The Amino Acid Sequence of Plastocyanin from French Bean

*(Phaseolus vulgaris)*

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The amino acid sequence of the plastocyanin from French bean (*Phaseolus vulgaris*) was determined. The protein consists of a single polypeptide chain of 99 residues, and the sequence was determined by characterization of CNBr, tryptic, chymotryptic and thermolysin peptides. When the sequence is compared with that from the plastocyanin of the unicellular green alga *Chlorella fusca*, the French-bean protein shows the deletion of the N-terminal residue, a two residue insertion and 53 identical residues. Detailed evidence for the sequence of the protein has been deposited as Supplementary Publication SUP 50037 (16pp., 1 microfiche) at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies may be obtained on the terms given in *Biochem. J.* (1973) 131, 5.

Plastocyanin is a small blue copper protein, first isolated from the unicellular green alga *Chlorella ellipsoidea* by Katoh (1960). It has been found in a wide range of photosynthetic organisms, including uni- and multi-cellular algae (Katoh *et al.*, 1961; de Kouchkovsky & Fork, 1964; Gorman & Levine, 1966) and higher plants (Katoh *et al.*, 1961, 1962; de Kouchkovsky & Fork, 1964; Wells, 1965, 1966; Ramshaw *et al.*, 1973). It has been found in at least one blue-green bacterium (*Anabaena variabilis*, Lightbody & Krosgmann, 1967), but not in any of the other classes of photosynthetic bacteria. It does not occur in the non-photosynthetic tissues of plants (Katoh *et al.*, 1961), although it is present in the leaves of etiolated barley seedlings (Plesincar & Bendall, 1970). Plastocyanin is involved in photosynthetic electron transport between photosystems I and II, although the precise details of its involvement are not yet clear (Boardman, 1968; Arnon *et al.*, 1970).

The plastocyanin from French bean (*Phaseolus vulgaris*) was isolated by Wells (1965) during the study of the plant carboxypeptidase phaseolain (Carey & Wells, 1972). French-bean plastocyanin consists of a single polypeptide chain, now known to contain 99 residues, to which one copper atom is bound. It is the smallest copper protein yet reported, with a molecular weight of about 10500 (Milne & Wells, 1970). The complete sequence of the plastocyanin from the green alga *Chlorella fusca* has been briefly reported (Kelly & Ambler, 1973), and the evidence for the amino acid sequence of this 98-residue protein is presented in an accompanying paper (Kelly & Ambler, 1974). The amino acid sequence of potato plastocyanin has been published by Ramshaw *et al.* (1974). French-bean plastocyanin has been crystalized and the tertiary structure is under investigation by X-ray methods (H. Freeman, personal communication).

We have been interested in the structure of plastocyanin for three reasons. First, its size and its involvement in photosynthesis indicate that the amino acid sequence will be useful for the investigation of evolutionary relationships between photosynthetic organisms. Secondly, we are interested to compare the primary structure of plastocyanin with that of the bacterial copper protein azurin (Ambler & Brown, 1967; Ambler, 1971). Preliminary results with spinach plastocyanin (R. P. Ambler, unpublished work) had indicated that the amino acid sequence around the single cysteine residue was similar to the sequence around the only free cysteine residue of the azurin. Evidence has been presented that the single free cysteine residues of both azurin and plastocyanin are involved in the binding of copper to the polypeptide chain (Katoh & Takamiya, 1964; Finazzi-Agro *et al.*, 1970). Finally, the small size of plastocyanin makes it appropriate for investigating the structural features that give rise to the unique blue colour and spectral characteristics of Type I Cu2+ proteins. All intensely blue copper proteins, such as caeruloplasmin, laccase, stellacyanin, azurin and plastocyanin, contain some copper in the 'Type I' form (Rist *et al.*, 1970; Andréasson & Vännård, 1970;
Malmström et al., 1968). Plastocyanin, azurin and stellacyanin contain copper only in the 'Type I' form, and the Type I Cu$^{2+}$ complex is apparently solely responsible for the unusual spectral properties of the intensely blue copper proteins. Although it has been the subject of much work and speculation, the identity of the copper-binding ligands has not yet been determined in any copper protein.

In this paper evidence is presented for the primary structure of the plastocyanin from French bean. Methods for preparing the protein, details of its properties and partial sequence studies have been published (Wells, 1965, 1966; Milne & Wells, 1970; Milne, 1971).

Experimental

The sequence work on French-bean plastocyanin was started in Adelaide and finished in Edinburgh. The methods and materials described below refer to the Australian part unless otherwise stated. The Edinburgh sequence experiments used protein prepared in Australia, examined by methods similar to those used by Kelly & Ambler (1974).

Materials

**Plants.** French bean (*Phaseolus vulgaris*) plants were obtained from a local Adelaide market gardener, and harvested when 6–8 weeks old.

**Chromatographic materials.** Whatman DEAE-cellulose DE-50 was obtained from W. & R. Balston Ltd., Maidstone, Kent, U.K. Sephadex was from Pharmacia, Uppsala, Sweden. Polyamide layers were from Cheng Chin Trading Co., Taipei, Taiwan.

**Enzymes.** 1-Chloro-4-phenyl-3-L-tosylamido-butane-2-one (TPCK)-treated trypsin (TRTPCK 8DA and 8GA), chymotrypsin (CC 16148-9) and carboxypeptidase A (COA-DFP 6138) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Methods

**Isolation of plastocyanin.** Plastocyanin was isolated as previously described (Milne & Wells, 1970). The usual yield was 60–120 mg of plastocyanin/10 litres of bean sap. This material was homogeneous by polyacrylamide-gel electrophoresis and N-terminal analysis, but to remove contaminating polyphenolic compounds, the preparations were normally further purified by repeated chromatography (Milne & Wells, 1970), until an absorption index value ($E_{278}/E_{597}$; Katoh et al., 1962) of 1.1–1.2 was obtained.

The amount of native plastocyanin in preparations could be determined from the molar extinction coefficient of the copper chromophore at 597 nm ($\varepsilon = 4.5 \times 10^{3}$ litre·mol$^{-1}$·cm$^{-1}$; Milne & Wells, 1970).

Quantities of apoplastocyanin and carboxymethyl-plastocyanin were determined from the molar extinction coefficient at 278 nm (5.0 × 10$^{3}$ litre·mol$^{-1}$·cm$^{-1}$) or by hydrolysis and amino acid analysis.

**Carboxymethylation of plastocyanin.** The method described by Milne & Wells (1970) was used.

**Apoplastocyanin.** Apoplastocyanin was prepared by trichloroacetic acid precipitation, as described by Kelly & Ambler (1974).

**Amino acid analysis.** In Australia, peptides were hydrolysed at 110°C for 20–24 h under vacuum in 1 ml of constant-boiling HCl. Large peptides were also analysed after longer times of hydrolysis, and from these analyses correction factors for the destruction/24 h for threonine (6%) and serine (11%) were deduced. In all the later hydrolysates, thioglycolic acid [10 μl of 10% (v/v) aqueous solution] was included, to decrease hydrolysis loss of tyrosine, methionine and S-carboxymethyl-cysteine (Sanger & Thompson, 1963; Dus et al., 1970). In Scotland, peptides were hydrolysed at 105°C for 24 h, and no correction made for serine or threonine destruction. Peptides which were believed to contain stable peptide bonds (Val-Val, Ile-Ile etc.) were generally also analysed after 96 h hydrolysis.

**Fractionation of tryptic peptides.** Carboxymethyl-plastocyanin (3 μmol) was digested with 0.6 mg of trypsin (batch TRTPCK 8DA) in 3 ml of 0.2M-NH$_4$HCO$_3$ at 37°C for 2 h. The digest was then at once passed through a column (110 cm × 2 cm diam.) of Sephadex G-50 (fine grade) equilibrated in 0.1M-NH$_4$ solution at room temperature, with a flow rate of 20 ml/h, and fractions of 5 ml volume were collected. Fractions were pooled on the basis of the u.v.-absorption profiles at 220 nm and 276 nm, and the examination of selected samples by high-voltage paper electrophoresis and N-terminal analysis. The smaller peptides were purified by high-voltage paper electrophoresis. The larger peptides were fractionated by chromatography on DEAE-cellulose (DE-50, 20 cm × 1.2 cm diam., starting buffer 0.02M-sodium phosphate, pH 7.2, eluted with a 250 ml linear gradient of NaCl from 0–1.0M), and finally purified by re-chromatography on a similar column with a 0–0.4M-NaCl linear gradient. The peptides were then de-salted by gel filtration through Sephadex G-50 into 0.1M-NH$_4$ solution.

**CNBr peptides.** The three peptides which resulted from CNBr cleavage of carboxymethyl-plastocyanin were isolated as described by Milne & Wells (1970).

**N-Terminal analysis of protein and peptides and dansyl–phenyl isothiocyanate degradation of peptides.** The dansyl method (Gray, 1972a) was used for qualitative investigations of protein and peptides. The conditions for phenyl isothiocyanate degradations were as described by Gray (1972b). In Australia, dansyl-amino acids were identified by t.l.c. on 3.75 cm square polyamide layers (as described by Hartley, 1974).
1970). In Scotland, primary identification was by high-voltage paper electrophoresis at pH 4.38, with confirmation by other paper systems (Ambler & Wynn, 1973).

Quantities of material used

Adelaide experiments. All experiments used carboxymethyl-plastocyanin. The amounts used were: tryptic digest I, 9 μmol; tryptic digest II (with high-specificity trypsin), 3 μmol; CNBr cleavage, 4 μmol; chymotryptic digest I, 2 μmol.

Edinburgh experiments. The amounts used were: thermolysin digest, 3 μmol of carboxymethyl-plastocyanin; chymotryptic digest II, 3 μmol of apoplastic cyanin; tryptic digest III, 3 μmol of apoplastic cyanin. Quantities of peptides used for analyses and sequence determination experiments were in the ranges given by Ambler & Wynn (1973).

Results

Previous work (Milne & Wells, 1970) had shown that French-bean plastocyanin consisted of a single polypeptide chain of about 100 amino acids to which was bound one copper atom. The protein had been shown to have the N-terminal sequence Leu-Glx-Val-Leu- and to be split into three fragments by CNBr. These fragments were considered to contain 58, 35 and 7 residues, and the longest was N-terminal and the shortest C-terminal. The complete amino acid sequence of the protein has now been determined, and the proposed sequence is shown in Fig. 1.

When the sequence of the French-bean plastocyanin is compared with that of Chlorella fusca plastocyanin (Fig. 2), the algal protein is found to have an extra residue at the N-terminus. To enable corresponding residues to be given the same number, the N-terminal residue of the French-bean protein is numbered 2 and so on in the rest of this paper.

Experimental approach

The initial sequence work was done in Adelaide. The protein contains no arginine and only 5 residues of lysine, so only 6 peptides are formed when the protein is digested with good trypsin. As the CNBr peptides X-1, X-2 and X-3 contained respectively 3, 1 and 1 residues of lysine, all the 6 tryptic peptides except T-2 and T-3 could be ordered by using simply the N-terminal and amino acid analysis results for the CNBr and tryptic peptides (Milne, 1971). In a preliminary experiment (trypsin digest I), with unsatisfactory trypsin (batch TRTPCK 8GA), several chymotrypsin-susceptible bonds were split, and secondary cleavage products of peptides T-1, T-4, and T-5 were isolated. The sequences of several of these peptides were either partially or completely determined by the dansyl–phenyl isothiocyanate method. The isolation of some lysine-containing chymotryptic peptides enabled the relative order of peptides T-2 and T-3 to be determined. These experiments gave sequence information on 66 of the 98 peptide bonds in the molecule, residues 2–11, 20–36, 55–64 and 72–100 (Milne, 1971). The sequence of residues 2–26 was subsequently determined with an automatic sequenator (P. Edman & G. Begg, personal communication).

The Adelaide sequence work was then completed in Edinburgh, by a thorough characterization of the peptides from a thermolysin digest of carboxymethyl-plastocyanin, and by a trypptic digest (III) and a chymotryptic digest (II) of apoplastic cyanin.

The amino acid composition of French-bean plastocyanin is shown in Table 1, which shows the values taken from the sequence (Fig. 1) together with the values deduced from a series of hydrolysis experiments (Milne & Wells, 1970). The values are in very good agreement except for one extra glutamic acid residue in the acid hydrolysis results. This may be due to an erroneously low colour factor for glutamic acid having been used, as this amino acid is the least stable component during storage of amino acid analyser calibration solutions.

Amide groups were assigned from a consideration of the electrophoretic mobilities at pH 6.5 of simple peptides containing the residue under examination. The results of carboxypeptidase digestion of appropriate peptides, and, for residues 2–26, the sequenator results (P. Edman & G. Begg, personal communication) were also used for these assignments.

Peptide nomenclature

Peptides are identified by a capital letter indicating the method used for the degradation of the protein, the letters used being: T, trypsin; X, CNBr; C, chymotrypsin; H, thermolysin; N, papain; S, subtilisin. For the tryptic and CNBr peptides, the letter is followed by a number indicating the order of the peptide in the polypeptide chain (thus peptide T-1 is the N-terminal tryptic peptide). A lower-case letter after the number for a trypptic peptide indicates that the peptide was formed by a secondary enzymic activity in the trypsin used, with peptide 'a' forming the N-terminal part of the primary peptide. For the more complex chymotryptic and thermolysin digests, and for the products of secondary digests with different enzymes, the nomenclature of Ambler & Wynn (1973) is used.

Evidence for the proposed sequence

The evidence for the proposed amino acid sequence is summarized in Fig. 1. This shows all the peptides isolated from each digest, together with peptides
Fig. 1. Amino acid sequence of French-bean plastocyanin

Peptides derived by CNBr cleavage (X) and thermolysin digestion (H) are shown above the sequence, and by tryptic (T) and chymotryptic (C) digestion below the sequence. Vertical arrows show peptides formed by further digestion. For each digest, peptides formed in best yield are shown nearest the sequence. Full lines indicate quantitative amino acid analyses (substandard if marked *). Broken lines indicate qualitative analyses. Under the sequence, → indicates residues identified (P. Edman & G. Begg, personal communication) after degradation of the protein in a sequenator. Under peptide lines, → indicates end-groups and subsequent residues revealed by phenyl isothiocyanate degradation, and identified by the dansyl method (substandard if shown as - ). ← indicates final residues of peptides identified as the free amino acid after removal of the remainder by phenyl isothiocyanate degradation. Peptides marked † were examined by carboxypeptidase A digestion. References to the text are given near regions that are specifically discussed. In the sequence, the N-terminal residue is numbered 2, so that corresponding residues in the French bean and Chlorella fusca plastocyanins should have the same numbers (Fig. 2).
Fig. 1. (continued)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
(a) Leu-Glu-VAL-Leu-LEU-GLY-Ser-Gly-Asp-GLY-Ser-LEU-Val-PHE-Val-PRO-Ser-Glu-Phe-Ser-Val-PRO-Ser-GLY-GLU-Lys-Ile-
(b) Leu-Asp-VAL-Leu-LEU-GLY-Gly-Asp-GLY-Ser-LEU-Ala-PHE-Ile-PRO-Gly-Asn-Phe-Ser-Val-Ser-Ala-GLY-GLU-Lys-Ile-
(c) Asp-Val-Thr-VAL-Lys-LEU-GLY-Ala-Asp-Ser-GLY-Ala-LEU-Val-PHE-Glu-PRO-Ser-Ser-Val-Thr-Ile-Lys-Ala-GLY-GLU-Thr-Val-

29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
(b) -Thr-Phe-Lys-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Val-VAL-PHE-ASP-GLU-ASP-GLU-ILE-PRO-Ala-GLY-Val-ASP-ALA-Ser-Lys-Ile-
(c) -Thr-Trp-Val-ASN-ASN-ALA-GLY-PHE-PRO-HIS-ASN-Ile-VAL-PHE-ASP-GLU-ASP-GLU-Val-PRO-Ser-GLY-ALA-Asn-ALA-Glu-ALA-Leu-

57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84
(a) -SER-Met-PRO-Glu-GLU-Leu-LEU-ASN-ALA-GLY-PRO-GLY-GLU-THR-Val-Val-Thr-Leu-ASP-Thr-Lys-GLY-THR-THR-Ser-Phe-Tyr-
(b) -SER-Met-Ala-Glu-GLU-Leu-LEU-ASN-ALA-GLY-GLU-THR-THR-Ser-Val-Thr-Leu-Ser-Glu-Lys-GLY-THR-THR-Thr-Phe-Tyr-
(c) -SER-----His-----GLU-Asp-Tyr-LEU-ASN-ALA-PRO-GLY-GLU-Ser-THR-Ser-ALA-Lys-Phe-Asp-Thr-ALA-GLY-THR-THR-Gly-THR-Phe-

85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
(a) -CYS-Ser-PRO-HIS-GLN-GLY-ALA-GLY-MET-Val-GLY-Lys-Val-THR-VAL-Asn
(b) -CYS-Ala-PRO-HIS-GLN-GLY-ALA-GLY-MET-Val-GLY-Lys-Val-THR-VAL-Asn
(c) -CYS-Glu-PRO-HIS-GLN-GLY-ALA-GLY-Met-Lys-GLY-Thr-Ile-THR-VAL-Gln

Fig. 2. Comparison of amino acid sequences of plastocyanins from (a) French bean, (b) potato (Ramshaw et al., 1974) and (c) Chlorella fusca (Kelly & Ambler, 1974)

Residues that are the same in all three sequences are shown in capitals.
formed by further digestion of selected peptides with other enzymes. Symbols show how much of the sequence of each peptide has been determined by the dansyl–phenyl isothiocyanate method, as well as peptides that were examined with carboxypeptidase. Cases in which the results of dansyl–phenyl isothiocyanate degradation or quantitative amino acid analysis were not considered wholly satisfactory are indicated.

Dansyl–phenyl isothiocyanate degradation results were considered unsatisfactory when more than one dansyl-amino acid was identified as present in significant amount (subjective visual estimate of more than 20%) at a step of the degradation, or when for some reason or other (such as accident or shortage of material) the dansyl-amino acid was not positively identified.

The criteria for satisfactory amino acid analyses are: (1) that no impurity should be present in amount larger than 0.20 mol/mol and (2) that when the relative amounts of amino acids present are calculated on the basis that the average amount is integral, no values should fall outside the limits 0.8–1.2, 1.8–2.2, 2.7–3.3, 3.7–4.3 etc. The peptides that did not meet these analytical criteria (marked * on Fig. 1) failed for the following reasons:

(1) Peptide C112 (mixture of residues 31–64 and 31–71): analysis gave Gly 2.3, Ala 3.3, Val 4.0, Leu 2.0, Ile 2.0, Ser 0.8, Asp 6.3, Glu 5.5, Phe 2.0, Met 0.9, Pro 3.0, Lys 1.9, His 0.9 (Thr 0.26, Tyr 0.19), for hydrolysis for 96 h at 105°C. The amino acid analysis and the amounts of amino acids released by carboxypeptidase digestion (Leu 1.5, Thr 0.31, Tyr 0.23, Val 0.13; no Glu or Gln) are consistent with the fraction being a mixture of about 75% of a peptide comprising residues 31–64 and 25% of a peptide comprising residues 31–71. The extra C-terminal sequence of the latter peptide (also found as peptide C311a) was...Asn-Ala-Pro-Gly-Glu-Thr-Tyr. These two big peptides would be expected to have very similar electrophoretic properties. As this part of the sequence had been well characterized by other experiments, it was not considered necessary completely to purify these peptides.

(2) Peptide T-3 (residues 32–55). From other evidence this peptide should contain four valine residues, including the sequence -Val-Val- at positions 40/41. Both the Australian and the Scottish analyses were of samples hydrolysed for only 24 h. The former (hydrolysed at 110°C) showed Val 3.6, and also failed the criteria by showing Gly 2.3 (=2), whereas the latter (at 105°C) showed Val 3.2.

(3) Peptide X-1 (residues 2–58; Milne & Wells, 1970, peptide CNBr-1): the analysis showed Glu 5.8, whereas all the other sequence evidence for this region indicated that there were only five residues of glutamic acid or glutamine. The other only piece of evidence that supports this analysis is the glutamic acid value for hydrolysates of the whole protein (Table 1 and Milne & Wells, 1970). A possible explanation for these high recoveries of glutamic acid has been suggested above.

(4) A few peptides slightly transgressed the stoichiometry or impurity criteria. Most of these offenders were minor peptides isolated in low yield, or the products of secondary digestions, but peptide T-5, a major trypptic peptide, showed Ser 1.7 (=2). The other offenders were peptides: H34bi (Ala 0.34 =0); H311b (Pro 1.3 =1); H310b (Ala 0.42 =0; Ser 0.24 =0; Phe 0.23 =0); T-1b (Ser 1.7 =2); T-5b (Pro 1.3 =1); T-3H2a (Ser 0.21 =0); H26N2 (Gly 0.21 =0; Ser 0.28 =0). The worst case, peptide H310b, could not be simply explained as contamination with a single known peptide of similar properties. The analysis for peptide H35a was satisfactory except that, through an instrumental fault, no quantitative value was obtained for histidine.
(5) Several peptides derived from carboxymethyl-
plastocyanin gave bad analysis values for S-carboxy-
methyl-cysteine (Cmc) and methionine. Thus peptide
X-2 showed Cmc 0.6 (=1), T-5b Cmc 0.5 (=1) (also
Pro 1.3 =1; see above). In some cases both cysteine
and S-carboxymethyl-cysteine, and both methionine
and S-carboxymethyl-methionine were detected,
although the combined values still did not give an
adequate recovery. These peptides were: H38f
(Cys+ Cmc 0.6 =1); H38bi (Cys + Cmc 0.34 =1;
Met+S-carboxymethyl-methionine 0.7 =1). Peptide
C26a gave an extremely low recovery of methionine
(0.3 =1), as well as defaulting values of Gly (2.6 =3),
Lys (0.7 =1) and His (0.7 =1). Completely satisfactory
values were obtained for the peptide (C26/9) from the
same region of the sequence from chymotryptic
digest II, in which apoplastocyanin was digested, and
the peptide oxidized with performic acid vapour on
paper in the course of its purification.

Supplementary publication

Detailed evidence for the amino acid sequence of
the protein has been deposited as Supplementary
Publication SUP 50037 with the British Library
(Lending Division) for storage on microfiche. The
evidence comprises:

(1) Tables showing the properties of all the peptides
in Fig. 1. The successive steps used in the purification
of each peptide are given, and where applicable the
values of V/V0 [elution volume/void volume for gel
filtration through Sephadex G-25 in 5% (v/v) formic
acid] and electrophoretic mobility on paper at pH6.5.
Absolute percentage yields are given for peptides
from primary digests, and relative yields for peptides
from secondary digests. Amino acid analyses are
given for all peptides, which show all impurities
present in amounts greater than 0.1 mol/mol peptide.

(2) Further tables show the individual sequence
evidence for each peptide. This consists of the
N-terminal group analysis results, and the results of
dansyl-phenyl isothiocyanate degradation experiments.
Details are given when these results are sub-
standard.

(3) The evidence for the presence or absence of
amide groups on each aspartic acid or glutamic acid
residue in the protein is given.

(4) Details are given of experiments with carboxy-
peptidase A on the peptides marked † in Fig. 1, and of
the effect of phaseolain [a carboxypeptidase C (Carey
& Wells, 1972)] on the whole protein.

Discussion

Problems in the elucidation of the sequence

The determination of the amino acid sequence of
the French-bean plastocyanin presented few major
problems, as adequate amounts of protein of high
purity were available. The protein contained suffi-
cient well-situated residues of methionine (two) and
lysine (five) to allow the isolation and characteriza-
tion of all the CNBr and tryptic peptides. This was in
contrast with the Chlorella fusca protein (Kelly &
Ambler, 1974), which had to be investigated by
chymotryptic and thermolysin digestion.

At the beginning of the investigation there was
difficulty in getting satisfactory tryptic digestion,
as the batch of commercial chymotrypsin-inhibitor-
treated trypsin available had much chymotrypsin-
like activity, so yields of the larger tryptic peptides
were low. This may have been due to actual contami-
nation with chymotrypsin, or to the presence of the
autolysis product pseudotrypsin (Keil-Dlouhá et al.,
1971). A second batch of the same product proved
satisfactory, and adequate results were also obtained
with trypsin that had been treated before use with
diphenylcarbamyl chloride (Erlanger & Cohen,
1962), even though the crystalline trypsin had been
stored for several years at 4°C with no special
attempts made to keep it dry.

French-bean plastocyanin lacks arginine, and con-
tains only a low proportion of lysine, so some of the
tryptic peptides are large and cannot be separated on
paper in good yield. Preliminary electrophoretic
experiments showed that these large peptides all had
net negative charges at pH6.5, and so would be ex-
pected to be separable on an anion-exchange resin.
This proved to be the case, and after initial removal of
the small peptides by gel filtration, the large peptides
were readily purified in satisfactory yield by chro-
matography on DEAE-cellulose eluted with a linear
NaCl gradient. The purification of the CNBr peptides
has been reported elsewhere (Milne & Wells, 1970).
After an initial gel filtration, the smaller tryptic pep-
tides, the thermolysin peptides and most of the ex-
pected chymotryptic peptides could be fractionated
satisfactorily by paper methods. The final tryptic
digest (III) was also fractionated by paper methods,
but the yields of two of the three large peptides were
much lower than by the ion-exchange method. In
digest III, peptide T-1 was recovered in 7% yield and
peptide T-4 in 5% yield, compared with 53% and 40%
respectively in digest II. Surprisingly, the similar-
sized peptide T-3 was recovered in 21% yield in digest
III compared with only 16% by the ion-exchange
method. The N-terminal chymotryptic peptide (resi-
dues 2–15) was not isolated, presumably because it
was too hydrophobic to separate on paper despite its
small size.

Some difficulty was experienced in the investigation
of the very acidic regions of the sequence. It proved
difficult to get unambiguous results for the region of
residues 43–46 (Asp-Glu-Asp-Glu), as the phenyl
isothiocyanate degradation tended to getragged
through this sequence. It was necessary to study pep-
tides from secondary digests of peptide H316

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(residues 40–50) and T-3 (residues 32–55) to establish this sequence conclusively. The proof of absence of amides in this sequence and for residues 60–62 (Glu-Glu-Glu) also posed problems. Evidence for the presence or absence of amide groups was generally based on the qualitative mobility at pH 6.5 of peptides containing only a single acidic residue, but this was not possible for these multiple acidic residue sequences. In these cases the mobilities of peptides formed by phenyl isothiocyanate degradation of peptides T-3H7 (residues 42–50) and H315c (residues 56–63) were measured, and the residues identified as acidic by the progressive decrease in mobility of the residual peptides as they were successively removed. The quantitative mobility values were compared with predicted values (Offord, 1966), but no assignments were made solely on the basis of such numerical results.

**Accuracy of proposed sequence**

Most of the peptide bonds in the sequence have been established by phenyl isothiocyanate degradation, many of them in two or more independent experiments. The method appears to have a generally high reliability, but errors (especially transpositions) are always possible, particularly from mislabelling. Except where specifically indicated, dansyl-amino acids were positively identified, no reliance being put on the known amino acid composition of the peptide under investigation to distinguish between dansyl-amino acids of similar chromatographic or electrophoretic properties.

The peptide bonds not established by this method were 27–28, 37–38, 39–40, 50–51, 53–54, 55–56, 71–72 and 92–93–94. The evidence for these bonds is primarily from amino acid compositions, in particular the composition differences between related peptides (e.g. between peptides H37e and H37di, and between peptides T-1b and C27ai for bond 27–28). Evidence from carboxypeptidase digestion was also used (e.g. to provide evidence for bonds 50–51 and 53–54 by the amino acids released from peptide T-3). The evidence for all the bonds seems to be adequate, and no region of the sequence can be singled out as being particularly likely to contain an error.

As mentioned above, the amino acid composition of the protein derived from hydrolysis experiments agrees well with that from the sequence (Table 1), apart from an extra residue of glutamic acid detected by hydrolysis in the whole plastocyanin and in the CNBr peptide X-1. The discrepancy is not much larger than the expected errors in the analytical method, and, as suggested above, may be due to inaccuracy in the amino acid analyser calibration mixture. There is no obvious site in the sequence into which an extra residue of glutamic acid or glutamine could be fitted that would not be inconsistent with sequence determination experiments and with what is known about protease specificity.

**Comparison of known plastocyanin sequences**

The amino acid sequences of the plastocyanins from potato (Solanum tuberosum; Ramshaw et al., 1974) and the unicellular green alga Chlorella fusca (Kelly & Ambler, 1973, 1974) are known (Fig. 2). The potato protein is of exactly the same length as that from French bean, and 80 of the 99 residues are identical. As compared with the higher plant proteins, the algal protein contains an extra residue at the N-terminus, and also has a two residue deletion at about residues 58–59. The plastocyanins show some sequence similarity to the bacterial azurins (Ambler, 1971; Ambler & Brown, 1967) around the single free cysteine residue.

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**References**


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