The Amino Acid Sequence of Plastocyanin from *Vicia faba* L. (Broad Bean)

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The amino acid sequence of plastocyanin from broad bean was determined. It consists of a single polypeptide chain of 99 residues. The sequence was determined by using a Beckman 890C sequencer and by dansyl-phenyl isothiocyanate analysis of peptides obtained by the enzymic cleavage of purified cyanogen bromide fragments. Some parts of the sequence depend on the results of Edman degradation of peptides for which amino acid analyses were not obtained. The evidence for one overlap is not strong.

Plastocyanin, a blue Type 1 copper protein which functions in photosynthesis (see Gregory, 1971), is present in a wide range of higher plants (see e.g. Katoh *et al.*, 1961; de Kouchkovsky & Fork, 1964; Ramshaw *et al.*, 1973). In most species from which it has been purified, it has a molecular weight of about 10 500 and contains one copper atom/molecule (see Ramshaw *et al.*, 1973). Its *N*-terminus is not blocked, making it suitable for automated sequencing techniques. This approach proved useful in the determination of the sequences of plastocyanin from potato (Ramshaw *et al.*, 1974) and from elder (Scawen *et al.*, 1974).

Experimental

Materials

Plastocyanin was prepared from broad-bean leaves which had been obtained locally. The methods of Ramshaw *et al.* (1973) were used, except that the leaves were stored at -20°C for at least 1 month before use instead of an acetone-dried powder being prepared.

Other materials were as described by Thompson *et al.* (1970) and Ramshaw *et al.* (1974).

Methods

The amino acid sequence of broad-bean plastocyanin was determined by using the methods described by Ramshaw *et al.* (1974) for the determination of the sequence of potato plastocyanin. *S*-Carboxymethylated plastocyanin was prepared and cleaved by treatment with CNBr; the resulting fragments were separated by gel filtration by using Sephadex G-50 equilibrated in 70% (v/v) formic acid (Milne & Wells, 1970). Peptides obtained by enzymic digestion

of the fragments were purified by high-voltage paper electrophoresis at pH 6.5 and 1.9 (Thompson *et al.*, 1970) and by paper chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.). The sequences of the purified peptides were determined by using the dansyl-phenylisothiocyanate method (Gray & Hartley, 1963) as described previously (Thompson *et al.*, 1970). Dansyl amino acids were identified by chromatography on polyamide sheets (Woods & Wang, 1967) by using the solvent systems described by Ramshaw *et al.* (1970). *C*-Terminal amino acids were determined by carboxypeptidase analysis as described by Ramshaw *et al.* (1974). Automatic sequence analysis was performed on a Beckman 890C sequencer. Fast protein (Quadrol) and fast peptide (Dimethylallylamine) programs were used as described in the instrument manual (Beckman, 1972). The sequencer grade chemicals used were obtained from Beckman-RIIC Ltd., Glenrothes, Fife, U.K. Phenylthiohydantoin amino acids were identified by t.l.c. on precoated silica gel layers (incorporating a fluorescent indicator) by using the solvent systems described by Jeppsson & Sjöquist (1967). They were also identified by g.l.c. with 10% (v/v) SP400 on Chromosorb W AW-DMCS 100-120 mesh (Phase Separations Ltd., Queensferry, Flint., U.K.) as described in the instrument manual (Beckman, 1972), and by hydrolysis in constant-boiling hydriodic acid (Inglis *et al.*, 1971), followed by dansylation. Amino acid analyses of the protein, the CNBr fragments and the peptides were performed on a Locarte amino acid analyser.

Quantities of material used. A 40 mg sample of *S*-carboxymethylated plastocyanin was cleaved with CNBr. Samples of each purified fragment were removed for amino acid analysis and automatic sequence analysis (approx. 300 nmol). The remaining material was used for enzymic digestions; approx. 1 μmol was used for each digestion. For automatic sequence analysis of the total protein 4 mg samples

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Fig. 1. Amino acid sequence of broad-bean plastocyanin

Peptides and fragments indicated by a solid line — are those for which amino acid-analysis results were obtained. Other purified peptides are indicated by a dashed line ----. Residues which were identified unambiguously are indicated by —; those for which some ambiguity existed, e.g. by overlap after incomplete Edman degradation, are indicated by ----; arrows — indicate that the free amino acid was identified, e.g. as its dansyl derivative. In the peptides marked * methionine was identified as homoserine.

of native plastocyanin (approx. 400nmol) were used.

Nomenclature. The CNBr fragments are labelled X, and have been numbered on Fig. 1 according to their position in the complete sequence. Peptides derived by enzymic digestion of these fragments are indicated by H for thermolysin peptides, P for papain peptides and T for trypsin peptides.

Results

The amino acid compositions of broad-bean plastocyanin as determined by analysis and from the sequence are shown in Table 1. The values obtained by amino acid analysis are in good agreement with the values obtained from the sequence (Fig. 1). However, the value obtained for proline after 20h hydrolysis was high, the values obtained for glutamic acid were consistently high and the value obtained for valine was still low even after 70h hydrolysis. No unusual amino acids were present in the analyses.

CNBr cleavage of *S*-carboxymethylated plastocyanin gave three fragments which were purified by

gel filtration on Sephadex G-50 in 70% (v/v) formic acid. The amino acid compositions of the purified fragments are given in Table 1 and are generally in good agreement with the values obtained from their sequences (Fig. 1). The sum of the amino acid compositions of these fragments accounts for the composition of the total protein.

The largest of the CNBr fragments, X-1, had a valine *N*-terminal residue and a homoserine *C*-terminal residue. In conjunction with the results of the automatic sequencer analysis of the total protein, this fragment was shown to represent the *N*-terminal region of the sequence and so account for the first 57 residues. The second CNBr fragment to be eluted from the Sephadex G-50 column, X-2, had a proline *N*-terminal residue and a homoserine *C*-terminal residue. It was therefore internal in the sequence and represented residues 58–92 (Fig. 1). The third and smallest CNBr fragment, X-3, had a valine *N*-terminal. It had an asparagine *C*-terminal residue, which is also the *C*-terminal residue of the native protein. Thus this fragment was from the *C*-terminal region of the sequence, residues 93–99 (Fig. 1).

Table 1. *Amino acid compositions of protein and CNBr fragments of broad-bean plastocyanin*

The results are expressed as residues/molecule and are based on 20h and 70h hydrolyses for the total protein and on 20h hydrolyses only for the CNBr fragments. Values for threonine, serine and tyrosine in the total protein are extrapolated to zero time, assuming first-order kinetics of destruction (Moore & Stein, 1963), and maximum values (70h) are taken for valine and isoleucine. Cysteine was determined as *S*-carboxymethylcysteine or as cysteic acid. The value for tryptophan was determined by using the method of Beaven & Holiday (1952), assuming that three tyrosine residues were present.

	Total protein				Fragment X-1		Fragment X-2		Fragment X-3	
	20h	70h	Average or extrapolated value	Sequence	20h	Sequence	20h	Sequence	20h	Sequence
Asp	12.93	12.74	12.84	13	7.95	9	*	3	1.03	1
Thr	3.61	3.51	3.66	4	0.98	1	1.72	2	0.86	1
Ser	6.76	6.33	6.93	7	4.42	5	1.78	2	0	0
Hse	0	0	0	0	0.61	0	0.48	0	0	0
Glu	9.45	9.47	9.46	9	4.23	4	3.97	4	1.00	1
Pro	6.72	6.13	6.42	6	3.18	3	2.80	3	0	0
Gly	10.66	11.01	10.84	11	5.92	6	3.68	4	0.96	1
Ala	8.94	8.96	8.95	9	5.91	6	3.08	3	0	0
Cys	0.6		0.6	1	0	0	*	1	0	0
Val	10.72	11.38	11.38	12	7.39	8	1.00	1	2.74	3
Met	1.76	1.84	1.80	2	0	1	0	1	0	0
Ile	2.71	2.99	2.99	3	2.87	3	0	0	0	0
Leu	6.23	6.06	6.14	6	3.41	3	2.82	3	0	0
Tyr	2.51	1.89	2.81	3	0	0	2.07	3	0	0
Phe	5.99	5.81	5.90	6	5.09	5	1.31	1	0	0
His	2.03	1.95	1.99	2	0.90	1	0.81	1	0	0
Lys	5.13	5.04	5.08	5	2.13	2	3.05	3	0	0
Arg	0	0	0	0	0	0	0	0	0	0
Trp			0.08	0		0		0		0
Totals				99		57		35		7

* Aspartic acid and *S*-carboxymethylcysteine were not adequately resolved for accurate integration.

Broad Bean Val-Glu-Val-Leu-LEU-GLY-Ala-Ser-Asp-GLY-Gly-LEU-Ala-PHE-
 Elder Val-Glu-Ile-Leu-LEU-GLY-Gly-Glu-Asp-GLY-Ser-LEU-Ala-PHE-
 Potato Leu-Asp-Val-Leu-LEU-GLY-Gly-Asp-Asp-GLY-Ser-LEU-Ala-PHE-
Chlorella Asp-Val-Thr-Val-Lys-LEU-GLY-Ala-Asp-Ser-GLY-Ala-LEU-Val-PHE-

20

Val-PRO-Asn-Ser-Phe-Glu-Val-Ser-Ala-GLY-Asp-Thr-Ile-Val-Phe-
 {Val-PRO-{Gly-Asn-Phe-Ser-Val-Pro-Ser-GLY-Glu-Lys-Ile-Thr-Phe-
 Ile-PRO-GLY-Asn-Phe-Ser-Val-Ser-Ala-GLY-Glu-Lys-Ile-Thr-Phe-
 Glu-PRO-Ser-Ser-Val-Thr-Ile-Lys-Ala-GLY-Glu-Thr-Val-Thr-Trp-

30

40

Lys-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Val-VAL-PHE-ASP-GLU-ASP-
 Lys-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Val-VAL-PHE-ASP-GLU-ASP-
 Lys-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Val-VAL-PHE-ASP-GLU-ASP-
 Val-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Ile-VAL-PHE-ASP-GLU-ASP-

50

GLU-Ile-PRO-Ser-GLY-Val-Asp-Ala-Ala-Lys-Ile-SER-Met-Pro-Glu-
 GLU-Val-PRO-Ser-GLY-Val-Asp-Ser-Ala-Lys-Ile-SER-Met-Ser-Glu-
 GLU-Ile-PRO-Ala-GLY-Val-Asp-Ala-Ser-Lys-Ile-SER-Met-Ala-Glu-
 GLU-Val-PRO-Ser-GLY-Ala-Asn-Ala-Glu-Ala-Leu-SER-----His-----

60

70

Glu-ASP-Leu-LEU-ASN-ALA-Pro-GLY-GLU-Thr-TYR-SER-Val-Lys-Leu-
 Asp-ASP-Leu-LEU-ASN-ALA-Pro-GLY-GLU-Thr-TYR-SER-Val-Thr-Leu-
 Glu-ASP-Leu-LEU-ASN-ALA-Ala-GLY-GLU-Thr-TYR-SER-Val-Thr-Leu-
 Glu-ASP-Tyr-LEU-ASN-ALA-Pro-GLY-GLU-Ser-TYR-SER-Ala-Lys-Phe-

80

Asp-Ala-Lys-GLY-THR-TYR-Lys-Phe-Tyr-CYS-Ser-PRO-HIS-GLN-GLY-
 Thr-Glu-Ser-GLY-THR-TYR-Lys-Phe-Tyr-CYS-Ser-PRO-HIS-GLN-GLY-
 Ser-Glu-Lys-GLY-THR-TYR-Thr-Phe-Tyr-CYS-Ala-PRO-HIS-GLN-GLY-
 Asp-Thr-Ala-GLY-THR-TYR-Gly-Tyr-Phe-CYS-Glu-PRO-HIS-GLN-GLY-

90

99

ALA-GLY-MET-Val-GLY-Gln-Val-THR-VAL-Asn
 ALA-GLY-MET-Val-GLY-Lys-Val-THR-VAL-Asn
 ALA-GLY-MET-Val-GLY-Lys-Val-THR-VAL-Asn
 ALA-GLY-MET-Lys-GLY-Thr-Ile-THR-VAL-Gln

Fig. 2. Alignment of four plastocyanin sequences

Numbering is based on the higher-plant plastocyanin sequences. Sources of information: broad-bean plastocyanin, this investigation; elder plastocyanin, Scawen *et al.* (1974); potato plastocyanin, Ramshaw *et al.* (1974); *Chlorella fusca* plastocyanin, Kelly & Ambler (1973). Residues common to all sequences are shown in capitals.

Table 2. *Properties of peptides from broad-bean plastocyanin*

Mobilities at pH 6.5 were measured from the position of the neutral amino acids relative to Dns-arginylarginine and at pH 1.9 from 5-dimethylaminonaphthalene-1-sulphonic acid relative to Dns-arginine. The mobility of Dns-Arg = $0.36 \times$ the mobility of Asp (Offord, 1966). Compositions are given as residues/molecule. Residues present in a molar ratio of less than 0.15 have been ignored.

Peptide	Mobility at pH 6.5	Mobility at pH 1.9	R_F in BAWP*	Peptide amino acid composition (residues/molecule)																
				Asp	Thr	Ser	Ser	Homo	Glu	Pro	Gly	Ala	Cys	Val	Ile	Leu	Tyr	Phe	His	Lys
X1H1	-1.08	0.47	0.79	0.4				1.4		0.3			1.7		0.9					
X1H1a	-1.77	0.69	0.38																	
X1H1b	0	0.70	0.82																	
X1H2	-1.11	0.40	0.26	1.3		1.1				2.8	0.9				0.8					
X1H3	0	0.78	0.67								1.0				0.9					
X1H4	0	0.43	—	1.0		0.9			1.1				1.1				0.9			
X1H5	-1.62	0.61	0.50	0.3				1.0		0.2							0.7			
X1H6	-1.18	0.45	0.20	1.0	0.8	0.8				1.2	1.0		1.0							
X1H7	0.89	0.70	0.30																	
X1H7a	1.03	0.80	0.37	0.6						0.3			0.7	0.7			1.0		1.1	
X1H7b	1.15	0.91	—										1.1	0.7			0.9		1.2	
X1H8	0.91	0.86	—	1.2		0.2		0.2	1.1	0.9	0.2						1.0	0.8		
X1H8a	0.85	0.80	0.26																	
X1H8b	0.77	0.68	0.23																	
X1H8c	0	0.78	0.18	1.0		0.2		0.2		0.3	1.0									
X1H9	-1.60	0.45	0.23	2.9		0.9		1.9	1.3	1.4	2.2		2.1	1.1			1.0		1.1	
X1H9a	-2.24	0.28	—	2.3		0.8		2.1	1.1	1.0	0.4		1.8	1.0			1.0			
X1H9b	0	0.90	0.15	1.3						0.2	2.1		1.0						1.0	
X1H9c	1.70	1.47	—																	
X1H9d	2.01	1.27	—																	
X1H10	0	0.61	—																	
X1P	1.74	0.70	—	0.3		1.0	‡			0.4				0.8						
	0	—	—																	
X2T1	0.87	—	—	1.3		1.2	0.6	0.2		0.3	1.9		0.8	0.8				0.6		
X2T2	-vet	0.44	—	2.0	0.9	0.8		2.8	1.9	1.0	1.1		1.0		2.1	0.6		1.0		
X2T3	0	0.97	0.28	1.0							1.0				0.9			1.0	1.1	
X2T4	1.30	—	—		1.0					0.9						1.0				
X2T4	0	0.48	—			0.9	0.3	1.1	1.0	2.2	1.1	1.1				1.0	0.9	1.0		
X2H1	0.48	—	—					2.0	1.0											
X2H1	-2.83	0.50	—	1.0																
X2H2	-vet	0.31	—																	
X2H2a	-vet	0.38	—																	
X2H2b	0	0.46	—																	
X2H3	1.82	1.30	0.30																1.0	

See Table 1

† Peaks present but unsuitable for integration.

Fragment X-3 was further purified by high-voltage paper electrophoresis at pH 6.5 before its sequence was determined by dansyl-phenyl isothiocyanate analysis. The electrophoresis also gave a peptide in low yield with the same amino acid composition but with an acidic mobility. This peptide zone was probably derived from the major peptide by deamidation of one of the two amide residues. Similar deamidation has been observed with the C-terminal asparagine residue in potato plastocyanin

(Ramshaw *et al.*, 1974) and with the C-terminal glutamine residue in *Chlorella fusca* plastocyanin (Kelly & Ambler, 1973).

The sequences of the three CNBr fragments account for the amino acid composition of the complete protein; no other CNBr fragments were found. Therefore, although the overlap of the three fragments has not been demonstrated, it is considered that these fragments give the complete sequence for broad-bean plastocyanin (Fig. 1). There was no evidence of heterogeneity as observed in the sequence of elder plastocyanin (Scawen *et al.*, 1974).

All the amide residues in the sequence have been assigned, either from the results obtained by using

the automatic sequencer or from the electrophoretic mobilities of the peptides at pH 6.5 (Offord, 1966).

The enzyme specificities which were observed in the digestions of fragments X-1 and X-2 were consistent with those expected and similar to those found with similar digests of potato (Ramshaw *et al.*, 1974) and elder (Scawen *et al.*, 1974) plastocyanins. The cases where thermolytic cleavage might have been expected but was not observed were probably due to the presence of neighbouring proline residues or to a clustering of the susceptible hydrophobic residues. This clustering also accounts for the low yields of hydrophobic residues (due to steric hindrance to hydrolysis) in certain cases when the amino acid composition of peptides was determined.

Discussion

The sequence of broad-bean plastocyanin (Fig. 1) is very similar to the sequences of potato (Ramshaw *et al.*, 1974), elder (Scawen *et al.*, 1974) and *Chlorella fusca* (Kelly & Ambler, 1973) plastocyanins (Fig. 2). In common with the other higher-plant sequences, both arginine and tryptophan residues are absent. All of the isoleucine residues are in fragment X-1 and all of the tyrosine residues are in fragment X-2. The positions of the two methionine residues are identical in all the higher-plant plastocyanins, which has allowed the preparation of equivalent CNBr fragments from each species. When the plastocyanins from higher plants are

Table 3. Amino acid differences between plastocyanins

The sequence of potato plastocyanin is from Ramshaw *et al.* (1974) and that of *Chlorella* from Kelly & Ambler (1973).

	Broad bean	Elder*	Potato	<i>Chlorella</i>
Broad bean	—	21½	22	42
Elder*	21½	—	18	47½
Potato	22	18	—	47
<i>Chlorella</i>	42	47½	47	—

* The non-integral values arise from the microheterogeneity observed in the elder plastocyanin (Scawen *et al.*, 1974).

Table 4. Comparison of the amino acid compositions of the CNBr fragments of broad-bean and French-bean plastocyanins

Broad-bean results are from this investigation (Fig. 1). French-bean results are from Milne & Wells (1970). Cysteine was determined as S-carboxymethylcysteine and methionine as homoserine.

	Fragment X-1		Fragment X-2		Fragment X-3		Total	
	<i>Phaseolus</i>	<i>Vicia</i>	<i>Phaseolus</i>	<i>Vicia</i>	<i>Phaseolus</i>	<i>Vicia</i>	<i>Phaseolus</i>	<i>Vicia</i>
Asp	7	9	2	3	1	1	10	13
Thr	0	1	4	2	1	1	5	4
Ser	6	5	2	2	0	0	8	7
Glu	6	4	5	4	0	1	11	9
Pro	4	3	3	3	0	0	7	6
Gly	6	6	4	4	1	1	11	11
Ala	3	6	2	3	0	0	5	9
Val	9	8	2	1	3	3	14	12
Cys	0	0	1	1	0	0	1	1
Met	1	1	1	1	0	0	2	2
Ile	3	3	0	0	0	0	3	3
Leu	4	3	3	3	0	0	7	6
Tyr	0	0	3	3	0	0	3	3
Phe	5	5	1	1	0	0	6	6
His	1	1	1	1	0	0	2	2
Lys	3	2	1	3	1	0	5	5
Arg	0	0	0	0	0	0	0	0
Trp	0	0	0	0	0	0	0	0
Totals	58	57	35	35	7	7	100	99

compared with *Chlorella* plastocyanin the regions 31–49 and 78–92, which had been noted previously as being highly conserved areas in the sequence (Ramshaw *et al.*, 1974), are still found to be conserved when the four sequences are compared. There are 46 positions that are invariant between these four species; 10 of these positions are glycine residues. The sequence differences between these four plastocyanins are given in Table 3. Comparison of the compositions of the CNBr fragments determined from the sequence of broad-bean plastocyanin with the analysis values given for the similar fragments obtained from French-bean (*Phaseolus vulgaris*) plastocyanin (Milne & Wells, 1970) (see Table 4) indicates a minimum of 12 differences between the two sequences. Sequence studies on French-bean plastocyanin (R. P. Ambler, personal communication) indicate that the actual number of differences is 20. By comparison with the differences shown between plastocyanins from plants of different families (Table 3) this suggests that these two members of the Leguminosae (the former from the semi-tropical region of the new world, the latter from the temperate region of the old world) may have had a very long and separate evolutionary history. Large differences were also observed between the sequences of the two legume ferredoxins, alfalfa and *Leucaena*, which have been studied (Keresztes-Nagy *et al.*, 1969; Benson & Yasunobu, 1969).

Comparison of the differences found between the various plastocyanins (Table 3) with the differences found between the cytochromes *c* isolated from species of similar families (Boulter *et al.*, 1972) indicates that plastocyanin may be a more rapidly evolving protein than cytochrome *c*. More extensive comparisons will be needed before a definite ratio relating the rates of evolution of these two proteins can be established. These data suggest, however, that plastocyanin will be suitable for extending the work of Boulter *et al.* (1972) on the elucidation of plant relationships by using protein-sequence data; the use of automatic sequencing methods will be of particular value in rapidly developing these studies.

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