fine and eluted with distilled water. Fractions absorbing at 280 nm were pooled and freeze-dried. The brown-red lyophilized protein was stored desiccated at -20 C.

C. Determination of the Molar Extinction Coefficient of *Sambucus* Ferredoxin

Sambucus ferredoxin was purified as rapidly as possible from fresh leaves. The concentrated protein from purification step (e) (see this chapter, section B3), was

dissolved quantitatively in 25 ml of 0.1 M Tris-HCl, 0.8 M NaCl, 1 mM β-mercaptoethanol, pH 7.5, and kept on ice. The optical densities at 420 nm, of two 3 ml samples, were read in a Unicam SP1800 redording spectrophotometer and the results averaged. The ferredoxin solution was then desalted and freeze-dried. The dry weight was used to calculate the concentration of the solution. The extinction coefficient could then be calculated directly.

D. Assay of *Sambucus* Ferredoxin Activity

The procedure of Crawford and Jensen (35) utilizing spinach chloroplasts was modified to determine the activity of *Sambucus* ferredoxin. Chloroplasts were prepared by homogenizing fresh spinach leaves (50 g) with 70 ml of 0.25 M Tris-HCl, 0.5 M sorbitol, 5 mM β-mercaptoethanol, pH 7.5, for 30 sec in a Waring Blendor and filtering the homogenate through 2-ply cheesecloth and glass wool. The chloroplasts were collected according to Keresztes-Nagy and Margoliash (33); chlorophyll content was determined according to Arnon (36). The sample of *Sambucus* ferredoxin, in 10 mM Tris-HCl, 0.2 M NaCl, 1 mM β-mercaptoethanol, pH 7.5, was taken from the Sephadex G-75 column effluent (see this chapter, section B3, purification step d) which showed maximum absorbance at 280 nm. The amount of protein used in the reaction mixture was calculated using the extinction coefficient for *Sambucus* ferredoxin (see this chapter, section C). The rate of light-

dependent reduction of NADP was measured in a cuvette containing spinach chloroplast suspension equivalent to 0.1 mg of chlorophyll, 0.1 mg of *Sambucus* ferredoxin, and 2 μM of NADP in 3.0 ml of 0.25 M Tris-HCl, 0.5 M sorbitol, 5 mM β-mercaptoethanol, pH 7.5 buffer. The reaction mixture and the blank, in which the ferredoxin sample was replaced with the above 10 mM Tris-HCl buffer, were illuminated at 20 C light from a 100 watt incandescent bulb, and were protected from excess heat by a 5-cm thick water filter. The absorbance of the reaction mixture at 340 nm was read against the blank after successive 3-min periods of illumination. Calculation of unit and specific activity followed the definitions of San Pietro (37). One unit of enzyme is that amount producing a change of 1.0 O. D. in 10 min at 340 nm, when the reaction mixture contains 0.1 mg chlorophyll per 3.0 ml. This unit represents the reduction of 4.8 μM of NADP per mg of chlorophyll in 10 min.

E. Polyacrylamide Disc Electrophoresis

The purity of *Sambucus* ferredoxin, prepared according to the procedure outlined in section B of this chapter, was assessed by disc electrophoresis in 7.7% polyacrylamide gels, pH 9.0. The general procedures and materials used were those described by Ornstein and Davis in their 1962 preprint(38). After small-pore and spacer gel polymerization, samples containing about 60 μg of ferredoxin in 0.1 ml of 0.1 M Tris-

HCl, 0.8 M NaCl, 1 mM β-mercaptoethanol, pH 7.5, were layered on top of the spacer gel in the absence of any further anti-convection agents, such as 10% sucrose. Samples were run at 4 C at 4 ma per tube for 10 min and then at 6 ma per tube for a further 65 min. After staining for 2 hr in a 1% solution of Buffalo Blue Black in 7% acetic acid, gels were destained and stored in 7% acetic acid.

F. SDS Electrophoresis

Estimation of molecular weight followed the procedure of Eng and Parkes (30). Ten microgram samples of lyophilized *Sambucus* ferredoxin were dissolved in 100 µliters of diluent buffer and complexed with SDS for 5 min at 100 C. Fluorescamine (5 µliters, 1 mg/ml acetone) was added and the mixture shaken. Protein standards (ovalbumin, chymotrypsinogen, myoglobin, lysozyme, and ribonuclease) were prepared the same way. Polyacrylamide gels, prepared using Cyanogum 41 and prerun for 30 min at 4 ma/gel, were used for electrophoresis of labeled proteins.

G. Amino Acid Analysis

(1) Automatic Amino Acid Analysis

The amino acid composition of *Sambucus* ferredoxin was determined on the Beckman Spinco Model 120C automatic amino

acid analyzer according to the method of Spackman, Stein, and Moore (39). The analyzer, equipped with an expanded range card, had a detection limit of 4.0-4.5 mv. Approximately 20 nmoles of each amino acid in the protein yielded peaks of adequate size. Freeze-dried ferredoxin was dissolved in distilled water to make a 0.4% solution. One hundred microliter samples of this solution were hydrolyzed with 100 µlitres of concentrated HCl (Baker Analyzed) in evacuated, sealed tubes at 104-108 C. Duplicate samples were hydrolyzed for 19, 38, and 62 hours to enable extrapolation to maximum yields for degradable residues and to enable an equally valid assessment of slowly hydrolyzed residues such as valine and isoleucine. After hydrolysis, one set of tubes was cooled, opened, and dried over NaOH pellets and P_2O_5 in a desiccator with oil pump vacuum. The dried hydrolyzates were dissolved in 2.0 ml of 0.2 N sodium citrate buffer, pH 2.2, according to standard procedure. The duplicate set of tubes was cooled, opened, and made up to 2.0 ml with the above sodium citrate buffer, according to Robel, to check for losses of certain amino acids due to adsorption to the glass tubes during desiccation (40). In each case, 200 µlitres of the sample was placed on the short column (5.7 cm) to resolve basic residues, and 200 µlitres was placed on the long column (57.7 cm) to resolve the neutral and acidic residues. Two hundred microlitres of internal standard solution (α-amino-β-guanido-propionic acid for the basic amino acids and norleucine for the acidic and neutral amino acids) was

included with each run to enable determination of ninhydrin deterioration.

Analysis of S-β-aminoethylcysteinylferredoxin (AECys-ferredoxin)(see this chapter, section I(2)) followed the above procedures, except that the short column resin height was 17 cm instead of 5.7 cm. S-β-aminoethylcysteine was eluted between the positions of lysine and histidine. The integration constant for AECys was taken to be 91.6% of that for lysine, as suggested by Hofmann (41).

(2) Determination of Tryptophan

The determination of the tryptophan content of *Sambucus* ferredoxin followed the method of Liu and Chang (42), as described by Liu (43). A solution of 3.0 N *p*-toluenesulphonic acid (PTSA) containing 0.2% 3-(2-aminoethyl)indole (AEI) was prepared by adding deionized water to the reagents in a 1 ml volumetric test tube. The mixture was heated in a boiling water bath until all of the AEI dissolved, whereupon the solution became pink. Freeze-dried ferredoxin (0.3 mg) was hydrolyzed with 100 µlitres of the above solution in an evacuated, sealed tube for 24 hours at 110 C. After hydrolysis, 0.4 ml of 0.5 M NaOH was added and the contents of the tube mixed. Aliquots (250 µlitres) were analyzed on the Beckman Spinco Model 120C automatic amino acid analyzer (see this chapter, section G1) using a 17 cm resin column. Using 0.35 N sodium citrate buffer, pH 5.25, tryptophan was eluted before lysine, with an elution volume of

81 ml. Calculations were based on the analysis of a 20 nM standard solution of tryptophan.

H. Determination of Non-Haeme Iron

The non-haeme iron content of *Sambucus* ferredoxin was determined by the *o*-phenanthroline method of Harvey, Smart, and Amis (44) as described by Matsubara (45). Samples, 0.2 ml of 0.1 M Tris-HCl, 0.8 M NaCl, 1 mM β-mercaptoethanol, pH 7.5, containing 0.01-0.02 µmole of ferredoxin, were heated with 1.3 ml of 1% HCl for 5 min at 80 C in 12 ml conical centrifuge tubes with glass marbles on top to reduce evaporation. The tubes were then centrifuged for 10 min at 800 x *g* and 1 ml of each supernatant was pipetted into 13 x 100 mm test tubes. These aliquots were mixed with 0.5 ml of 0.2 M potassium hydrogen phthalate in 0.3 M NaOH, 1 ml of 0.5% 1,10-phenanthroline in water, and 1 µlitre of β-mercaptoethanol. The absorbance at 511 nm was measured. Ferrous ammonium sulphate, dissolved in the above Tris-HCl buffer, was used to obtain a standard curve. Carried through this procedure, 0.1 µg atom of iron gave an absorbance of 0.42 in 2.5 ml reaction volume.

I. Protein Modification

(1) Trichloroacetic Acid Treatment

The procedure of Matsubara, Sasaki, and Chain was fol-

lowed to remove iron and labile sulphur from native *Sambucus* ferredoxin (46). Freeze-dried protein (50 mg) was dissolved in 5 ml of 0.05 M NH_4OH, pH 10.3, in a 14 ml Sorvall centrifuge tube, and treated with 5 ml of cold 1.2 M trichloroacetic acid (TCA). After standing in the refrigerator for 1 hr, the precipitate was collected by centrifugation at 27,000 x *g* for 10 min at 4 C. The supernatant was removed carefully. The pale yellow brown precipitate, resuspended using a thin glass stirring rod, was washed with 10 ml of cold 0.6 M TCA and then redissolved in 5 ml of 0.1 M NaOH. Treatment with 1.2 M TCA was repeated, and the precipitate was successively washed with 10 ml each of 0.6 M TCA, acetone, and anhydrous ethyl ether. The ether supernatant was carefully removed and the protein was dried in a desiccator. The precipitate was pulverized with the stirring rod into a fine, dry powder during drying.

(2) Reduction and S-β-Aminoethylation

To convert native *Sambucus* ferredoxin to the S-β-aminoethylcysteinyl (AECys) derivative, the method of Raftery and Cole (47), modified by Matsubara *et. al.* (46), was used as described below. Trichloroacetic acid-treated ferredoxin (44 mg) was dissolved in 3.3 ml of 7 M urea (titrated to pH 8.05 with 0.01 M NaOH) in a 50 ml Nalgene centrifuge tube. Octanol (5 µlitres) was added and nitrogen was bubbled through the solution for 5 min, at which time 0.55 ml of β-mercaptoethanol was added. The tube was flushed with nitrogen for a

further 5 min, sealed with Parafilm, and the solution was incubated at 40 C for 4.5 hr. The pH was brought to 9.8 by adding 1.144 g of 2-amino-2-methyl-1,3-propanediol. A thin combination electrode connected to a Radiometer pH meter, was inserted into the solution. The reduced ferredoxin was treated with 0.8 ml of ethyleneimine in 0.2 ml aliquots over a period of 10 min, during which time the solution was being flushed with nitrogen, stirred with a small magnetic bar, and titrated with concentrated HCl to maintain pH 9.0 (ca. 0.55 ml added in total). The reaction was allowed to proceed for a further 20 min at 40 C in a nitrogen atmosphere. Eleven ml of 0.8 M trichloroacetic acid, and enough 1.6 M TCA (ca. 2.5 ml) were added in order to precipitate the derivative. After 30 min at 4 C, the precipitate was collected by centrifugation at 27,000 x *g* for 10 min at 4 C. The pellet was resuspended in and washed with 25 ml of 0.6 M TCA. It was resuspended in deionized water and brought to pH 10.1 with 6 M NH_4^+OH, in a total volume of 35 ml. The dissolved protein was dialyzed against 2 x 4 litres of deionized water for 18 hr and lyophilized.

J. Digestive Procedures with Thermolysin and Trypsin

(1) Digestion of TCA-Treated Ferredoxin with Thermolysin

Titani *et. al.* reported that commercial preparations of thermolysin were not homogeneous by either disc gel elec-

trophoresis or NH_2-terminal analysis (48). To minimize possible contamination of ferredoxin peptides on high voltage electrophoresis papers, thermolysin was purified, under conditions which prevented autolysis, before it was used in digestive procedures. However, trial digests with enzyme purified by gel filtration through Sephadex G-75 in 0.2 M ammonium acetate pH 6.0, and subsequent separation of peptides, showed no marked improvement in results. Indeed, an apparent decrease in the rate of digestion of ferredoxin was observed with this thermolysin preparation, when compared with the original commercial preparation. Thermolysin (Sigma protease type X, lot 23C-0280) was therefore used directly, without further purification.

The enzyme:substrate ratio (1:120, w/w) of Rao and Matsubara was used (49). A typical digestion was done in 3.0 ml of 0.2 M ammonium acetate, pH 8.05, containing 18 mg of TCA-treated *Sambucus* ferredoxin and 0.15 mg of thermolysin. The sample was incubated at 40 C for 4 hr in a 14 ml Sorvall centrifuge tube. At 1 hr intervals, 4 µlitre aliquots were spotted on pH indicator paper, and the reaction mixture was adjusted to pH 8.0 with 2 M NH_4OH if necessary. Digestion was stopped by adding one drop of acetic acid. The digest was chromatographed directly on Sephadex G-25 Fine.

(2) Tryptic Digestion of AECys-Ferredoxin

Matsubara and Sasaki reported a tryptic digestion of spinach AECys-ferredoxin, in which the substrate (180 mg) was

dissolved in 20 ml of water, adjusted to pH 8.2 with 0.1 M NaOH, and digested with 5 mg of enzyme for 4.5 hr. The pH was maintained at 8.2 during the reaction (50). In contrast, *Sambucus* AECys-ferredoxin proved insoluble at pH 8.2. Accordingly, digestion was carried out with the following modifications. Deionized water (2 ml), brought to pH 8.0 with 0.1 M NaOH, was added to 27 mg of AECys-ferredoxin, in a 13 x 100 mm test tube. After raising the pH to 10.0 with 1 M NaOH, most of the protein dissolved, and the mixture was stirred magnetically for 2 hr. The pH was lowered slowly to 8.9 with 0.1 M HCl, and considerable precipitation occurred. TPCK-trypsin (0.27 mg) was added to the stirred solution and the digest was incubated at 37 C. After 3.5 hr, the solution had cleared slightly, and the pH had fallen to 8.2. The solution was adjusted to pH 8.9 with 0.1 M NaOH, and another 0.27 mg quantity of TPCK-trypsin was added. Digestion was finally stopped after a further 2.5 hr by lowering the pH to 5 with acetic acid. A voluminous precipitate appeared after 30 min at 20 C. The sample was centrifuged at high speed in a clinical centrifuge. Both pellet and supernatant were retained - the latter being applied directly to Sephadex G-25 Fine for gel filtration.

K. Column Chromatography of Enzyme Digests

Thermolytic and tryptic digests were fractionated by the following procedure. Samples (1 ml charge volume) were made 5% (w/v) in sucrose; layered on a 1.4 x 97 cm column of

Sephadex G-25 Fine, and eluted with 10 mM NH_4OH, pH 10. The flow rate was 8 ml/hr with a hydrostatic pressure of 29 cm. Fractions (2.5 ml) were collected using an ISCO Model 270 fraction collector, and their absorbance at 220 and 280 nm was measured. All of the digest was fractionated by repeating chromatographic runs in this way. Appropriate fractions were pooled and concentrated by rotary evaporation in 50 ml flasks at 30 C. Concentrated samples (ca. 3 ml) were then ready for preparative peptide purification by high voltage paper electrophoresis.

L. High Voltage Paper Electrophoresis of Peptides

High voltage electrophoresis of peptides was used as the final purification step in the protein digest isolation procedures. The vertical strip high voltage electrophoresis apparatus used was custom-built and of the Michl type. The operation of this system was similar to that described by Ryle *et. al.* (51). Electrophoresis at both pH 6.5 and pH 1.9 was performed in glass Chromotanks utilizing xylene and varsol respectively as inert coolants. Buffers used were as follows:

	Constituents	Ratios by volume
pH 6.5	Pyridine-acetic acid-water	(100:4:900)
pH 1.9	Formic acid-acetic acid-water	(9:12:179)

Whatman 3MM filter paper was used for separation of peptides. The sample loads (100 nmoles/cm) suggested by Ambler (52) were

considered to be maximum. For the first dimension, at pH 6.5, electrophoresis was carried out on full length sheets of 3MM for 40 min at 3 KV. The sheets were dried and strips cut from them were sewn to new sheets of 3MM. The second dimension, at pH 1.9, was run perpendicularly to the first for 35 min at 3 KV. The internal fluorescent markers of Brown and Hartley were included with each run to act as guides for location of peptide spots (53). The papers were dried and the fluorescent bands outlined in pencil. Peptides were initially located by dipping papers in cadmium-ninhydrin reagent (54), and heating them in a warm oven for about 5 min, until the initial colours of the peptide spots developed. The papers were then stored in individual plastic bags in the dark at 20 C overnight (55). Changes in spot colour gave some indication of NH_2-terminal residues. Peptide spots on unstained papers were located by circling areas of corresponding mobilities. These spots were cut out and stitched to short strips of 3MM paper. Purified peptides were then eluted into 10 x 75 mm test tubes using the eluting solvents of Shotton and Hartley (55). Peptides were evaporated to dryness, covered with Parafilm, and stored in the freezer.

M. NH_2-Terminal and Sequence Determination

The general procedure of Gray (56) as modified by Hartley (57) was used for NH_2-terminal determination and sequence of peptides and protein. This "Dansyl-Edman" method allowed for

minimum loss of material for NH_2-terminal determination and maximum speed of degradation.

(1) Dansylation of Peptides

The method of Gray was followed (58), although some of the volumes of reactants were modified. Dansyl tubes were cleaned before use by heating them at 500 C in a muffle furnace overnight and then cooling. After each addition, the dansyl tubes were centrifuged in a bench-top centrifuge at high speed for 30 sec to ensure that all of the reactant was at the bottom of the tubes. The peptide, in the 10 x 75 mm tube (see this chapter, section L), was taken up in 200 µlitres of aqueous pyridine (50% v/v, flushed with N_2) and an appropriate sample (5-50 µlitres), depending on the concentration, was removed and transferred to a dansyl tube. The sample was evacuated to dryness in a heated aluminum block which was fitted with a desiccator cover, according to Laycock (59). Five microlitres of 0.2 M $NaHCO_3$ were added and the sample was dried down again. Deionized water (5 µlitres) and 5 µlitres of dansyl chloride solution (2.5 mg/ml in acetone) were added to the sample which was then covered with Parafilm and incubated at 45 C for 20 min. The sample was evaporated and then hydrolyzed with 20 µlitres of 6 N HCl. The tube was evacuated and sealed to prevent any possible oxidative degradation. Hydrolysis at 105 C was performed routinely for 6 hr, although valine- and isoleucine-containing peptides often required 20 hr. Tubes were opened and the contents were dried under vacuum.

(2) Dansylation of Protein

The procedure recommended for proteins by Gray (58) was followed with slight modifications. Native ferredoxin was dissolved in 50% aqueous pyridine, whereas AECys-ferredoxin was suspended in N-ethylmorpholine-pyridine-water (2:9:9, by volume). Approximately 50-100 µg of protein was transferred to a dansyl tube and dried as above. After adding 50 µlitres of 1% (w/v) SDS, the sample was heated in a boiling water bath for 5-7 min. N-Ethylmorpholine (50 µlitres) was added to the cooled solution, and mixed thoroughly with a Vortex mixer. A dansyl chloride solution (2.5 mg/100 µlitres of dimethylformamide) was prepared and 75 µlitres of this was added with thorough mixing. The dansyl tube was covered with Parafilm and left at 20 C overnight. Acetone (0.5 ml) was added and the labeled protein precipitated at 4 C for 1 hr. The material was centrifuged and the supernatant was carefully poured off. The precipitate was washed with 0.5 ml of 90% acetone, centrifuged, and dried. Hydrolysis was carried out as described for peptides in section M(1)b above.

(3) Identification of Dansyl Amino Acids

Thin-layer chromatography of dansyl amino acids on polyamide sheets was performed essentially according to the method of Hartley (57) using some modifications of Olafson (60) and Weiner, Platt, and Weber (61). Each dried dansyl hydrolyzate was taken up in 2.5 µlitres of 50% aqueous pyridine. Using a 1 µlitre Drummond capillary pipet, 0.5 µlitres

of sample were applied to both sides of a 5 x 5 cm polyamide sheet, 8 mm in from the edge. The origin spot was not allowed to exceed 2.5 mm in diameter. Standard marker solution (0.2 µlitres) was applied to one side. Plates were developed in 250 ml beakers (two plates per beaker) with about 5 ml of solvent in each. Ascending chromatography in two dimensions was performed using the standard solvent systems.

System 1: 1.5% formic acid (v/v)

System 2: Benzene-acetic acid (9:1, v/v)

System 3: Ethyl acetate-methanol-acetic acid (20:1:1) by volume

System 4: 0.1 M ammonia-ethanol (9:1, v/v)

System 4 was sometimes used to resolve α-dansyl histidine, dansyl arginine, and dansyl lysine (62). Systems (3) and (4) were run in the same direction as system (2), which was perpendicular to the direction of system (1). Unknown dansyl amino acids were identified in ultra-violet light by the relationship of their mobilities to those of the standards.

(4) Edman Degradation

a. Coupling with Phenylisothiocyanate (PITC)

The modified Edman degradation procedure described by Gray (63) was used. The volume of the peptide solution was restored to 200 µlitres (see section M(1) above) with 50% aqueous pyridine. If protein was being degraded, the sample (in a 13 x 100 mm test tube) was made up to 500 µlitres with the appropriate solvent (see section M(2) above). A half-

volume of 10% PITC in pyridine (prepared fresh every two weeks, stored under N_2 in the freezer) was added, and the tubes were flushed with N_2, covered with Parafilm, and incubated at 50 C for 30 min. The reaction was stopped by evaporating the samples to dryness in a 60 C desiccator with NaOH pellets and P_2O_5. The desiccator was evacuated using a dry ice-acetone trap and an oil pump. When the tubes were dry, the P_2O_5 was replaced, and the evacuated desiccator was kept at 60 C for a further 10 min. Water, pyridine, unreacted PITC, and volatile by-products were removed in this way.

b. Cleavage of Phenylthiocarbamyl-Peptide or -Protein

Anhydrous trifluoroacetic acid (50 µlitres for peptides, 75 µlitres for protein) was added to the above dried residues. The tubes were flushed with N_2, sealed with Parafilm, and incubated at 45 C for 10 min. TFA was then removed by drying the samples again in vacuo, as in section M(4)a above.

c. Removal of Diphenylthiourea (DPTU)

Dried peptide residue was dissolved in 1 ml of water-saturated ethyl acetate. Ethyl acetate-saturated water (0.25 ml) was added, mixed vigorously with a Vortex mixer, and the tubes centrifuged. The supernatant was carefully pipetted off and the aqueous phase was washed twice more with 1 ml aliquots of the ethyl acetate. The aqueous samples were then dried in vacuo at 60 C, and the peptide residues taken up in

200 µlitres of 50% pyridine. Samples were removed for dansylation (see section M(1) above) and the degradation cycle repeated (see section M(4)a above).

Dried protein residue was washed free of DPTU according to the method of Percy and Buchwald (64). The residue was washed twice with 1-ml portions of 95% ethanol to make it less adherent to the walls of the tube. After drying, the residue was broken up finely in 2 ml of chloroform-benzene (1:1, v/v) with a thin glass stirring rod, and washed three times with this solvent. The protein was washed again and then suspended in 0.4 ml of deionized water. After three extractions with 2-ml aliquots of butyl acetate, and drying in vacuo, the sample was taken up in 500 µlitres of 50% aqueous pyridine (or 50% aqueous pyridine made 10% in N-ethylmorpholine). A sample was removed for dansylation (see section M(2) above) and the degradation cycle repeated (see section M(4)a above).

CHAPTER III

RESULTS

1. CHARACTERIZATION STUDIES

A. Preparative Work

At the start of this investigation in 1970, only a few procedures for the preparation of ferredoxin from leaves were available in the literature. The basic method of acetone fractionation of an aqueous leaf extract (65) had been modified to purify ferredoxin from some plants [e.g. spinach (46, 66, 67, 68, 69), parsley (70, 71), *Leucaena* (72), and taro (73)]. A radically different procedure which involved exhaustive extraction of green cotyledons with carbon tetrachloride-hexane (2:1, v/v) and various treatments of the subsequent residue had been used for the isolation of cotton ferredoxin (74). However, the above methods were initially avoided because their reported yields were not as large as those needed for sequence studies, or because of the discomfort and health hazards involved when working with organic solvents in an unventilated 4 C cold room.

In contrast, Keresztes-Nagy and Margoliash had prepared the protein from alfalfa by a method which did not involve organic solvents: the cheesecloth filtrate of an aqueous homogenate of alfalfa stems and leaves was directly passed through a bed of ion-exchange resin; the resin was washed, the adsorbed ferredoxin was then eluted, and the protein was

further purified by precipitation and several chromatographic steps (33). However, when this method was attempted with *Sambucus* leaves, numerous difficulties were encountered. More than the reported amount of Tris was needed to buffer the initial extract at pH 7.5 (3.1 g of Tris per 1.3 litre H_2O instead of 1.0 g). Four layers of cheesecloth instead of only two were necessary to contain the thick homogenate when being squeezed. The extract from even one lot of leaves, when filtered through cheesecloth, did not pass through a 16 (diam) x 3 cm bed of DEAE-cellulose with the reported flow rate. In fact, large amounts of chlorophyllous slime in the solution interfered with the DEAE-cellulose filtration (even when suction was applied) to such a degree that flow through the bed ceased. A modification was then sought which would quickly remove particulate and lipid-like residues prior to DEAE-cellulose filtration. After filtration through cheesecloth, continuous centrifugation of the extract using a dairy cream separator was slow and inefficient because of the voluminous precipitate. Also, prior filtration of the cheesecloth filtrate on a Buchner funnel with Celite filter aid, according to Lee, Travis, and Black (75), proved time-consuming and expensive due to the large amount of Celite needed to adsorb most of the interfering material. Because of these results, further attempts at preparation of ferredoxin from *Sambucus* leaves did not involve the direct filtration of the aqueous extract through ion-exchange resin. Instead, batch methods developed more recently were tried (34, 77).

Leaves (5 kg) were homogenized in Tris buffer contain-

ing 1% Tween 80 (76). The homogenate was squeezed through cheesecloth and a 200 ml batch of well-settled DEAE-cellulose was added to the 12 litres of filtrate (34, 77). After stirring for 1 hr the resin was allowed to settle and the supernatant was siphoned off. The resin was collected and repeatedly washed with buffer by batch technique using a 1 litre centrifuge. Further washing of the resin in a column and elution of adsorbed protein with 1 M NaCl in Tris buffer was also slow because of poor flow rate properties of the cellulose at that stage of the preparation. The ferredoxin-containing solution was then treated with ammonium sulphate and the precipitate was removed by centrifugation. Preparation of the supernatant (ca. 2.5 litres) for dialysis (34) was cumbersome because of the large volume involved. The dialyzed ferredoxin solution was concentrated and further purified by DEAE-cellulose chromatography (34). Amino acid analysis of the product indicated a very low yield (ca. 5 mg/kg leaf material), possibly due to degradative losses during the long extraction period.

The initial stages of aqueous extraction procedures were slow partly because it appeared that the wax content of *Sambucus* leaves interfered with DEAE-cellulose chromatography. This suggested that completely aqueous methods would not allow optimal rates of ferredoxin extraction. Therefore, efforts turned to the use of acetone in the homogenizing medium. It was thought that the resultant decrease in the surface tension of the extracts would increase the rate of treatment with DEAE-cellulose by improving flow rate properties. The

method of Crawford and Jensen, which described the isolation of ferredoxin from corn leaves, seemed promising (35).

When *Sambucus* leaves were homogenized in buffered 50% acetone-water, filtered through cheesecloth, and centrifuged, the supernatant extract passed through a bed of DEAE-cellulose very rapidly [see Chapter II, section 2 B(3)]. Also, ferredoxin, unlike many leaf proteins, is soluble in 50% acetone, so the centrifugation step was an efficient method of eliminating unwanted precipitate and particulate matter. Although the procedure described by Crawford and Jensen worked well with *Sambucus* leaves, the yield of pure protein from one such extraction (10 mg/400 g leaves) was considered inconveniently low for purposes of accumulating large enough amounts of material for sequence studies. Therefore, large-scale preparations of ferredoxin using modifications of the basic procedure of Crawford and Jensen were attempted. However, if volumes of solutions were increased too much, those steps involving column chromatography became unworkable. The method outlined in Chapter II, section 2 B(3), utilizing common laboratory apparatus, proved to be the most efficient adaptation. The extraction procedure (to the end of purification step d) was accomplished in 48 hr. The yield was 18-20 mg of freeze-dried ferredoxin from 800 g fresh weight of leaves (22.5-25.0 mg/kg leaf material). The reported yields of ferredoxin from leaves of other plants are shown in Table I. The large difference in the yields of ferredoxin from plants picked at various times of the year, which was observed with alfalfa (33), was not noticed with *Sambucus*.

TABLE I

YIELD OF FERREDOXIN FROM *SAMBUCUS* AND COMPARISON TO YIELDS FROM OTHER PLANTS

Source	Yield (mg/kg fresh wt)
Sambucus leaves	22255-25.0
Equisetum (78)	2.0-5.0
Leucaena leaves (72)	20.0
Spinacea leaves	22.0
Medicago stems and leaves (33)	20.0-25.0
Colocassia leaves (73)	25.0
Zea leaves (35)	37.5
Gossypium cotyledons (74)	200.0[a]

a: Fresh weight not reported. Calculation based on yield of ferredoxin from lyophilized, solvent-extracted dried cotyledons.

However, one seasonal difference was pronounced. During chromatography on the 2.5 x 45 cm column of DEAE-cellulose (see Chapter, section 2 B(3), purification step c), extracts of leaf shoots and very young leaves obtained in early spring showed more varied and intense polyphenol-like colouration than extracts of late spring and summer leaves. The migration of the ferredoxin-containing yellow red band down the column was therefore more difficult to see. In most preparations though, ferredoxin-containing fractions collected from the 2.5 x 45 cm DEAE-cellulose column could be identified by their UV spectra. An absorption peak or shoulder at 330 nm and a small peaks at 410-414 nm and 464-466 nm were characteristic of the protein.

During the final preparative step - gel chromatography of the concentrated ferredoxin-containing solution through a 4.7 x 100 cm column of Sephadex G-75 - ferredoxin migrated ahead of two slower moving fluorescent bands. A typical elution profile is shown in Figure 4. Crawford and Jensen found only one fluorescent band at this stage during preparation of corn ferredoxin (35). The fluorescent contaminants gave a negative test for flavonoids and appeared to be polyphenol condensation products. The ferredoxin band collected from the Sephadex G-75 column was pure, as indicated by disc electrophoresis and other criteria (see this chapter, section 1 D).

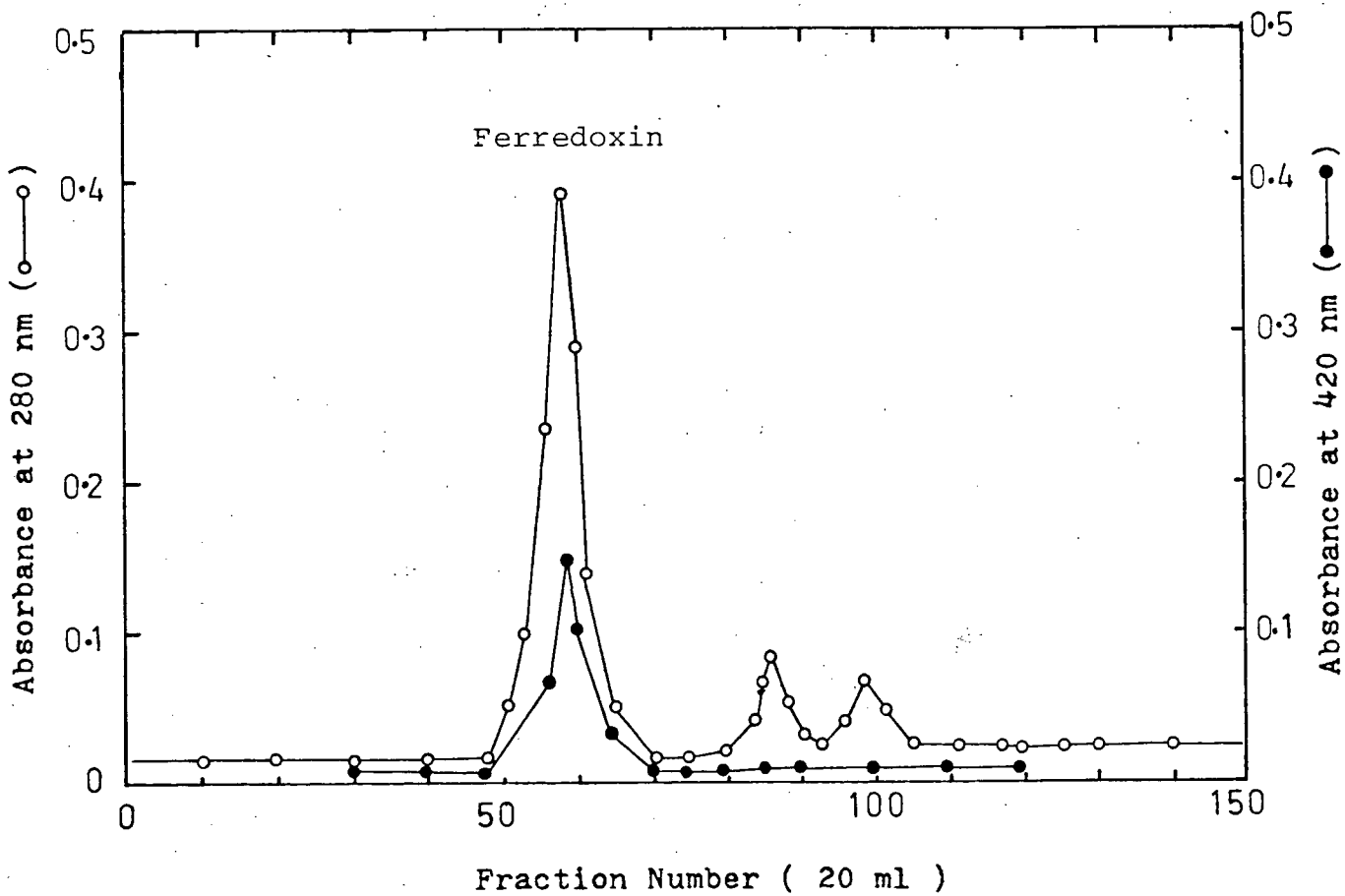

Fig. 4 Sephadex G-75 chromatography of ferredoxin-containing solution previously fractionated on DEAE-cellulose. The sample was applied to a 4.7 x 100 cm column equilibrated with 10 mM Tris-HCl, 0.2 M NaCl, pH 7.5. Fractions were collected at 60 ml/hr.

B. Spectral Properties

The UV and visible absorption of purified *Sambucus* ferredoxin was measured on a Unicam SP1800 dual beam recording spectrophotometer. Spectra were recorded immediately after purification. Samples were either taken directly from the Sephadex G-75 column eluate, or after the eluate was concentrated (see Chapter II, section 2 B(3), purification step e). The absorption spectrum is shown in Figure 5 (Curve A). Absorption maxima occur at 277, 331, 423, and 466 nm. These average positions are based on the spectra of eight different preparations of *Sambucus* ferredoxin and have a range of ±1 nm. Table II compares the absorption maxima of other plant ferredoxins with those of *Sambucus*. The ratios ("critical ratios") of the absorbances at 466, 423, and 331 nm to that at 277 nm are 0.44, 0.49, and 0.67. Table III presents these values together with the absorbance ratios of other ferredoxins. The molar extinction coefficient at 423 nm (ε_{423}), calculated from a molecular weight of 11,000, varied from 10.8 to 12.9 $mM^{-1}cm^{-1}$, when the concentration of the ferredoxin solutions was determined on a dry weight basis. When the concentration was determined by direct iron analysis, assuming 2 atoms of iron per molecule of ferredoxin, ε_{423} was 9.1 mM^{-1} cm^{-1}. The molar extinction coefficients of plant ferredoxins are given in Table IV.

C. Electron-Transfer Activity

The purified protein exhibited ferredoxin activity by

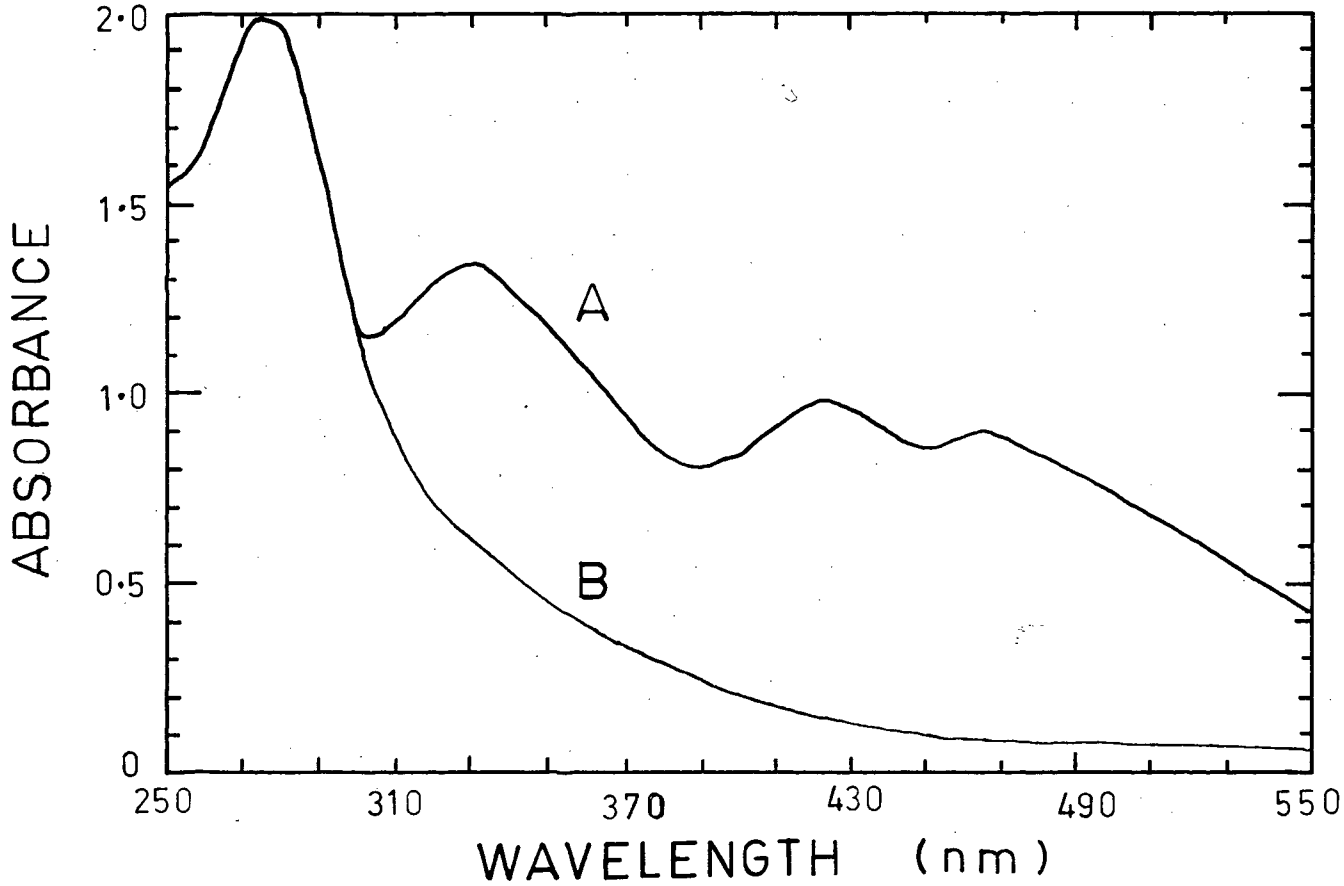

Fig. 5 Absorption spectrum of native and deteriorated ferredoxin from *Sambucus racemosa*. *Curve A* is the spectrum of a ferredoxin preparation with the highest absorbance ratio (423/277 nm) of 0.49. Ferredoxin (ca. 1 mg/ml) was in 0.1 M Tris-HCl, 0.8 M NaCl, 1 mM β-mercaptoethanol, pH 7.5. *Curve B* is the spectrum of apoferredoxin. Ferredoxin was desalted on Sephadex G-25 with distilled water.

TABLE II

ABSORPTION MAXIMA OF PLANT FERREDOXINS

Source of Ferredoxin	Wavelength (nm) of Absorption Maxima (M)			
	M_1	M_2	M_3	M_4
Angiosperms				
Sambucus	277	331	423	466
Leucaena (72)	277	325	420	463
Spinacea (79)	274	325	420	463
Medicago (33)	277	331	422	465
Colocassia (73)	277	330	420	465
Zea (35)	277	330	423	463
Gossypium (74)	280	325	419	460
Fern				
Polystichum (80)	276	330	420	465
Primitive Vascular Plant				
Equisetum (78)	276	330	421	465
Green Algae				
Scenedesmus (12)	276	330	421	464
Euglena (81)	276	328	422	465
Blue-green Algae				
Nostoc (82)	276	331	423	470
Microcystis (83)	276	330	422	464

TABLE III

CRITICAL ABSORBANCE RATIOS OF PLANT FERREDOXINS

Source of Ferredoxin	Ratio of absorbance at different maxima (M) to that at M_1 (cf. TableIII)		
	M_4	M_3	M_2
Angiosperms			
Sambucus	0.44	0.49	0.67
Leucaena (72)	0.43	0.49	0.65
Medicago (33)	0.43	0.48	0.65
Colocassia (73)	0.39	0.43	0.64
Zea (35)	0.43	0.48	0.60
Green Algae			
Scenedesmus (45)	nr	0.65	0.88
Euglena (81)	0.60	0.68	0.87
Blue-green Alga			
Nostoc (82)	nr	0.57	nr

nr: Not included in reference.

TABLE IV

MOLAR EXTINCTION COEFFICIENTS OF PLANT FERREDOXINS

Source of Ferredoxin	Wavelength (nm)	ε ($mM^{-1} cm^{-1}$)
Angiosperms		
Sambucus	423	9.1[a]
Spinacea (12)	420	9.7
Medicago (12)	422	9.1
Colocassia (73)	420	9.7
Zea (35)	423	10.0
Gossypium (74)	419	7.6
Petroselinum (84)	422	9.2
Primitive Vascular Plant		
Equisetum (78)	421	8.8
Blue-green Alga		
Spirulina (85)	420	9.7

a: Value calculated from absorbancy measurement and direct iron analysis of the same ferredoxin solution with an absorbance ratio (423 nm/277 nm) of 0.49.

mediating the photoreduction of NADP by spinach chloroplasts. Figure 6 shows the resultant increase in the absorption of the reaction mixture at 340 nm due to the formation of $NADPH_2$. *Sambucus* ferredoxin sustained an initial reduction rate of 86 μmoles NADP per mg chlorophyll per hour.

D. Homogeneity

The homogeneity of ferredoxin preparations was assessed by disc electrophoresis. In 7.7% polyacrylamide gels, ferredoxin migrated together with the bromophenol blue front marker dye as a single, thin, fast moving, coloured band. All samples examined showed no evidence of contamination. Figure 7 depicts the typical pattern of stained protein in the gels.

A useful index of purity is the comparison of the light extinction of iron-sulphur proteins at characteristic wavelengths (absorbance maxima). Absorbance ratios have almost always been used as one criterion of homogeneity in studies involving ferredoxin (4, 33). In *Sambucus* ferredoxin preparations, the 423 nm to 277 nm absorbance ratio varied from 0.45 to 0.49. This is comparable to the ratios of other pure ferredoxins (Table III) and indicates a high level of purity. Also, similarity of the spectrum (Figure 5), absorption maxima (Table II), and molar extinction coefficient (Table IV) of *Sambucus* ferredoxin to the corresponding spectral properties of other purified ferredoxins was used to judge the protein preparations homogeneous.

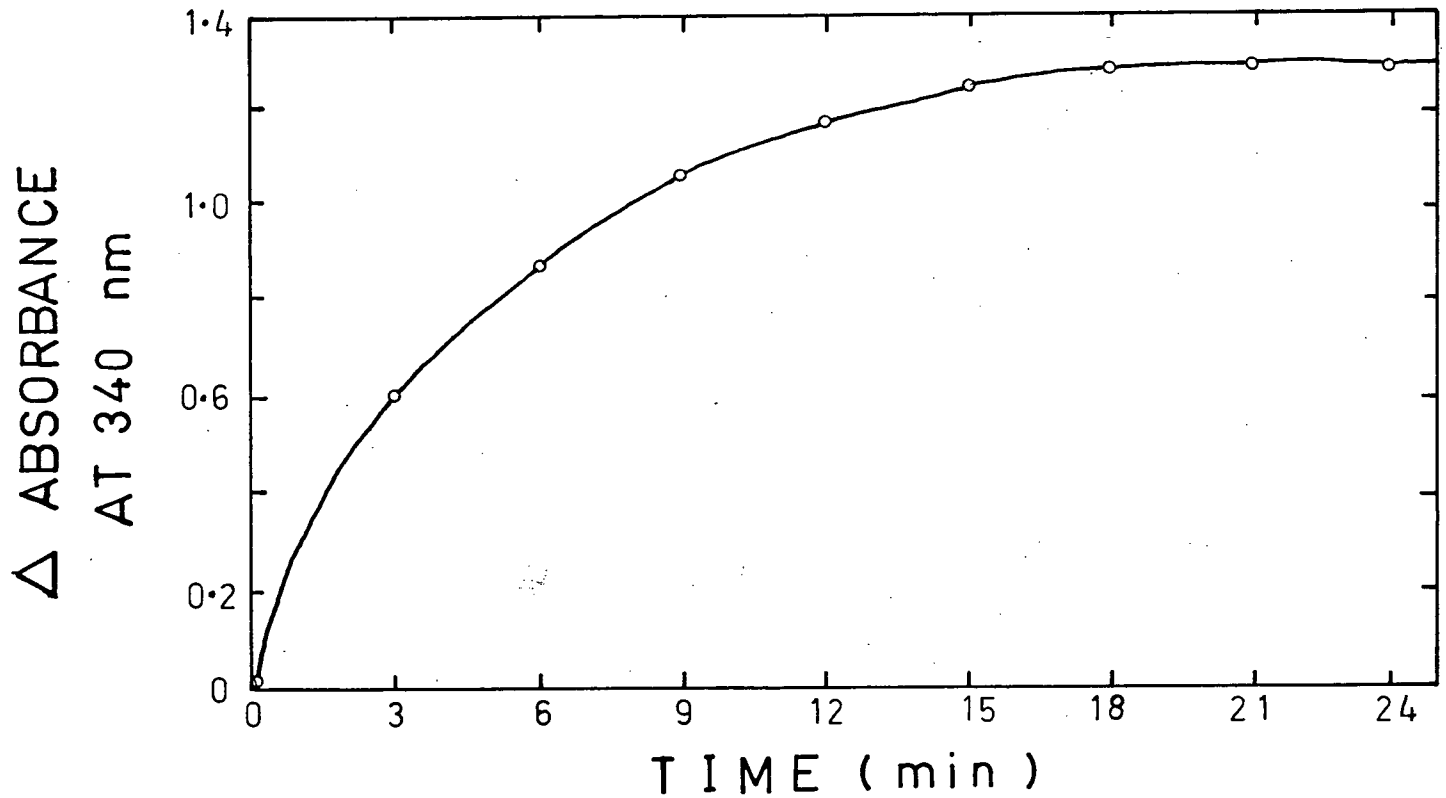

Fig. 6 *Sambucus* ferredoxin-mediated photoreduction of NADP. The reaction mixture contained 2 μmole of NADP, chloroplast suspension with 0.1 mg of chlorophyll, 0.1 mg of ferredoxin, and 0.25 M Tris, 0.5 M sorbitol, 5 mM β-mercaptoethanol at pH 7.5, in a final volume of 3.0 ml. The blank cuvette contained the same components except for ferredoxin. The ferredoxin sample had an absorbance ratio (423/277 nm) of 0.44. See Chapter II, section 2 D for conditions of assay.

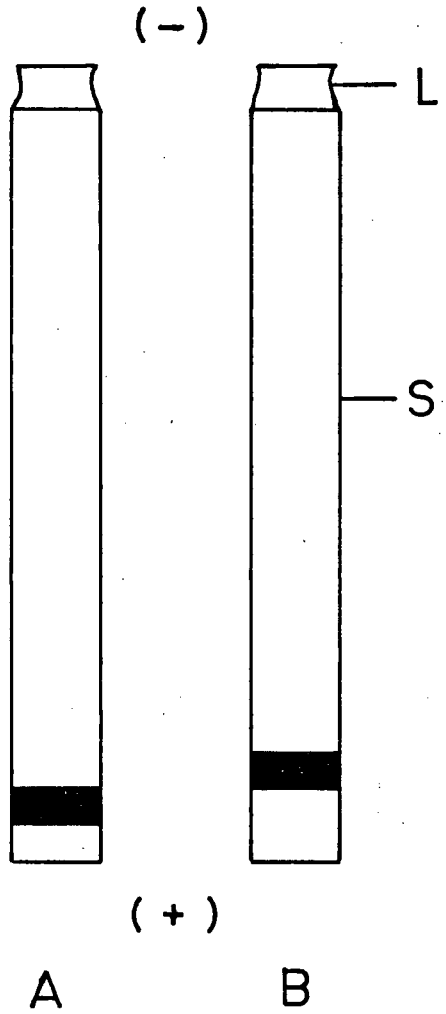

Fig. 7 Electrophoretic pattern of *Sambucus* ferredoxin. L: large-pore spacer gel. S: 7.7% polyacrylamide small-pore separating gel. A: 60 µg of ferredoxin (with a 423/277 nm absorbance ratio of 0.268) was applied to spacer gel. B: 200 µg of ferredoxin (423/277 nm ratio of 0.47). In all gels, ferredoxin moved with the electrophoretic front. See Chapter II, section 2 E for conditions of electrophoresis.

The final step for purification of ferredoxin was gel filtration through a 4.7 x 100 cm column of Sephadex G-75 [see Chapter II, section 2 B(3)]. The elution profile indicated a clean sharp peak corresponding to the ferredoxin band (Figure 4). Symmetry of the peak suggested the presence of a single component. Other close peaks, shoulders, or skewness, indicative of possible contamination, were absent. The visible light absorbance at 420 nm, due to the iron chromophore (33), coincided with the UV light absorbance at 280 nm, due to protein aromatic amino acids. This further showed that the peak represented only ferredoxin.

Electrophoresis of fluorescamine-labeled samples on polyacrylamide gels in the prescence of SDS was performed to estimate the molecular weight of *Sambucus* ferredoxin (see this chapter, section F). In all gels, only one thin fluorescent band was visible.

On no occasion during the sequence studies was there any evidence of heterogeneity in the ferredoxin preparations. The NH_2-terminal determinations performed on the whole protein as well as on peptides always showed the presence of only one amino terminus.

Furthermore, high stoichiometry among amino acid residues was evident in the amino acid analyses.

E. Amino Acid Composition

The results of the amino acid analyses of *Sambucus* ferredoxin are given in Tables V, VI, VII, and VIII. Three samples of protein were hydrolyzed with 6 N HCl in vacuo for 19,

TABLE V

AMINO ACID ANALYSES OF DESICCATED HYDROLYZATES OF *SAMBUCUS* FERREDOXIN[a] (nmole amino acid/mg protein)

Amino acid	Values obtained after hydrolysis for: 19 hr	38 hr	62 hr	Best estimate[b]
Lysine	282.9[c]	304.8	309.9	309.9
Histidine	72.6	119.0	127.8[c]	127.8
Arginine	58.1	69.7	73.4	73.4
Aspartic acid	650.4	752.4	771.8	771.8
Threonine	262.2	336.2	337.9[c]	337.9
Serine	415.9	472.7	494.9[c]	494.9
Glutamic acid	1002.8	1150.7	1144.2	1150.7
Proline	257.9	355.4	338.1	355.4
Glycine	428.9	475.3	498.5[c]	498.5
Alanine	379.2	429.9	445.9	445.9
Half cystine	176.3	266.4[c]	262.1	266.4
Valine	247.1	478.2[c]	495.6	495.6
Methionine	0	0	0	0
Isoleucine	222.5	346.3[c]	355.2	355.2
Leucine	326.4	440.3	454.1	454.1
Tyrosine	187.8	235.3[c]	248.6[c]	248.6
Phenylalanine	117.3	144.4	148.2	148.2

a: See Chapter II, section 2 G(1) for details of analysis procedures.

b: Maximum value.

c: Higher than corresponding value from undesiccated hydrolyzate (Table VI).

TABLE VI

AMINO ACID ANALYSES OF UNDESICCATED HYDROLYZATES OF *SAMBUCUS* FERREDOXIN[a] (nmole amino acid/mg protein)

Amino acid	Values obtained after hydrolysis for: 19 hr	38 hr	62 hr	Best estimate[b]	Percent increase[c]
Lysine	260.5[d]	308.2	391.5	391.5	7.0
Histidine	79.3	139.5	106.4[d]	139.5	1.8
Arginine	66.6	73.8	75.4	75.4	7.2
Aspartic acid	743.8	812.6	838.8	838.8	10.1
Threonine	291.6	355.3	296.3[d]	355.3	0.7
Serine	470.1	551.9	481.2[d]	551.9	8.6
Glutamic acid	1071.1	1206.7	1249.2	1249.2	7.0
Proline	316.8	384.5	439.8	439.8	19.9
Glycine	435.1	485.8	486.6[d]	486.6	0.3
Alanine	404.4	448.7	465.3	465.3	5.0
Half cystine	212.5	261.5[d]	277.2	277.2	6.6
Valine	284.8	468.6[d]	619.7	619.7	12.5
Methionine	0	0	0	0	-
Isoleucine	252.5	342.8[d]	381.7	381.7	5.7
Leucine	365.8	446.6	461.6	461.6	4.4
Tyrosine	201.8	232.8[d]	239.1[d]	239.1	0.3
Phenylalanine	153.8	165.9	173.8	173.8	20.4
Average Percent Increase					7.3

a: Hydrolyzates were not desiccated according to the method of Robel (40). See Chapter II, section 2 G(1) for details of the analysis procedures.

b: Maximum value.

c: The increase in total amino acid recovered from the 3 undried hydrolyzates (this Table), expressed as a percentage of total amino acid recovered from the 3 dried hydrolyzates (see Table V).

d: Lower than corresponding value from dried hydrolyzates (Table).

TABLE VII

AMINO ACID COMPOSITION OF *SAMBUCUS* FERREDOXIN

Amino acid	Average of best estimates[a]	Residues per molecule[b]	Nearest integer
Lysine	350.7	5.00	5
Histidine	133.7	2.00	2
Arginine	74.4	1.00	1
Aspartic acid	805.3	11.48	11
Threonine	346.6	5.00	5
Serine	523.4	7.46	7
Glutamic acid	1200.0	17.11	17
Proline	397.6	5.67	6
Glycine	492.6	7.00	7
Alanine	455.6	6.50	6-7
Cysteine[c]	271.8	3.88	4
Valine	557.7	8.00	8
Methionine	0	0	0
Isoleucine	368.5	5.25	5
Leucine	457.9	6.53	7
Tyrosine	243.9	3.48	3
Phenylalanine	161.0	2.30	2
Tryptophan	92.1[d]	1.31	1
Total residues			97-98

a: Best estimates (nmole amino acid/mg protein) from desiccated and undesiccated hydrolyzates are shown in Table V and Table VI, respectively.

b: Assuming 1 arginine, 2 histidine, 5 lysine, 5 threonine, 7 glycine, and 8 valine per molecule of ferredoxin.

c: Also determined as AECys. Analysis of undried 24 hr hydrolyzate of AECys-ferredoxin showed 229.1 nmole/mg protein.

d: Determined according to the method of Liu (43). See Chapter II, section 2 G(2) for details of analysis.

TABLE VIII

AMINO ACID COMPOSITION OF PLANT FERREDOXINS

	Lys	His	Arg	Trp	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Total
Sambucus	5	2	1	1	11	5	7	17	6	7	6-7	4	8	0	5	7	3	2	97-98
Leucaena (72)	5	1	2	1	11	4	7	16	5	6	7	5	6	0	4	10	3	3	96
Medicago[a] (86)	5	2	1	1	9	6	8	16	3	7	9	5	9	0	4	6	4	2	97
Spinacea[a] (50)	4	1	1	1	13	8	7	13	4	6	9	5	7	0	4	8	4	2	97
Zea mays (35)	3	2	1	1	13	5	8	14	4	8	8	4	10	0	5	8	5	1	100
Colocassia[a] (49)	5	1	1	1	10	6	8	15	4	9	6	5	10	0	4	6	4	2	97
Polystichum (80)	4	2	1	0	14	6	8	9	5	9	7	5	5	2	6	7	3	4	97
Equisetum (78)	4	1	1	0	9	7	8	16	4	9	6	4	6	1	5	8	2	4	95
Scenedesmus[a] (87)	4	1	1	0	12	10	8	10	4	7	10	6	5	1	3	7	4	3	96
Microcystis (83)	3	1	1	0	13	7	6	13	4	12	9	5	4	1	6	9	3	1	98

a. Amino acid sequences known.

38, and 62 hr. After the tubes were opened the hydrolyzates were desiccated, dissolved in 0.2 N sodium citrate buffer, and then analyzed according to standard procedure. The recovery of each amino acid from these three hydrolyzates is shown in Table V. Three duplicate hydrolyzates were not dried; instead, they were directly diluted with 0.2 N sodium citrate buffer [see Chapter II, section 2 G(1)]. Table VI presents the results from these latter samples. Comparison of the 48 pairs of corresponding values (16 amino acids x 3 different hydrolysis times) in Tables V and VI shows that only ten values were higher in the desiccated hydrolyzates. In most cases, higher values were recorded for the undesiccated hydrolyzates. The total difference between the recovery of each amino acid from the two types of hydrolyzates is expressed as a percentage in Table VI. Every amino acid was recovered in a higher average yield from the undesiccated hydrolyzates than from those hydrolyzates which were dried before analysis, and the increase varied with each amino acid from 0.3% (tyrosine and glycine) to 20.4% (phenylalanine). Aspartic acid (10.1%), valine (12.5%), proline (19.9%), and phenylalanine (20.4%) showed the highest increases. The average increase in recovery of amino acids from hydrolyzates not dried before analysis was 7.3%.

Methionine was not detected. All other amino acids, including serine and threonine, were recovered in larger amounts from the 38 hr hydrolyzates than from the 19 hr hydrolyzates. Most amino acids yielded their highest values after

62 hours of hydrolysis. For this reason the results from different hydrolysis times were not averaged; instead, the maximum value of each amino acid was assumed to be the best estimate of its actual occurence in the native protein. The best estimate values from desiccated hydrolyzates (Table V) and undesiccated hydrolyzates (Table VI) were averaged. The average values are shown in Table VII, together with the result of the tryptophan analysis which was performed by the method of Liu (43). The average value for cysteine was higher than the value obtained from analysis of S-β-aminoethylcysteinylferredoxin; and therefore the average value probably approximates the maximum occurence of cysteine in the protein. The amino acid composition was calculated with arginine normalized to one residue per molecule of ferredoxin, histidine normalized to two residues, lysine normalized to five, threonine to five, glycine to seven, and valine to eight. Table VII gives the resultant amino acid composition. The calculations show that alanine may occur as six or seven residues per molecule of protein. The calculations also show four other amino acids (aspartic acid, serine, leucine, and tyrosine) with poor integral stoichiometry. Therefore, the actual total number of residues may vary from 97 to 102. But if homology with other plant ferredoxins is assumed (see Table VIII for comparison), 97 or 98 residues is probably correct. The amino acid composition of *Sambucus* ferredoxin presented in this thesis includes aspartic acid, serine, and tyrosine rounded off to the nearest integer.

The contents of various amino acids of *Sambucus* ferredoxin resemble those of algae and other higher plant ferredoxins (Table VIII). The numbers of lysine, tryptophan, aspartic acid, serine, alanine, and tyrosine residues are identical to *Leucaena* ferredoxin. The content of lysine, histidine, arginine, tryptophan, glycine, and phenylalanine are identical to *Medicago* ferredoxin. The total basic amino acid content (9 residues) is identical to that of *Leucaena*, *Medicago*, and *Cyperus* (75). The relative proportions of aspartic acid and glutamic acid are remarkably similar to those found in other plant ferredoxins: *Leucaena*, *Medicago*, *Colocassia*, *Gossypium* (74), *Equisetum*, *Cyperus* (75), and *Cladophora* (88). The occurence of 6 proline and 17 glutamic acid residues in *Sambucus* ferredoxin has not been reported for other ferredoxins (5, 20).

F. Molecular Weight

The minimum value for the molecular weight of *Sambucus* ferredoxin calculated from the amino acid composition reported in Table VII is 10,610 daltons. Including the two atoms of iron and two atoms of inorganic sulphur in the active centre (see this chapter, section 1 G), the minimum molecular weight is 10,786.

The molecular weight was also determined from the electrophoretic mobility of fluorescamine-labeled ferredoxin in SDS gels relative to molecular weight standards. The protein

standards used were oyalbumin (45,000), chymotrypsinogen (25,000), myoglobin (17,800), lysozyme (14,300), and ribonuclease (12,700). Figure 8 shows the results of a typical electrophoretic run. Values for ferredoxin repeatedly ranged from 1.02×10^4 to 1.12×10^4 daltons. The average molecular weight of ferredoxin from this method is 10,700 ± 500 daltons.

The values from the two different methods are consistent and give an average molecular weight of *Sambucus* ferredoxin of 10,740 ± 600. This is similar to the values reported for other plant ferredoxins. For example, the formula molecular weights of *Leucaena* (72) and *Spinacea* (50) ferredoxins are 10,770 and 10,646, respectively.

G. Iron Content

The iron content of ferredoxin was determined by the *o*-phenanthroline colorimetric method. Table IX shows the results from three typical analyses. The values suggest that two iron atoms are present in one molecule of *Sambucus* ferredoxin, as in all well characterized plant ferredoxins (5). From a preliminary analysis, the content of inorganic labile sulphide seemed to be 2 g atom per molecule. Together with the results of the iron analysis, this suggests the active centre of *Sambucus* ferredoxin to be the same as in other plant ferredoxins.

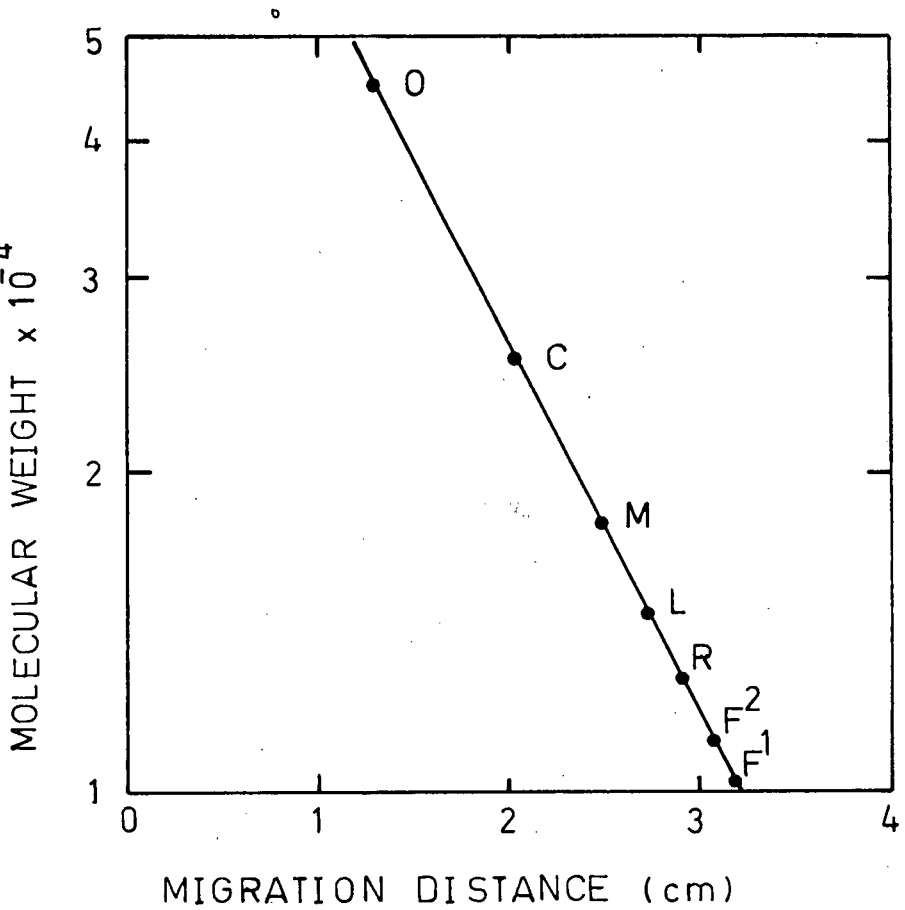

Fig. 8 SDS electrophoresis of fluorescamine-labeled ferredoxin and molecular weight standards on 10% polyacrylamide gels. Plot of migration distance versus logarithm of molecular weight. Distances are normalized to a bromophenol blue front migration distance of 5 cm. Abbreviations: O, ovalbumin; M, myoglobin; C, chymotrypsinogen; L, lysozyme; R, ribonuclease; F^1, F^2, samples of *Sambucus* ferredoxin from two different preparations.

TABLE IX

IRON CONTENT OF *SAMBUCUS* FERREDOXIN

Protein[a] (μmole)	Iron (μmole)	Ratio (g atom/molecule)	Nearest integer
0.0154	0.0347	2.256	2
0.0173	0.0291	1.682	2
0.0224	0.0546	2.438	2

a: Determined on dry weight basis, assuming molecular weight of 10,700. Samples were from different preparations.

2. PRELIMINARY SEQUENCE STUDIES

The NH_2-terminal amino acid residue of *Sambucus* ferredoxin was determined by the dansyl method (58). Protein was denatured by heating in 1% SDS and buffered with N-ethylmorpholine. Dansyl chloride, dissolved in dimethylformamide, was then added to the protein and allowed to react. When reagent grade dimethylformamide was distilled and used as the solvent for the dansyl chloride, no dansylated products were detected after hydrolysis of the protein. It seemed that even small traces of water interfered with the labelling of the protein. This problem was solved by the use of anhydrous silylation grade dimethylformamide as specified in Chapter II, section 1 B. After reaction, the dansylated ferredoxin was precipitated with acetone and washed free of excess reagents. If the labeled protein was washed with 80% acetone as suggested by Gray (58), the ferredoxin redissolved and only precipitated again on long standing at 4 C. Instead, 90% acetone was used to wash the dansylated ferredoxin which, in this case, remained precipitated as tiny flocculi.

The dansyl amino acid liberated from the dansyl derivative of native ferredoxin upon hydrolysis was identified as dansyl alanine by thin-layer chromatography. ε-Dansyl lysine was also detected on the polyamide sheet due to the content of five lysine residues in the protein. Attempts at dansylating the NH_2-terminus of AECys-ferredoxin failed. Only ε-dansyl lysine was detected. The apparent reason was the insolubility of the AECys-derivative of *Sambucus* ferredoxin

(see below) which did not dissolve even in the 1% SDS-N-ethylmorpholine-dimethylformamide (2:2:3 by volume) dansyl reaction medium; and that the NH_2-terminus was therefore not exposed to the dansyl chloride and so unable to react with it.

After one cycle of Edman degradation performed on native ferredoxin, the second amino acid residue was identified as dansyl threonine. Although AECys-ferredoxin was insoluble in the coupling buffer, it was carried through one cycle of degradation nevertheless, with the hope that during the subsequent dansylation procedure, the NH_2-terminus of the polypeptide chain would be exposed to and react with the dansyl chloride. However, no new dansyl amino acid was detected, apparently for the same reason as stated above.

These results suggest that the amino-terminal sequence of native *Sambucus* ferredoxin is Ala-Thr.

Apoferredoxin, prepared by trichloroacetic acid treatment, appeared to be well hydrolyzed by thermolysin under the digestion conditions described in Chapter II, section 2 J(1). The thermolytic peptide mixture was fractionated by gel filtration through Sephadex G-25 Fine. The elution profile is shown in Figure 9. On the basis of light absorbancy at 220 nm (peptide bonds) and 280 nm (aromatic amino acids), the column eluate was pooled into five fractions. Two-dimensional electrophoresis showed that each fraction was substantially heterogeneous: fractions A, B, C, D, and E contained 32, 38, 22, 14, and 9 ninhydrin-positive spots respectively. The first three fractions shared a considerable number of spots with identical mobilities. Six thermolytic peptides (Th-1 to Th-6) were iso-

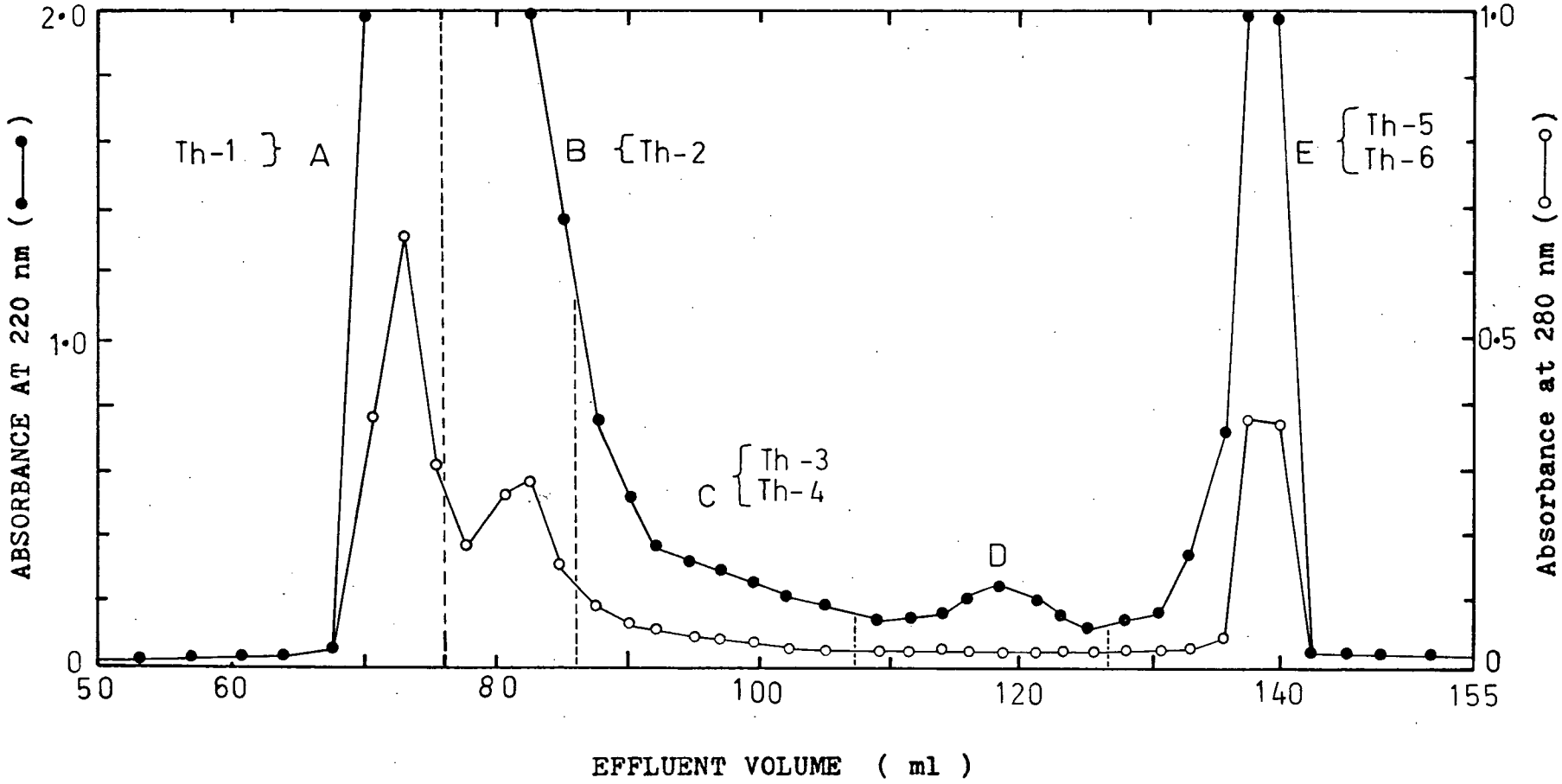

Fig. 9 Elution pattern of peptides from the thermolytic digest of TCA-treated ferredoxin chromatographed on a column of Sephadex G-25 Fine (1.4 x 97 cm). Letters refer to the fractions pooled which are indicated by the vertical lines. Thermolytic peptides studied (Th-1 to Th-6) were isolated from the pooled fractions indicated. See Chapter II, sections 2 J(1) and 2 K for experimental details.

lated and partially studied. The amino acid compositions of three peptides (Th-1, -3, and -4) are presented in Table X.

Peptide Th-1: Leu-Asx (Asx, Glx, Glx). This was an acidic peptide isolated from Fraction A. It stained red with ninhydrin. Mobility (m) with respect to dansyl arginine was 0.39 at pH 6.5 and 0.45 at pH 1.9. Having an approximate molecular weight of 600, these mobility characteristics indicate that the peptide has a net charge of +1 at pH 1.9 according to Bailey and Ramshaw (89). Two dansyl-Edman steps indicated the amino-terminal sequence to be Leu-Asx.

Peptide Th-2: neutral peptide from Fraction B, stained red, $m_{6.5}$ = 0.90, $m_{1.9}$ = 0.74. Two dansyl-Edman steps indicated the amino-terminal sequence to be Leu-Gly.

Peptide Th-3: Leu-Thr (Ala). A neutral peptide from Fraction C, stained red, $m_{6.5}$ = 0.91, $m_{1.9}$ = 0.65. Amino-terminal sequence was Leu-Thr. The complete sequence must therefore be Leu-Thr-Ala.

Peptide Th-4: Ile-Asx (Glx, Gly, Trp, Val). An acidic peptide from Fraction C, $m_{6.5}$ = 0.17, $m_{1.9}$ = 0.39. Fluorescence in UV light indicated the presence possibly of ferredoxin's single tryptophan residue. It stained light red with ninhydrin but became dark red overnight indicating valine or isoleucine as the NH_2-terminal residue (55). The molecular weight of the peptide and its mobility at pH 1.9 with respect to dansyl-arginine indicate a net charge of +1. Two dansyl-Edman steps indicated the amino-terminal sequence to be Ile-Asx.

Peptide Th-5: basic peptide from Fraction E, stained

TABLE X

AMINO ACID COMPOSITION OF THERMOLYTIC PEPTIDES[a]

OF TCA-TREATED FERREDOXIN

The composition of each peptide is given as the molar ratios of the amino acids, calculated without correction for destruction during 6 N HCl hydrolysis for 24 hr. The integral values of major constituents are given in parentheses.

Amino acid	Th-1	Th-3	Th-4
Lysine	0.03		
Aspartic acid	2.15 (2)	0.14	1.27 (1)
Threonine		0.73 (1)	
Serine		0.06	
Glutamic acid	2.18 (2)		1.27 (1)
Glycine		0.21	1.19 (1)
Alanine		1.13 (1)	
Valine		0.18	1.00 (1)
Isoleucine			1.06 (1)
Leucine	1.00 (1)	1.00 (1)	
Total Residues	5	3	5

a: See Chapter II, sections 2 J(1), 2 K, and 2 L for experimental details. Purified peptides were eluted from paper and divided into two equal halves for amino acid analysis and dansyl-Edman sequencing.

red, $m_{6.5} = 1.60$, $m_{1.9} = 1.14$. Amino-terminal sequence was Tyr-Leu.

Peptide Th-6: neutral peptide from Fraction E, stained red, $m_{6.5} = 0.90$, $m_{1.9} = 0.84$. Amino-terminal sequence was Leu-Gly, the same as for peptide Th-2. The increased mobility of Th-6 at pH 1.9 as compared to that of Th-2, and its larger elution volume from the Sephadex G-25 column both suggest that peptide Th-6 may be a shorter version of the neutral peptide Th-2.

S-β-aminoethylcysteinylferredoxin was insoluble at pH 8.2, the pH optimum of trypsin. The digest was performed at pH 8.9 instead, for six hours. When the digest was clarified by centrifugation the pellet appeared to contain most of the original protein, an indication that little digestion had occurred. Nevertheless, the supernatant was chromatographed on Sephadex G-25 Fine, to obtain a fractionation pattern of the tryptic peptides. Figure 10 shows the elution profile. The eluate was pooled into three fractions, A, B, and C, which were shown to contain 29, 24, and 11 ninhydrin-positive spots respectively, by two-dimensional high voltage paper electrophoresis. Due to their low yield, these tryptic peptides were not studied.

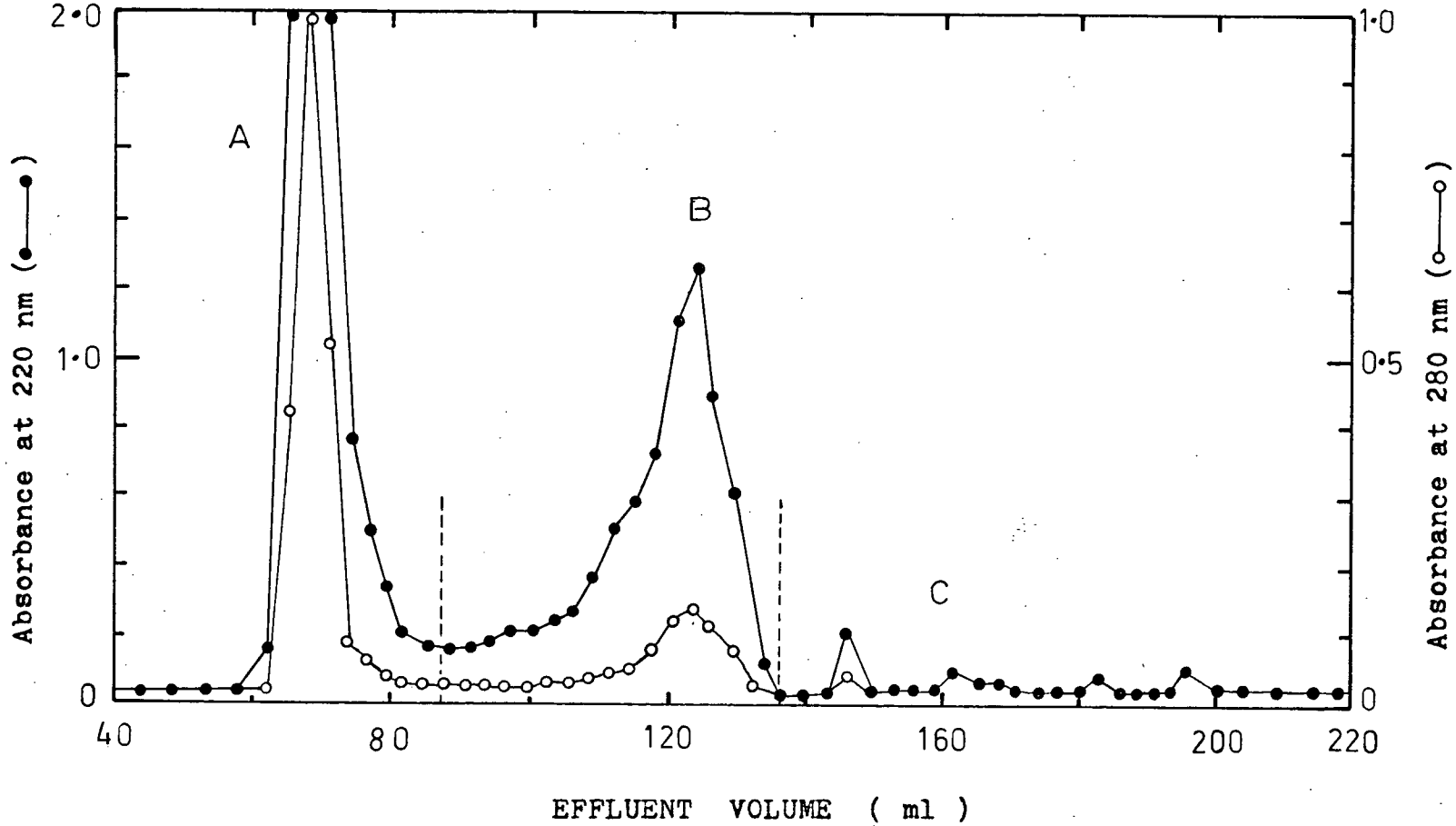

Fig. 10 Elution pattern of peptides from the tryptic digest of *Sambucus* AECys-ferredoxin chromatographed on a column of Sephadex G-25 Fine (1.4 x 97 cm). Letters refer to the fractions pooled which are indicated by the vertical lines. See Chapter II, sections 2 J(2) and 2 K for experimental details.

CHAPTER IV

DISCUSSION

A satisfactory method was developed for the isolation and purification of ferredoxin from leaves of *Sambucus racemosa*. The method, based on the techniques of Crawford and Jensen (35), was modified to process 800 g of leaf material at one time as rapidly as possible.

Initially, aqueous extraction methods (33, 34, 75, 77) were tried with *Sambucus* leaves but these proved unsuitable. The filtration and chromatographic steps were made difficult by the presence of chlorophyllous slime and wax-like material, the content of which in *Sambucus* leaves appeared to be greater than in *Medicago* or *Spinacea*, with which the aqueous methods were developed. Others have encountered similar problems. Crawford and Jensen reported that the aqueous extraction method of Keresztes-Nagy and Margoliash (33) was unsatisfactory for the isolation of ferredoxin from leaves of *Zea mays* (35). Margoliash's group was unable to recover any ferredoxin whatsoever when large quantities of the dark acidic oxidation product of phenoloxidase were present (Dr. E. Margoliash, personal communication). Finally, extraction of *Sambucus* leaves with 50% acetone-water allowed the preparation of ferredoxin to proceed in a fast and efficient manner.

The extraction procedure is described in detail in Chapter II, section 2 B(3). Although it worked well with

Sambucus leaves, *Viburnum* and *Lonicera* leaves failed to yield any ferredoxin when this method was used. However, the applicability of the procedure to other plant sources was not extensively investigated.

The yield of ferredoxin (22.5 to 25.0 mg per kg leaf material) from *Sambucus* is very similar to the yields from other higher plants (Table I). Although the methods of ferredoxin extraction have differed, the reported yields from leaves of *Spinacea, Leucaena, Colocassia, Medicago,* and *Sambucus* are all 20.0 to 25.0 mg per kg leaf material. Crawford and Jensen used 20 to 35 day-old seedlings of *Zea mays* and obtained 37.5 mg of ferredoxin per kg of leaves (35). The yield reported from *Equisetum* (2.0 to 5.0 mg of ferredoxin per kg leaves) is much lower. This value may represent the actual ferredoxin content in this group of primitive vascular plants. Such a low yield from *Equisetum* may also be a result of the specific extraction method which was applied to this plant material (78). If the ferredoxin content of ferns were investigated, the result might indicate an increasing trend from the primitive vascular plants to the Angiospermae. In this respect it is unfortunate that in the study of *Polystichum* ferredoxin, the yield was not reported (80).

In a recent study of ferredoxin development in *Phaseolus vulgaris*, the leaves of 14 day-old etiolated plants contained 1.42 to 1.76 nmol/g fresh weight (90). Assuming a molecular weight of 11,000, this represents 15.6 to 19.4 mg of ferredoxin per kg leaf material. During subsequent illumina-

tion of the plants at high light intensity (2500 lx) for 50 hr, the amount of ferredoxin per leaf increased approximately eight-fold. However, the weight of the leaves during this period was not monitored. After 50 to 70 hours the net synthesis of ferredoxin levelled off. In the present case with *Sambucus* leaves, the yield of ferredoxin at various developmental stages was not studied.

The yield of ferredoxin from leaves of higher plants is much greater than the yield of other proteins used in amino acid sequence studies of plant relationships. Boulter and coworkers have reported yields of cytochrome *c* to vary from 0.2 to 0.45 mg per kg dry seeds (91). Young inflorescences of *Brassica oleracea* and whole plants of *Arum maculatum* yielded 0.2 and 0.09 mg of cytochrome *c* per kg fresh weight respectively (92). This same group has sequenced plastocyanin from *Sambucus nigra* and *Solanum tuberosum* (26, 27). The yield of purified protein from the latter source was 3.5 mg per kg of leaves.

Sambucus ferredoxin, prepared by the extraction procedure presented in this thesis, was shown to be pure by disc electrophoresis in 7.7% polyacrylamide gels at pH 9.0. The protein, due to its low molecular weight and high content of acidic amino acids, exhibited the same high anionic mobility (Figure 7) as reported for other ferredoxins (33, 34, 45, 75, 93). Electrophoresis at an additional pH [e.g. pH 4.3 (75)] offers a further test of homogeneity, but to conserve ferredoxin material, this was not performed. Keresztes-Nagy and Margoliash noted that deteriorated preparations of

alfalfa ferredoxin showed a slow moving colourless material migrating behind the fast moving coloured native ferredoxin (33). This was not observed with *Sambucus* ferredoxin (Figure 7, Gel A). A partially deteriorated preparation with a 423/277 nm absorbance ratio of only 0.268 still showed a single band. The appearance of an additional trailing band in 16% polyacrylamide gels was reported for pure spinach ferredoxin (34). This effect was observed with *Sambucus* ferredoxin during preliminary experiments with higher acrylamide concentrations, but was not investigated further.

In this study ferredoxin was detected in the acrylamide gels by its native red brown colour and by the general protein stain, Buffalo Blue Black (Amido Schwartz). In a polyacrylamide electrophoretic study of ferredoxin and other leaf proteins, Rougé (93) used the putatively more sensitive stain, Coomassie Brilliant Blue (94). A recently reported stain is specific for iron-containing proteins. Brill *et. al.* have shown that ferredoxin, separated in polyacrylamide gels and stained with 0.7% α,α-dipyridyl containing 8% mercaptoacetic acid, can be detected as a thin pink band (95).

Fluorescamine-labeled preparations of *Sambucus* ferredoxin, when fractionated in SDS polyacrylamide gels by electrophoresis, showed only one fluorescent band. This was further convincing evidence of homogeneity since Fluorescamine [4-phenylspiro(furan-2[3H],1'-phthalan)-3,3'-dione] is a highly sensitive reagent for the detection of peptides and proteins in the picomole range (96). Contaminating moieties with free

primary amino groups were not present.

Purified *Sambucus* ferredoxin was found to be similar to other plant ferredoxins in its spectral properties, biological activity, amino acid composition, molecular weight, and active centre.

The UV and visible spectrum of native *Sambucus* ferredoxin (Figure 5, curve A) is almost identical to those of other plants (Table II). The position of each absorption maximum (peak) is within 1 to 6 nm of the corresponding maxima of other ferredoxins. The "critical ratios" of absorbances at these maximal positions are also similar to the ratios reported for other angiosperm ferredoxins (Table III).

From extensive studies of the spectral properties of ferredoxin from alfalfa, Keresztes-Nagy and Margoliash concluded that the protein, the iron, and the labile sulphur account for the entire spectrum (33). No prosthetic groups such as flavin exist. The aromatic amino acid residues (Trp, Phe, Tyr) and the two iron atoms account for the entire absorption at 277 nm. Angiosperm ferredoxins all contain seven aromatic residues (Table VIII, and ref. 20). Their critical absorbance ratios are similar to each other and differ from those of fern (80) and algae (Table III) which lack tryptophan and which have different total amounts of phenylalanine and tyrosine. The aromatic amino acid content of *Sambucus* ferredoxin was calculated to be only six residues: 1 Trp, 3 Tyr, 2 Phe (Table VII). The spectral similarity of the protein to other angiosperm ferredoxins may indicate that it instead contains seven aromatic amino acid residues. Tyrosine may

actually occur as four residues because its value of 3.48 residues per molecule in Table VII shows poor integral stoichiometry. However, the content of phenylalanine is also suspect and must await further confirmation because during the amino acid analyses it showed the greatest difference in recovery (20.4%) between the dried and undried protein hydrolyzates (Table VI). Such anomalous recovery behaviour gives reason for further study. The true amino acid composition of *Sambucus* ferredoxin ascertained from its complete sequence would indicate whether the UV absorption at 277 nm is in fact due to six or seven residues, together with the two atoms of iron.

The two labile sulphur atoms from the active centre of ferredoxin account for the structure of the near ultraviolet and visible portions of the spectrum (33). Deterioration of the protein in ion-low water involves the loss of the labile sulphur which results in the almost complete lack of absorption in this region. Deteriorated *Sambucus* ferredoxin (Figure 5, curve B) shows a spectrum not unlike that of deteriorated ferredoxin from *Medicago* and *Zea mays* (33, 35).

The molar extinction coefficient at 423 nm varied depending on the method of calculation of the concentration term. Dry weight analysis indicated a range of 10.8 to 12.9 $mM^{-1}cm^{-1}$ for ε_{423}. Iron analysis showed ε_{423} as 9.1 $mM^{-1}cm^{-1}$, a value similar to other plant ferredoxins (Table IV). Amino acid analysis of an aliquot of the solution being used for absorption measurement could give a more accurate estimate of the concentration of the solution, but limited amounts of the material

precluded this. More extensive evidence regarding the extinction at 423 nm should also involve an accurate determination of the labile sulphur content of *Sambucus* ferredoxin as this is the source of the absorption in this region of the spectrum.

Ferredoxins are defined as low molecular weight protein electron carriers that transfer to appropriate enzyme systems some of the most "reducing" electrons in cellular metabolism (15). A useful test to identify ferredoxins is their ability to catalyze the photoreduction of NADP by washed chloroplasts. In this respect, the protein purified from *Sambucus* leaves behaved in a manner similar to other plant ferredoxins (Figure 6). Spinach and corn ferredoxins both give initial reduction rates of 100 µmoles of NADP per mg chlorophyll per hr (35). *Sambucus* ferredoxin exhibited a somewhat lower rate of 86 µmoles NADP per mg chlorophyll per hr. This may have been caused by the ferredoxin sample being slightly denatured. The absorbance ratio (423/277 nm) was only 0.44 (Figure 6). This represents a proportional 0.12 "degree of deterioration" based on the maximal (0.49, Table III) and the minimal (0.075, Figure 5) ratios obtained for the native and completely deteriorated protein respectively (33). Using this as a correction value, the rate for native *Sambucus* ferredoxin may be 98.2 µmoles of NADP per mg chlorophyll per hour, very similar indeed to that of corn and spinach.

The amino acid compositions of all ferredoxins reported to date have been determined from analyses of desiccated or rotary evaporated hydrolyzates. This removal of excess acid

prior to analysis has been the usual procedure (97). In a recent study of this step using standard amino acid solutions, Robel has shown that the majority of amino acids incur significant losses through adsorption to the glass surface of the hydrolysis tubes (40). The errors were eliminated by omitting the drying step and diluting the acid hydrolyzates directly with buffer. The acid hydrolyzates of *Sambucus* ferredoxin were treated both ways, as a preliminary investigation of the effect of drying on protein samples. The overall result was an increase in the recovery of amino acids with undesiccated hydrolyzates (Table VI). However, the relative average overall amino acid increase of 7.3% is difficult to relate to Robel's findings, as his average overall amino acid loss due to evaporation (2.09%) was expressed in relation to the absolute initial amounts of standard amino acids. Robel also found that aspartic acid, glutamic acid, glycine, and alanine were adsorbed more than other amino acids. With ferredoxin, aspartic acid, proline, valine, and phenylalanine gave the greatest increases in recovery. The reason for these differences is unknown. Protein hydrolyzates may behave differently from standard amino acid solutions. As the silicon-oxygen bridges of the glass surface are implicated in the adsorption phenomenon, the composition of the Pyrex glass used for the hydrolysis tubes may have an effect. The reults presented for *Sambucus* ferredoxin are only preliminary and should be considered tentative. The effect of drying on hydrolyzates may be better understood when studies with other proteins become available.

The amino acid compositions of ferredoxins (e.g. *Medi-*

cago (33), *Scenedesmus* (45), *Leucaena* (72), *Cyperus* (75)) have been based on average or extrapolated data from hydrolyzates of different times. Extrapolation to zero time corrects for the usual decomposition of labile amino acids such as serine, threonine, cysteine, and tyrosine (97). Valine, leucine, and isoleucine, with hydrophobic side chains, form peptide bonds less susceptible to hydrolysis than other amino acids and are released in increasing amounts with time. The results with *Sambucus* ferredoxin showed that most of the amino acids, including the labile ones, were recovered in maximal amounts after the longest period of hydrolysis (Tables V, VI). The presence during hydrolysis of the iron and sulphur from the active centre of the protein may have caused this unusual pattern of recovery. The maximal values were used for the calculations.

The amino acid composition presented in the results was based on the combined data from both dried and undried hydrolyzates because the limited number of replicates did not allow a rigorous assessment of either method. Also for comparative purposes, it was felt necessary to base the amino acid composition of *Sambucus* ferredoxin partly on the analyses of dried hydrolyzates because all previous reports of ferredoxin composition have involved the analyses of dried hydrolyzates.

The amino acid composition was calculated using an average value (70.13 nmoles amino acid per mg protein) from those amino acid residues whose integral stoichiometry most closely resembled that of the residue occurring in the smallest amount (arginine). The compositions of other ferredoxins have been calculated in a similar manner (33, 35, 75). Determination

of the best integer ratios of amino acid residues can now be facilitated by a computer method published recently (98).

Sambucus ferredoxin has an amino acid composition nearly identical to those of other plants (Table VIII, and ref. 20), which in general show characteristics similar to those found for the bacterial ferredoxins (13). In all cases the acidic and aliphatic amino acids are preponderant, in contrast to the low content of basic and aromatic residues . Tryptophan is missing in all ferredoxins from non-angiosperm plants. The occurrence of methionine is sporadic. Thus far only one residue of methionine per molecule has been reported for *Cyperus*, *Amaranthus*, and *Gossypium* of the angiosperms, and *Microcystis*, *Bumelleriopsis*, *Scenedesmus*, and *Equisetum* of the lower plants. The ferredoxins of *Cladophora* and *Polystichum* contain two residues of methionine (20). Methionine is absent from *Sambucus* ferredoxin as it is from five other angiosperms (Table VIII). An important aspect of the amino acid composition is the presence of only four cysteine residues, which are sufficient to form the iron-sulphur cluster of the proposed form (99). *Gossypium*, *Cyperus*, and *Zea mays* also contain ferredoxins with only four cysteine residues.

The systematic comparison of amino acid compositions of ancestrally related proteins is greatly aided by the use of multivariate analysis methods. One such method which is simple to use is that of Metzger *et. al.* (100). A "difference index" inversely proportional to the compositional relatedness between two proteins is calculated by obtaining the difference in the molar fractional contents of each amino acid, summing the

absolute values of those differences, and multiplying that sum by fifty. Two proteins with no amino acid in common have a difference index (DI) of 100. Two proteins with the identical composition have a DI of zero. This method has been used extensively during recent years in comparisons of a wide variety of proteins [e.g. 30S ribosomal proteins (101), dehydrogenases (102), *Brassica* seed globulins (103)]. Both Slobin (101) and Fondy and Holohan (102) have used amino acid sequence data from cytochromes *c* to show that compositional DI up to a level of approximately 10 correlates very closely with sequence similarity. Not enough sequences of ferredoxins are available to thoroughly test the method with this group of proteins. However, a preliminary analysis of the amino acid compositions of *Sambucus* ferredoxin and sixteen other plant ferredoxins (data from ref. 20) showed that *Sambucus* ferredoxin is most closely related to that of *Leucaena* (Rosales, DI = 7.797), then *Colocassia* (Arales) and *Medicago* (Rosales, both with DI = 8.247), and then *Zea mays* (Graminales, 9.484). The composition most distantly related to *Sambucus* ferredoxin is that of *Anacystis* (blue-green alga, DI = 18.046). The remaining ten ferredoxins compared to *Sambucus* give DI values ranging from 10.429 to 17.235.

The five plant ferredoxins for which the complete primary structure is known possess sequence differences ranging from 16% to 35% (19). The correlation between their difference indices and their percent sequence differences might be used to "predict" the structural relationship of *Sambucus* ferredoxin to those ferredoxins already sequenced. The successive comparison of all five sequenced ferredoxins shows that *Sambucus*

ferredoxin may exhibit a 16, 17, 18, 25, and 38% sequence difference with *Colocassia*, *Leucaena*, *Medicago*, *Spinacea* (Chenopodiales), and *Scenedesmus* (green alga) respectively. It must be stressed that these results are highly speculative and only are presented here to illustrate the remarkable similarity of the amino acid composition of *Sambucus* ferredoxin to those of other angiosperm ferredoxins. The limitations and values of Metzger's DI method have been discussed (102). However, the compositional relatedness of ferredoxins deserve further study, especially in view of the difficulty of obtaining sequence data, and the use of other multivariate techniques should also be investigated with this class of proteins (104, 105, 106).

The molecular weight of *Sambucus* ferredoxin (10,740) is similar to those of other plant ferredoxins (20) and almost identical to the formula molecular weights of those plant ferredoxins which have been sequenced (19). Ultracentrifugation, Sephadex G-75 gel filtration, and determination of iron content have been commonly used methods to estimate the molecular weights of ferredoxins (35, 73, 20). Besides being calculated from the amino acid composition, the molecular weight of *Sambucus* ferredoxin was determined by the recently developed technique of SDS electrophoresis of fluorescent proteins. The excellent agreement between the values derived from these two sources indicates the sensitivity of the latter method. The linear relationship between the logarithm of the molecular weights of the standards and their electrophoretic mobility (Figure 8) was taken as adequate evidence of the reli-

ability of the method. However, it was recognized that the migration of an additional standard such as parathyroid hormone (molecular weight 9,000) ahead of the unknown samples could have lent a further degree of accuracy to the estimation of the molecular weight of *Sambucus* ferredoxin.

The iron content presented in the results was based on analyses of ferredoxin samples whose concentration was determined by dry weight (Table IX). Others have used amino acid analysis of an aliquot of the sample or the molar extinction coefficient to determine the ferredoxin concentration used in the calculation of the iron content (45, 80, 81). The values obtained for *Sambucus* (2 g atoms of iron per molecule) are in good agreement with those for other plants (5). To obtain further evidence of the typical nature of the active centre in *Sambucus* ferredoxin, the content of labile sulphur must be accurately determined. Also, electron paramagnetic (EPR) and electron nuclear double resonance (ENDOR) spectroscopy of *Sambucus* ferredoxin would yield invaluable information concerning physico-chemical properties of its active centre (107).

The sequence data presented in the results also show that *Sambucus* ferredoxin is very similar to other angiosperm ferredoxins. The amino-terminal residue is alanine. All higher plant ferredoxins investigated have the same amino-terminal residue except *Cyperus* in which it is serine (19, 74, 75). The amino-terminal sequence of *Sambucus* ferredoxin is Ala-Thr. The same sequence has only been reported for *Colocassia* and *Scenedesmus* (19). Thermolytic peptide Th-1, Leu-Asx(Asx, Glx, Glx), showed a net charge of +1 at pH 1.9. This peptide

appears to be homologous to the peptide chain of *Colocassia* and *Leucaena* at position 64-68 where the sequence is Leu-Asp-Asp-Glu-Gln (see Appendix). The placement of this peptide is consistent with the cleavage specificity of thermolysin (50) because residue 69 is isoleucine in all four sequenced angiosperm ferredoxins. The sequence at position 64-68 in both *Spinacea* and *Medicago* is almost identical, Leu-Asp-Asp-Asp-Gln (19). The amino-terminal sequence of peptides Th-2 and Th-6 was Leu-Gly. Position 31-32 in *Leucaena* is the only occurrence of this sequence in the five ferredoxins which have been sequenced. The composition of peptide Th-3 suggested that it was tripeptide so the complete sequence must be Leu-Thr-Ala. This sequence or a similarily substituted one only occurs in ferredoxins at position 95-97 where the actual sequence is Leu-Thr-Ala in all four angiosperms. This strongly suggests that peptide Th-3 represents the carboxyl-terminal sequence of *Sambucus* ferredoxin. Homology with other ferredoxins allows the assignment of peptide Th-4, Ile-Asx (Glx, Gly, Trp, Val) to position 69-74. The sequence in *Spinacea* is Ile-Asp-Glu-Gly-Trp-Val. The placement of peptide Th-4 is also consistent with thermolytic specificity as residue 75 in all sequenced ferredoxins is leucine. The Tyr-Leu sequence at the amino-terminus of peptide Th-5 is unique to *Sambucus* ferredoxin. Although the above tentative sequence relationships considered together indicate a close similarity between *Sambucus* ferredoxin and other plant ferredoxins, it is clear that the ferredoxin sequence from *Sambucus* cannot be identical to any of the known sequences. Only the elucidation of the

complete primary structure can reveal the degree of divergence of *Sambucus* ferredoxin from those of other plants. To this end, the sequencing of peptides from thermolytic digests of TCA-treated ferredoxin appears to be a promising approach. Rao and Matsubara obtained 25 thermolytic peptides in good yield from performic acid-oxidized ferredoxin (49).

The strategies used for sequencing other ferredoxins have had much in common (49, 50, 72, 86, 87). Generally, tryptic and chymotryptic peptides from TCA-treated or carboxymethylcysteinyl (CMCys) ferredoxin were separated by ion-exchange chromatography on columns of Dowex AG 1-X2 and purified by high voltage paper electrophoresis. Due to the low content of basic and aromatic residues in the protein, most of these peptides were large and required further cleavage with additional enzymes. But conversion of the four or five cysteine residues in ferredoxin to AECys makes these sites also available for tryptic cleavage (47). Matsubara and Sasaki characterized 17 tryptic peptides from spinach AECys-ferredoxin (50). It was thought that a similar digest of *Sambucus* AECys-ferredoxin would yield an adequate number of small peptides easily separated by gel filtration and paper electrophoresis, thus obviating the difficulties of ion-exchange chromatography with volatile buffers. However, the use of AECys-ferredoxin was not fruitful as this derivative was insoluble.

These results suggest that the use of performic acid-oxidized and carboxymethylcysteinyl (CMCys) derivatives may prove more useful in the sequencing of *Sambucus* ferredoxin.

The separation of tryptic and chymotryptic peptides from these sources may require ion-exchange chromatography. The advantage of following a strategy similar to that used with other ferredoxins is the increased ease with which nonhomologous peptides can be identified and sequenced.

Ferredoxin from *Sambucus racemosa* (Caprifoliaceae) resembles other plant ferredoxins in its spectral properties, molecular weight, iron content, and electron-transfer activity. The amino acid composition, typical of this group of proteins, is highly similar to those of ferredoxins from other angiosperms, and most closely related to that of *Leucaena* (Leguminosae). The amino-terminal amino acid sequence is identical to that of *Colocassia* and *Scenedesmus*. The carboxyl-terminal amino acid sequence is probably identical to that of *Leucaena*, *Colocassia*, *Spinacea*, and *Medicago*. Partial internal amino acid sequences are also characteristic of angiosperm ferredoxins. Elucidation of the complete amino acid sequence will determine the actual degree of similarity between *Sambucus* ferredoxin and other plant ferredoxins. The isolation and purification methods developed in the course of the present research will allow the preparation of *Sambucus* ferredoxin in quantities large enough to facilitate the further characterization of the molecule and the determination of its complete amino acid sequence.

APPENDIX

THE AMINO ACID SEQUENCE OF FERREDOXIN FROM THREE HIGHER PLANTS[a]

	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	20
Spinacea	Ala	ALA	TYR	Lys	Val	THR	Leu	VAL	Thr	Pro	THR	Gly	ASN	VAL	Glu	Phe	GLN	Cys	Pro	Asp-
Colocassia	Ala	THR	TYR	Lys	Val	LYS	Leu	VAL	Thr	Pro	SER	Gly	GLN	GLN	Glu	Phe	GLN	Cys	Pro	Asp-
Leucaena	-	ALA	PHE	Lys	Val	LYS	Leu	LEU	Thr	Pro	ASP	Gly	PRO	LYS	Glu	Phe	GLU	Cys	Pro	Asp-

	21	2	3	4	5	6	7	8	9	30	1	2	3	4	5	6	7	8	9	40
Sp.	Asp	Val	Tyr	Ile	Leu	Asp	ALA	Ala	Glu	Glu	GLU	Gly	Ile	ASP	Leu	Pro	Tyr	Ser	Cys	Arg-
Co.	Asp	Val	Tyr	Ile	Leu	Asp	GLN	Ala	Glu	Glu	VAL	Gly	Ile	ASP	Leu	Pro	Tyr	Ser	Cys	Arg-
Le.	Asp	Val	Tyr	Ile	Leu	Asp	GLN	Ala	Glu	Glu	LEU	Gly	Ile	GLU	Leu	Pro	Tyr	Ser	Cys	Arg-

	41	2	3	4	5	6	7	8	9	50	1	2	3	4	5	6	7	8	9	60
Sp.	Ala	Gly	Ser	Cys	Ser	Ser	Cys	Ala	Gly	Lys	LEU	LYS	THR	Gly	SER	LEU	ASN	Gln	ASP	Asp-
Co.	Ala	Gly	Ser	Cys	Ser	Ser	Cys	Ala	Gly	Lys	VAL	LYS	VAL	Gly	ASP	VAL	ASP	Gln	SER	Asp-
Le.	ALA	GLY	SER	CYS	SER	SER	CYS	ALA	GLY	LYS	LEU	VAL	GLU	Gly	ASP	LEU	ASP	Gln	SER	Asp-

	61	2	3	4	5	6	7	8	9	70	1	2	3	4	5	6	7	8	9	80
Sp.	GLN	Ser	Phe	Leu	Asp	Asp	ASP	Gln	Ile	ASP	Glu	Gly	Trp	Val	Leu	Thr	Cys	ALA	Ala	Tyr-
Co.	GLY	Ser	Phe	Leu	Asp	Asp	GLU	Gln	Ile	GLY	Glu	Gly	Trp	Val	Leu	Thr	Cys	VAL	Ala	Tyr-
Le.	GLN	Ser	Phe	Leu	Asp	Asp	GLU	Gln	Ile	GLU	Glu	Gly	Trp	Val	Leu	Thr	Cys	ALA	Ala	Tyr-

	81	2	3	4	5	6	7	8	9	90	1	2	3	4	5	6	97
Sp.	Pro	VAL	Ser	Asp	VAL	THR	Ile	Glu	Thr	His	Lys	Glu	Glu	Glu	Leu	Thr	Ala
Co.	Pro	VAL	Ser	Asp	GLY	THR	Ile	Glu	Thr	His	Lys	Glu	Glu	Glu	Leu	Thr	Ala
Le.	Pro	ARG	Ser	Asp	VAL	VAL	Ile	Glu	Thr	His	Lys	Glu	Glu	Glu	Leu	Thr	Ala

a: References: *Spinacea* (50), *Colocassia* (49), *Leucaena* (72).

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