The Amino Acid Sequence of Plastocyanin from *Solanum tuberosum* L. (Potato)

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The amino acid sequence of plastocyanin from potato was determined. It consists of a single polypeptide chain of 99 residues, of molecular weight 10332. The sequence was determined by using a Beckman 890c sequencer and by dansyl-Edman analysis of peptides derived from purified CNBr fragments. The sequence shows considerable similarity with that of *Chlorella fusca*, and also with the C-terminal region of bacterial azurins.

Plastocyanin is a blue Type 1 copper protein which was first isolated from *Chlorella ellipsoidea* by Katoh (1960); it has since been shown to be widely distributed in other green algae and higher plants (Gorman & Levine, 1966; Milne & Wells, 1970; Scawen & Hewitt, 1971; Ramshaw *et al.*, 1973). Plastocyanin is located in the chloroplast (Katoh *et al.*, 1961) and is involved in photosynthetic electron transport (see Gregory, 1971). A copper protein whose properties resemble those of plastocyanin has also been isolated from a prokaryote, *Anabaena variabilis* (Lightbody & Krogmann, 1967).

Plastocyanin has been purified from several higher plants (Ramshaw *et al.*, 1973) and shown in most cases to have a molecular weight of about 10500 and to contain 1 copper atom/molecule. Plastocyanins from spinach and from *Chenopodium album*, however, are reported to have molecular weights of 21000 and 11500 respectively and to contain two copper atoms/molecule (Katoh *et al.*, 1962; Blumberg & Peisach, 1966). The amino acid sequence of plastocyanin from the green alga *Chlorella fusca* has been determined (Kelly & Ambler, 1973) and gives a sequence molecular weight of 10300.

The aim of the present investigation was to determine the amino acid sequence of plastocyanin from a higher plant to obtain additional evidence of its size and structure.

Since plastocyanin is a small protein which can be readily purified from a wide range of higher plants, the determination of additional sequences may prove useful in confirming and extending the work of Boulter *et al.* (1972) on the elucidation of plant relationships by using protein-sequence data.

Materials

Potato leaves (20 kg) were obtained locally. Trypsin (EC 3.4.21.4) (1-chloro-4-phenyl-3-L-tosylamido-butan-2-one-treated), α -chymotrypsin (EC 3.4.21.1) and papain (EC 3.4.22.2) (twice recrystallized) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Thermolysin (EC 3.4.24.4) (crystalline) was obtained from Daiwa Kasei K.K., Osaka, Japan. Carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) (EC 3.4.12.2) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.

Other materials were as described by Thompson *et al.* (1970, 1971).

Methods

Preparation of plastocyanin

Plastocyanin was purified by the method described by Ramshaw *et al.* (1973), except that the leaves were frozen at -20°C for at least 1 month before use, instead of an acetone-dried powder being used. The yield of purified plastocyanin was 3.5 mg/kg of leaves.

Preparation and carboxymethylation of apoplastocyanin

Plastocyanin was denatured in 6M-guanidine hydrochloride and the copper removed by Chelex 100 resin by the method described by Milne & Wells (1970). Apostocyanin was carboxymethylated by the method described by Milne & Wells (1970).

Amino acid analysis

Amino acid analyses were done by using a Locarte amino acid analyser. Hydrolysis of protein samples and of CNBr fragments was carried out for 20 and 70 h

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Fig. 1. Amino acid sequence of potato 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 that were unambiguously identified 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. In the peptides marked * homoserine was identified as the derivative from CNBr cleavage at the methionine residue. For peptide X1H10 the mobilities at pH6.5 of the peptides on successive steps of Edman degradation are indicated.

Table 1. *Amino acid compositions of potato plastocyanin and its CNBr fragments*

The results are expressed as residues/molecule and are the results of 20h and 70h hydrolyses. Values for threonine and serine are extrapolated to zero time, assuming a first-order rate of destruction (Moore & Stein, 1963). Cystine was determined as either cysteic acid or S-carboxymethyl cysteine. For valine and isoleucine maximal values (70h hydrolysis) were taken. The value for tryptophan was determined by using the method of Beaven & Holliday (1952), assuming that three tyrosine residues were present.

	Total protein			X-1 fragment			X-2 fragment			X-3 fragment			
	20h	70h	Average	Sequence	20h	70h	Average	Sequence	20h	70h	Average	Sequence	
Asp	12.71	12.18	12.45	13	8.98	12.15	10.56	10	*	2.34	2.34	2	1
Thr	5.73	5.11	6.00	6	1.10	0.97	1.16	1	3.50	3.19	3.64	4	1
Ser	6.73	5.85	7.11	7	4.35	3.79	4.61	5	1.92	1.57	2.09	2	0
Hse	0	0	0	0	0.78	0.81	0.79	1	0.60	0.64	0.62	1	0
Glu	8.42	8.17	8.30	8	3.37	3.26	3.31	3	5.02	5.14	5.08	5	0
Pro	4.03	4.03	4.03	4	3.02	2.96	2.99	3	1.14	1.06	1.10	1	0
Gly	11.91	11.56	11.74	12	6.88	6.89	6.89	7	4.04	4.02	4.03	4	1
Ala	9.93	9.97	9.95	10	5.15	5.05	5.10	5	4.78	4.74	4.76	5	0
Cys	0.6	0.6	0.6	1	0	0	0	0	*	0.91	0.91	1	0
Val	8.04	8.67	8.67	9	4.38	4.77	4.77	5	1.05	1.09	1.09	1	2.98
Met	1.86	1.70	1.78	2	0	0	0	0	0	0	0	0	0
Ile	3.79	4.14	4.14	4	3.93	4.03	4.03	4	0	0	0	0	0
Leu	7.00	7.00	7.00	7	4.11	4.14	4.12	4	2.93	2.96	2.95	3	0
Tyr	2.84	2.78	2.81	3	0	0	0	0	2.69	2.60	2.65	3	0
Phe	5.95	5.95	5.95	6	4.91	5.00	4.96	5	1.04	1.05	1.04	1	0
His	1.98	1.73	1.86	2	0.96	0.94	0.95	1	0.92	1.02	0.97	1	0
Lys	5.68	5.64	5.66	5	3.31	2.93	3.12	3	1.09	1.01	1.05	1	0.95
Arg	0	0	0	0	0	0	0	0	0	0	0	0	0
Trp	0	0	0.06	0	0	0	0	0	0	0	0	0	0
Totals				99				57				35	7

* Aspartic acid and carboxymethylcysteine were not adequately resolved for accurate analysis.

and that of peptides for 20h, as described by Thompson *et al.* (1970). Tryptophan was measured in the whole protein by the method of Beaven & Holliday (1952).

Cleavage with CNBr and separation of fragments

S-Carboxymethylated plastocyanin (40mg) was cleaved by a 5-fold excess (w/w) of CNBr by using the method of Steers *et al.* (1965). Fragments produced by the cleavage were separated by using Sephadex G-50 (fine grade) in 70% (v/v) formic acid as described by Milne & Wells (1970). The elution profiles were followed by measuring the E_{280} and by *N*-terminal analysis by using the dansyl technique (Gray & Hartley, 1963a).

Enzymic digestions

Digestion by thermolysin was with a 2% (w/w) ratio of enzyme to substrate at 37°C in 0.2M-*N*-ethylmorpholine-acetate buffer, pH8.5, containing 5mM-CaCl₂. Digestion by trypsin or chymotrypsin was with a 2% (w/w) ratio of enzyme to substrate at 37°C in 0.2M-*N*-ethylmorpholine-acetate buffer, pH8.5. Digestion by papain was with a 2% (w/w) ratio of enzyme to substrate at 37°C in 0.2M-pyridine-acetate buffer, pH6.5, containing 0.1% 2,3-dimercapto-propan-1-ol.

Digestion was terminated by adjusting to pH2-3 with 10% (v/v) acetic acid and digests were then frozen and freeze-dried.

Carboxypeptidase A was made soluble by using method (i) of Ambler (1972). Digestion was with a 2 to 5% (w/w) ratio of enzyme to substrate at 37°C for periods up to 1h in 0.2M-*N*-ethylmorpholine-acetate buffer, pH8.5.

Separation and purification of peptides

Peptides were purified by high-voltage paper electrophoresis at pH6.5 (pyridine-acetic acid-water, 25:1:225, by vol.) and at pH1.9 (acetic acid-formic acid-water, 4:1:45, by vol.), as described by Thompson *et al.* (1970) and by chromatography in butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.). Peptides were detected by using cadmium-ninhydrin reagent (Heilman *et al.*, 1957).

Electrophoretic mobilities at pH6.5 and at pH1.9 were determined as described by Thompson *et al.* (1970).

Peptides with *C*-terminal homoserine or homoserine lactone were interconverted when necessary, by using the methods of Ambler (1965).

Amino acid sequence methods

Peptide sequences were determined by using the dansyl-phenyl isothiocyanate method (Gray & Hart-

ley, 1963b) as described by Thompson *et al.* (1970). Separation of dansyl amino acids was on polyamide sheets (Woods & Wang, 1967) by using the solvent systems described by Ramshaw *et al.* (1970). In addition, electrophoresis at pH1.9 was used to identify α -dansyl-histidine (Gray, 1967). This identification was confirmed by eluting the fluorescent spot after electrophoresis, labelling it further with dansyl chloride, and the bis-dansyl-histidine derivative was identified by using polyamide-sheet chromatography.

After carboxypeptidase A digestion, the *C*-terminal amino acid(s) released were identified as their dansyl derivatives (Thompson *et al.*, 1970). In certain cases labelling with ³H (Matsuo *et al.*, 1966) was also used. The *C*-terminal amino acid of the sample was labelled with ³H₂O (100mCi/ml) by using method 'C' of Holcomb *et al.* (1968). After digestion with carboxypeptidase A the released amino acids were identified as their dansyl derivatives by polyamide-layer chromatography. The radioactive *C*-terminal amino acid was then identified by using a radiochromatogram scanner (Betagraph; Panax Equipment Ltd., Redhill, Surrey, U.K.). Automated sequencing was performed on a Beckman 890C sequencer by using the fast-protein and fast-peptide programmes. Phenylthiohydantoin amino acids were identified, (1) by t.l.c. on precoated silica-gel sheets incorporating a fluorescent indicator (Eastman Kodak Ltd.) by using the solvent systems of Jeppsson & Sjöquist (1967); (2) by g.l.c. on SP-400/Chromosorb W; and (3) by hydriodic acid regeneration (Inglis *et al.*, 1971), followed by conversion into dansyl derivatives.

Quantities of material used

For CNBr cleavage, 40mg of *S*-carboxymethylated plastocyanin was used. One-quarter of the material of the smallest resulting CNBr fragment (X-3) was taken for further analysis (see below). After removal of approx. 300-400nmol of material from each of the two larger fragments (X-1, X-2) for amino acid analysis and sequencer analysis (only one run with fragment X-2), the remaining material in each case was divided into three equal parts; each fraction was then used for an individual enzymic digest (see below). Sequencer analysis on the native protein was performed on approx. 4mg (400nmol) samples.

Nomenclature

The CNBr fragments are numbered on the basis of their position in the complete sequence and are prefixed by the letter X. Peptides derived by digestion of the fragments are numbered on the basis of their position in the parent fragment. The following abbreviations are used: H, thermolytic peptides; P, papain peptides; T, tryptic peptides; C, chymotryptic peptides.

Table 2. *Mobilities and amino acid compositions of peptides from potato plastocyanin*

The mobilities of peptides during electrophoresis at pH 6.5 were measured from Dns-arginine relative to Dns-arginylarginine, and at pH 1.9 from 1-dimethylaminonaphthalene-5-sulphonic acid relative to Dns-arginine.

Peptide	Mobility at pH 6.5	Mobility at pH 1.9	R_F in BAWP*	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Hse	CM-Cys
XIH1	-1.22	0.47	0.83	1.4		0.3	0.3		0.4	1.0	1.0		1.6						
XIH1a	-1.80	0.64	0.53																
XIH1b	0	0.75	0.82																
XIH2	-1.95	0.37	—	2.1		0.8			3.0				1.1						
XIH3	0	0.81	—	0.2					0.2	1.1			0.9						
XIH4	0	0.42	—	1.0		0.1		1.0	1.0						0.9				
XIH5	0	0.70	—			0.8									1.0				
XIH6	0	0.86	0.18	0.3		0.9	1.0		1.0	1.1	0.8						1.1		
XIH7	0	0.75	0.57	0.2	0.9	0.2	0.1		0.3			1.0							
XIH8	1.04	0.79	0.17	2.1						0.7					0.9		1.3		
XIH8a	1.27	1.03	—																
XIH9	0.90	0.85	0.24	1.2				0.7	1.2						0.9	0.8			
XIH10	-2.15	0.25	—	2.0			1.9	1.0	1.1	1.0	1.2	1.0			0.9				
XIH10a	-1.50	0.43	—	3.0		1.0	1.9	1.0	1.2	2.0	2.3	†			†		1.3		
XIH11	0	0.90	0.18	1.1		0.8	0.1		0.2	1.0	1.0						1.1		
XIH11a	-1.89	0.67	0.38																
XIH11b	1.69	1.21	0.15																
XIH12	{ 0 1.71	0.62 0.71	— 0.61	0.2		1.0			0.2			1.0						0.4	
XIP1	-1.77	0.26	—																
XIP1a	-vet	0.35	—																
XIP1b	-2.05	0.37	—																
XIP2	0	0.27	—																
XIP2a	0	0.48	—																
XIP2b	0	0.39	—																
XIP3	0.83	0.95	—																
XIP4	-vet	0.34	—																
XIP5	0	0.66	—																
X2T1	-1.53	0.38	—	2.1	1.8	1.7	4.0		1.1	2.9	1.1		3.0	0.9			1.1		
X2T2	0	0.43	—		2.0	0.6	1.5	1.0	2.9	2.0	0.3	0.1	0.3	1.3	1.0	0.8	0.3	0.3	1.2
X2C1	-2.05	0.22	—																
X2C2	0	0.57	—																
X2C2a	0	0.51	—																
X2C2b	0	0.72	—																
X2C3	0	0.45	—																
X2C4	0.49	0.63	—																

Results

The elution behaviour of potato plastocyanin on Sephadex G-75 during purification suggested that it had a molecular weight of approx. 10500, which is similar to those reported for most other plastocyanins (see Ramshaw *et al.*, 1973). The complete sequence (see Fig. 1) contained 99 residues, giving a sequence molecular weight of 10332.

CNBr cleaved *S*-carboxymethylated plastocyanin into three major fragments, which were purified by gel filtration on Sephadex G-50, as found for the plastocyanin from French bean (Milne & Wells, 1970). The three fragments are named X-1, X-2 and X-3 in their order of elution from the column (this is coincidentally the same as their order in the complete sequence). Fragment X-3 did not absorb at 280nm and was detected by *N*-terminal analysis. A very small additional peak was also eluted before fragment X-1. It had *N*-terminal leucine and contained tyrosine, so probably represents uncleaved protein; it was not analysed further.

The amino acid compositions of potato plastocyanin and its CNBr fragments, 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 sequence values, and the compositions of the three CNBr fragments account for the composition of the total protein. However, in the analysis of the total protein, the value obtained for aspartic acid was slightly low, whereas the value obtained for lysine was a little high. Both arginine and tryptophan were shown to be absent. In the analysis of fragment X-1 there was poor agreement in the aspartic acid values obtained. The values obtained for homoserine were consistently low; this amino acid was present only in fragments X-1 and X-2. The analyses of the CNBr fragments indicated that the four residues of isoleucine were all present in fragment X-1 and that the three tyrosine residues were all present in fragment X-2.

The *N*-terminal sequence of potato plastocyanin, as far as residue 40, was determined on the sequencer with 400nmol of native protein (see Fig. 1). Repetition of the analysis by using a similar quantity of protein did not enable any additional residues to be determined. Carboxypeptidase analysis indicated that the protein had a *C*-terminal asparagine residue.

From the results with the sequencer, and from the *N*-terminal and *C*-terminal analyses, the largest CNBr fragment, X-1, was shown to be the *N*-terminal fragment of the protein. The analysis of the peptides from the two thermolysin (3h and 1h) and one papain (1h) digests were used in conjunction with the sequencer results to establish its sequence. The peptides analysed are shown in Fig. 1 and their mobilities and amino acid compositions in Table 2.

X2H1	-2.76	0.38	—
X2H1a	-3.21	0.41	—
X2H2	-0.69	0.29	—
X2H2a	-1.02	0.36	0.41
X2H2b	0	0.62	0.51
X2H2c	-0.98	0.36	0.35
X2H3	0	0.77	0.56
X2H4	0	0.65	0.42
X2H5	0.34	0.53	—
X2H5a	0	0.58	—
X2H5b	0	0.77	0.28
X3	0.97	—	—

* Abbreviation: BAWP, Butan-1-ol-acetic acid-water-pyridine (15:3:12:10, by vol.).
† Acidic peptides whose mobilities were not accurately determined.
‡ Peaks present but unsuitable for integration.

See Table 1

Peptides from the region residues 29–38 were obtained in low yield from the first thermolysin digest (for 3 h). A second digest for only 1 h, however, gave peptides from this region of the sequence in good yield. In the second digest very little cleavage was observed at Val-3, Val-50 and Ala-52; that apart, the two digests were very similar. No cleavage was observed in either digest at Leu-4, Ile-15, Phe-35, Val-40, Phe-41 and Ile-46. The lack of activity at Ile-15, Phe-35 and Ile-46 is probably a result of the proline residues that are on the C-terminal side of each of these residues (Matsubara *et al.*, 1969).

The order of the peptides X1H1–X1H10 was placed by comparison with the sequencer results, which gave the first 40 residues of the protein. Peptide X1H10a, which was obtained from the second thermolysin digest only, demonstrated the overlap between peptides X1H10 and X1H11, which were obtained from the first thermolysin digest. The papain

peptide X1P5 demonstrated the overlap between thermolytic peptides X1H11 and X1H12. Since peptide X1H12 had C-terminal homoserine this peptide was the C-terminal peptide of the fragment X-1. The other papain peptides verify the results obtained from the sequencer and the thermolytic peptides. All the amide residues were assigned from the mobilities of the peptides at pH 6.5 (Offord, 1966). All the acid/amide residues present in the sequence up to residue 40 were also confirmed by the results of the sequencer analysis, except for Asn-38. The mobility of peptide X1H10 indicated that it had a charge of –4. Since predicted charges on peptides of greater than ± 3 are not always reliable the presence of the four acidic residues were confirmed by following the changes of the peptide mobilities on successive steps of Edman degradation.

The second CNBr fragment, X-2, had N-terminal alanine and C-terminal homoserine; it was therefore

Fig. 2. Comparison of potato and *Chlorella fusca* (Kelly & Ambler, 1973) plastocyanins (numbering is based on potato plastocyanin)

Residues common to both sequences are shown in capital letters.

internal in the sequence. Digestion by trypsin for 5min gave only two peptides resulting from cleavage at the single lysine residue, so their order was directly established.

Digestion by chymotrypsin for 5min gave six peptides, resulting from cleavage at the three tyrosine residues and partial cleavage at Leu-74. No cleavage was observed at Phe-82.

Digestion with thermolysin for 1h gave five major peptides. Additional peptides were also found because of partial cleavage at Leu-63, Ala-65, Tyr-70 and Ala-90 (see Fig. 1). The purified peptides from these three digests were analysed by the dansyl-Edman method and established the sequence of the X-2 fragment. The amide residues were all deduced directly from the mobility of the purified peptides at pH6.5 (Offord, 1966). In addition, our analysis on the sequencer, by using 200nmol of the fragment, confirmed the presence of Glu-59, Glu-60, Glu-68, Asp-61 and Asn-64.

The smallest CNBr fragment, X-3, had C-terminal asparagine and therefore represented the C-terminus of the protein. This fragment was purified by pH6.5 electrophoresis before analysis by the dansyl-Edman method. The mobility at pH6.5 confirmed that residue 99 was asparagine. A neutral peptide was also present after electrophoresis; it had the same amino acid composition as the major peptide and was present in about 20% of the yield of the latter, and was probably the result of deamidation of Asn-99.

Some peptides had their amino acid compositions determined (see Table 2). The results obtained were generally in good agreement with the determined sequence, although the short hydrolysis time (20h) gave, in some cases, a low yield of hydrophobic residues. Some peptides were contaminated with amino acids probably derived from the paper used for electrophoresis and chromatography. Certain peptides, for example X1H1 and X1H7, showed an indication of impurity from the amino acid analysis results which could not be accounted for by the presence of amino acids eluted from the paper used for electrophoresis and chromatography, although the dansyl-phenyl isothiocyanate analysis of these peptides did not show the presence of any recognizable contaminating peptides.

Discussion

The amino acid sequences and compositions of the three CNBr fragments examined account for the amino acid composition of the total protein. Although overlap through the two methionine residues has not been shown, it is considered that these three fragments give the information for the complete sequence. The sequences of the fragments were mainly obtained by

Fig. 3. Comparison between the C-terminal regions of plastocyanins and azurins

The data on Chlorella plastocyanin and the azurins are taken from Kelly & Ambler (1973). The site XXX has been filled by various amino acids.

dansyl-Edman analysis. However, residues 1-40 and 58-73 were also identified as phenylthiohydantoin derivatives from the sequencer analyses. Peptides that account for every residue in the sequence were also analysed by using an amino acid analyser. No evidence for the presence of any unusual amino acid was found in these analyses, nor in the analyses of the total protein and the CNBr fragments.

The peptide bonds which are C-terminal to both of the methionine residues were cleaved in high yield by CNBr. This method was chosen for the sequence analysis as Milne & Wells (1970) had shown that plastocyanin from French bean, which also contained two methionine residues, was cleaved into three readily separable fragments by CNBr.

Thermolysin was used to digest fragment X-1, since this enzyme had proved of use in the sequence analysis of plastocyanin from *Chlorella fusca* (Kelly & Ambler, 1973). Also, chymotryptic digestion of a similar fragment from *Vicia faba* plastocyanin had given several insoluble peptides, which were difficult to purify (J. A. M. Ramshaw, unpublished work), and tryptic digestion of a similar fragment from lettuce plastocyanin had given two large peptides that could not be readily purified (J. A. M. Ramshaw, unpublished work).

Papain digestion of the potato plastocyanin fragment X-1 gave peptides which were easily separated and which were analysed to confirm the sequence. Trypsin, chymotrypsin and thermolysin were all used for examining fragment X-2. The enzyme specificities observed were all generally consistent with those expected.

All the amide residues in the sequence were determined from the mobilities at pH 6.5 of the purified peptides (Offord, 1966). Some of these assignments were confirmed by the sequencer analyses.

When the sequences of *Chlorella fusca* and potato plastocyanins are compared, they have 53 residues in common (Fig. 2). The regions 31-47 and 78-92 are very similar in the two sequences and may be highly conserved areas in this protein. The potato plastocyanin sequence fits in well with the similarity proposed by Kelly & Ambler (1973) between plastocyanins and bacterial azurins (Fig. 3).

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