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Hydrolysis of Proteins Using Dipeptidyl Aminopeptidases: Analysis of the N-Terminal Portion of Spinach Plastocyanin†

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ABSTRACT: The exopeptidases dipeptidyl aminopeptidases I and IV were used to hydrolyze the N-terminal portion of spinach plastocyanin to dipeptides. The enzymes were used individually as well as in a mixture and the dipeptides were analyzed by combined gas chromatography–mass spectrometry. Data are presented for native plastocyanin and the S-methylated protein. Of the 98 residues which make up this protein, the first 44 were released in the form of 22 dipeptides by the combined action of DAP I and DAP IV. These dipeptides were aligned by homology to other plastocyanins of known sequence. The results demonstrate the versatility of the two enzymes in hydrolyzing proteins to obtain information on their primary sequence.

Gutman and Fruton (1948) first described a chymotrypsin-like proteolytic enzyme isolated from bovine spleen which catalyzed the deamidation of hydrophobic dipeptide amides. This enzyme was later termed cathepsin C (Tallan et al., 1952). Several papers concerned with the properties of this enzyme were then published (Izumuja and Fruton, 1956; Metrione et al., 1966) which concluded that the enzyme had extremely limited substrate specificity. McDonald et al. (1965) published the first of a series of papers investigating the properties of a chloride-activated sulfhydryl-dependent enzyme from bovine pituitary gland similar in action to bovine spleen cathepsin C. This enzyme was later found (McDonald et al., 1969) to catalyze the sequential release of dipeptides from polypeptides and was termed dipeptidyl aminopeptidase I (EC 3.4.14.1, DAP I).[1] Subsequent work with the spleen cathepsin C lead to the conclusion that it was identical with the pituitary enzyme (McDonald et al., 1972). With the necessary activators it was found that DAP I showed a very broad substrate specificity in that it was able to hydrolyze dipeptides from the N termini of β-corticotropin, glucagon, secretin, oxidized bovine insulin B-chain, and angiotensin-II. The studies showed that all unsubstituted amino-terminal dipeptidyl groups can be hydrolyzed by DAP I with the exception of those in which arginine or lysine is the N-terminal residue or which involve hydrolysis of a peptide bond involving proline (McDonald et al., 1971).

The use of DAP I in sequence studies was first proposed by McDonald et al. (1969) where they utilized paper chromatography to identify the dipeptides released in the time course reaction for hydrolysis of several small polypeptides. Subsequent work by Callahan et al. (1972) applied several physical methods for the separation and identification of the released dipeptides, including paper chromatography, ion-exchange chromatography, thin-layer chromatography on both polyamide and microcrystalline cellulose, and membrane diffusion.

Other enzymes having specificities much narrower in scope than DAP I have been isolated from a number of mammalian tissues. Hopsu-Havu and Sarimo (1967) isolated an enzyme from rat liver and hog kidney which hydrolyzed Gly-Pro-β-naphthylamide. Based on the substrate specificity reported for this enzyme, McDonald et al. (1971) named the enzyme dipeptidyl aminopeptidase IV (DAP IV). DAP IV preferentially hydrolyzes peptide bonds in dipeptide β-naphthylamides and small peptides in which proline is in the penultimate position, although it is not limited to these substrates. Caprioli and Seifert (1975) showed that DAP IV will also hydrolyze dipeptides from polypeptides containing arginine and lysine in the N-terminal positions, but will not hydrolyze the imide bond of a prolyl residue.

The use of a mixture of DAP I and DAP IV for the hydrolysis of polypeptides was first demonstrated by Caprioli and Seifert (1975). Since the two enzymes have somewhat complementary activities, the mixture provides a convenient way of circumventing many of the specificity problems associated with either enzyme, except that of the prolylimide bond.

Although many analytical methods have been employed to identify the dipeptides, the most effective method involves their simultaneous separation and identification utilizing combined gas chromatography/mass spectrometry (GC/MS). The use of GC/MS techniques in combination with DAP I hydrolyses was reported earlier by Ovchinnikov and Kiryushkin (1972) and Caprioli et al. (1973). Subsequent work by Caprioli and Seifert (1975) demonstrated the versatility and reliability of

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[1] Abbreviations used: DAP, dipeptidyl aminopeptidase; GC/MS, combined gas chromatography/mass spectrometry; Pth, phenylthiohydantoin.

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the GC/MS method for the identification of dipeptides released from these enzyme hydrolyses. The strategy proposed by these authors involves hydrolysis of the original polypeptide and the des-N-terminal polypeptide obtained from one round of the Edman degradation. Each set of dipeptides is derivatized and identified by GC/MS methods, providing overlapping dipeptide data which contain sufficient information to provide the sequence. In addition, it provides a nearest neighbor analysis for polypeptides to identify specific residues in a sequence, as needed, for example, in determining the extent of modification of a given residue.

The present work was undertaken to test the viability and versatility of these techniques and the effectiveness of the DAP I/IV mixture. Since in the past only polypeptides of known sequence have been used in initial development of the methods, it was necessary to test the technique utilizing a protein of reasonable size whose sequence was unknown. Spinach plastocyanin with a molecular weight of approximately 10 500 was chosen as a suitable substrate. Shortly after the completion of this work, the sequence of spinach plastocyanin was published by Scawen et al. (1975) using classical sequencing methods. These results are discussed later since they provide a basis of comparison and confirmation of the work described here.

Experimental Section

Enzyme Preparation. DAP I was isolated from freshly obtained beef spleens and assayed by the method of McDonald et al. (1972). Gel filtration chromatography was performed using a 10 × 90 cm Sephadex G-200 column with an upwards flow rate of 14 mL/h. Low levels of Ser-Met dipeptidase and catheptic carboxypeptidase C reported to be present in the G-200 fraction were eliminated by treatment with diisopropyl fluorophosphate (to inactivate the carboxypeptidase) and EDTA (to inhibit the dipeptidase). The final preparation had a specific activity of 12.1 units/mg. The enzyme was dispensed into glass ampoules in 1–2 mg quantities, lyophilized, sealed under vacuum, and stored in the refrigerator. When needed, an ampoule was reconstituted in 1% NaCl (200 μL/mg of enzyme). A fluorometric procedure was used in which the rate of release of β-naphthylamine from Gly-Phe-β-naphthylamide was measured. Assay mixtures contained 1.0 mL of 31.5 mM 2-mercaptoethylamine hydrochloride/26.3 mM sodium cacodylate/HCl buffer, pH 6.0, and 2.0 mL of 0.3 mM Gly-Phe-β-naphthylamide (Fox Chemical Co.). After equilibration at 37 °C, the reaction was initiated by the addition of 1 μL of DAP I solution. One unit of activity is defined to be the release of 1 μmol of β-naphthylamine per min.

DAP IV was prepared by the method of Hopsu-Havu et al. (1968) from freshly obtained hog kidneys. Ion-exchange chromatography was performed on a 21 × 2.5 cm column of DEAE-cellulose. The final preparation had a specific activity of 9.1 units/mg. The enzyme was dispensed in 2.4-mg quantities into glass ampoules, lyophilized, sealed under vacuum, and stored in the refrigerator. When needed, the enzyme was reconstituted in 1 mL of deionized water. DAP IV was assayed by the method of Caprioli and Seifert (1975) in 2.0 mL of 0.2 M Tris-HCl buffer, pH 7.8, containing 1.0 mL of 0.5 mM Gly-Pro-β-naphthylamide (Fox Chemical Co.). After equilibration at 37 °C, the reaction was initiated by the addition of 1 μL of DAP IV solution.

Hydrolysis of Plastocyanin. DAP I digests were carried out in the following manner: to 20–50 nmol of substrate dissolved in 40 μL of 4% pyridine were added 79 μL of water, 40 μL of 0.5% acetic acid, 32 μL of 0.1 N HCl, 8 μL of 0.375 M 2-mercaptoethanol, and 2 μL of 0.1 M Na_2EDTA. The solution was adjusted to pH 5.0 with acetic acid or pyridine, if necessary. These reagents formed a volatile buffer which could be removed under vacuum without leaving a substantial salt residue. After equilibration at 37 °C, digestion was initiated by the addition of 3–5 units of DAP I per μmol of substrate. The digestion was carried out for 2–3 h at 37 °C and stopped by freezing and lyophilization.

Hydrolyses of polypeptides with a mixture of DAP I and DAP IV were carried out by dissolving 20–50 nmol of substrate in 30 μL of 5% 2,6-dimethylpyridine and diluted with 79 μL of water. To this solution were added 40 μL of 0.5% acetic acid, 47 μL of 0.1 N HCl, 8 μL of 0.375 M 2-mercaptoethanol, and 2 μL of 0.1 M EDTA. The pH was adjusted to 6.5 with 2,6-dimethylpyridine or acetic acid, if necessary. After equilibration at 37 °C, digestion was initiated by the addition of 3–5 units of DAP I and 2–3 units of DAP IV per μmol of substrate. The mixture was incubated at 37 °C for 2–3 h. The digestion was stopped by freezing and lyophilization.

Edman Degradation. The N-terminal amino acid was removed by the isothiocyanate degradation of Edman according to the procedure of Blömback et al. (1966) as modified below. Substrate peptide (~30 nmol) was dissolved in 100 μL of pH 9.0 buffer composed of 0.4 M *N*-ethylmorpholine in 60% pyridine. The pH was brought to 9.0 with trifluoroacetic acid. This peptide solution was degassed, flushed with N_2, then 10 μL of phenyl isothiocyanate (Sequanal grade, Pierce Chemical Co.) were added through the N_2 barrier. After reacting for 4 h at 45 °C, the mixture was extracted five times with 2 volumes of benzene and the aqueous layer removed and lyophilized. The vessel containing the lyophilized residue was degassed and flushed with N_2, 100 μL of trifluoroacetic acid was added through the N_2 barrier, and the reaction was allowed to continue for 1 h at 45 °C. Approximately 200 μL of 1,2-dichloroethane was added and the reaction mixture evaporated to dryness in a stream of N_2. The residue was extracted two times with 100 μL of 1,2-dichloroethane, the extracts pooled, and the peptide residue was dried in vacuo.

S-Methylation. The free sulfhydryl group of spinach plastocyanin was blocked by the methylation procedure of Heinrikson (1971), modified as follows. Spinach plastocyanin (1.7 mg) was dissolved in 2.0 mL of a denaturing solvent at pH 8.6 consisting of 6 M guanidine hydrochloride, 0.25 M Tris, 3.3 mM Na_2 EDTA, and 25% (v/v) of acetonitrile. Approximately 60 mg of Chelex 100 resin (Bio-Rad Laboratories) was added and allowed to react at room temperature for 2 h with occasional mixing. The solution was centrifuged and the supernatant was removed and passed through a Millipore filter. The filtered solution was placed in a 3-mL Reacti-vial fitted with a Mininert valve (Pierce Chemical Co.). The solution was flushed with N_2 for 15 min, and 2 μL of 2-mercaptoethanol added through the N_2 barrier. The solution was incubated at 50 °C for 1 h under N_2. After cooling at 37 °C, a solution of 6.3 mg of methyl-*p*-nitrobenzenesulfonate (Aldrich Chemical Co.) in 0.5 mL of acetonitrile was added to the reaction mixture through the N_2 barrier, and the mixture was allowed to incubate at 37 °C for 4 h. It was then dialyzed extensively against 0.001 M NH_4HCO_3 using dialysis tubing with a 6000–8000 molecular weight cut-off and finally lyophilized.

GC/MS Analysis. The lyophilized residue from the enzyme hydrolysis was treated in the following manner to convert the dipeptide to the *N,O*-perfluoropropionyl, methyl ester derivatives for GC/MS analysis. Approximately 200 μL of dry methanol was added to the residue in a 1-mL Reacti-vial fitted with a Teflon-lined cap, the suspension was cooled in a dry ice–ethanol bath, and 40 μL of thionyl chloride was slowly added. The solution was allowed to come to room temperature and then heated in a constant temperature block at 45 °C for

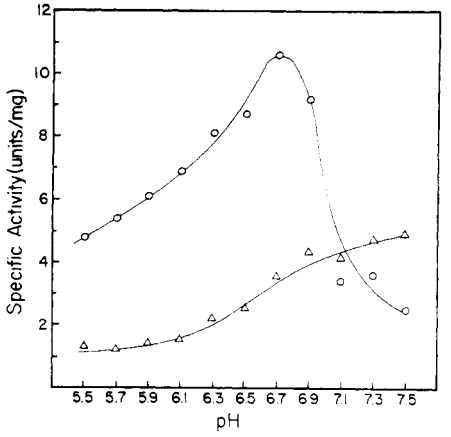


FIGURE 1: Effect of pH on the activity of the dipeptidyl aminopeptidase I/IV mixtures; (○) Gly-Phe-β-naphthylamide substrate; (△) Gly-Pro-β-naphthylamide substrate.

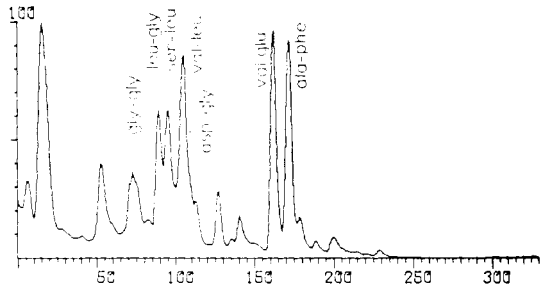


FIGURE 2: Total ion chromatogram from the GC/MS analysis of dipeptides released from spinach plastocyanin after digestion with dipeptidyl aminopeptidase I.

30 min. The reagents were removed in vacuo and 100 μL of perfluoropropionic acid anhydride (Pierce Chemical Co.) was added. After 15 min at room temperature, the reagents were removed in vacuo and the residue was dissolved in 10–50 μL of either dry dioxane or dry acetone. After thorough mixing, 1–2 μL of this solution was injected into the inlet of the GC/MS. Under these derivatization conditions, the side chain amides are converted to their acids.

A Finnigan Model 3200 GC/MS equipped with a Finnigan Model 6000 data system was employed for the analysis of the derivatized dipeptide products. Separation of the dipeptides was effected using a glass column (2 mm i.d. × 150 cm) packed with 3% OV-1 on 80/100 mesh Gas-Chrom Q (Applied Science Laboratories). On-column injection into the gas chromatograph was used with the injector at 220 °C and a linear temperature program from 100 to 300 °C at 10 °C/min. Helium was used as the carrier gas at a flow rate of approximately 30 mL/min. The separator oven was held at 280 °C. Mass spectra were obtained using an electron-impact source operated at 70 eV with an analyzer temperature of 100 °C. Ion chromatograms, plots analogous to normal gas chromatograph traces, were obtained by plotting ion intensity for each mass spectrum taken in the analysis vs. mass spectrum number.

Results

pH Optimum of DAP I/DAP IV Mixtures. It was essential to establish the optimal pH for hydrolysis of polypeptides by the DAP I/IV mixture since each enzyme has a different pH optimum: approximately 6.0 for DAP I and 7.8 for DAP IV (McDonald et al., 1971). Individual assays were performed at various pH values using Gly-Phe-β-naphthylamide (DAP I substrate) and Gly-Pro-β-naphthylamide (DAP IV substrate). The results are presented in Figure 1. From these data, it appears that the pH optimum for the DAP I/DAP IV mixture is 6.5–6.9. For the peptide hydrolyses, pH 6.5 was used routinely. It has been reported (Nilsson and Fruton, 1964) that, at about pH 7–8, DAP I exhibits considerable dipeptide transferase activity toward dipeptide amides, leading to formation of peptide tetramers and oligomers. However, McDonald et al. (1971) have reported that below pH 7 little, if any, transpeptidation occurs with dipeptides.

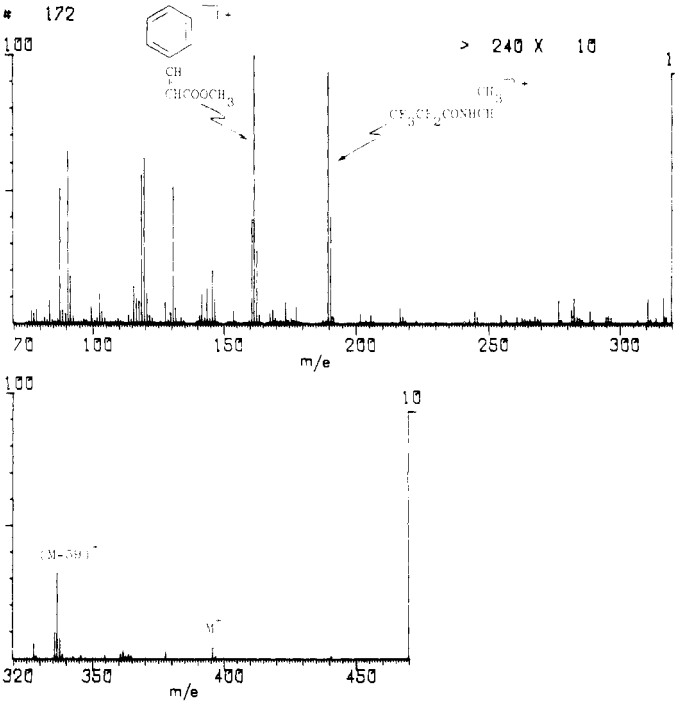


FIGURE 3: Mass spectrum of perfluoropropionyl-Ala-Phe methyl ester obtained from the dipeptide analysis shown in Figure 2.

Control determinations were performed using normal hydrolytic conditions with (i) no polypeptide substrate; and (ii) horse heart cytochrome *c*. In the first case, we were testing for evidence of any autodigestion of either enzyme and also for the presence of contaminating reagents which might unnecessarily complicate identification of the dipeptides. GC/MS analysis showed that no dipeptides were present in the reaction mixture and that there was no significant contamination from exogenous compounds. In addition, the stability of the enzymes in this system is good. After 3 h of incubation, the activities of each enzyme were greater than 95% of their value at time zero. The control substrate was horse heart cytochrome *c* which is N-acetylated and which should not be degraded unless the DAP I/IV mixture contained endopeptidase activity. GC/MS analysis showed that no dipeptides were released during incubation.

Hydrolysis of Spinach Plastocyanin. Active, unmodified spinach plastocyanin was hydrolyzed according to the procedures given above. The buffer solution contained EDTA which complexed the single Cu^{2+} ion present in the active form as seen from the change in color from blue to a colorless solution on addition of the EDTA. Analysis of the DAP I digest of this preparation of spinach plastocyanin showed seven dipeptides to be present; Gly-Gly, Leu-Gly, Ser-Leu, Val-Leu, Asp-Gly, Val-Glu, and Ala-Phe. The total ion chromatogram obtained from the GC/MS for this analysis is given in Figure 2. As an illustrative example, the mass spectrum of Ala-Phe (derivatized) from this analysis is given in Figure 3. The molecular ion (M) appears at *m/e* 396 with a major fragment at *m/e* 337

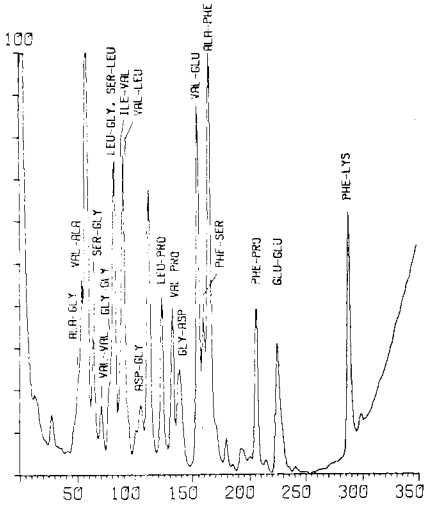


FIGURE 4: Total ion chromatogram from the GC/MS analysis of dipeptides released from spinach plastocyanin after digestion with the dipeptidyl aminopeptidase I/IV mixture.

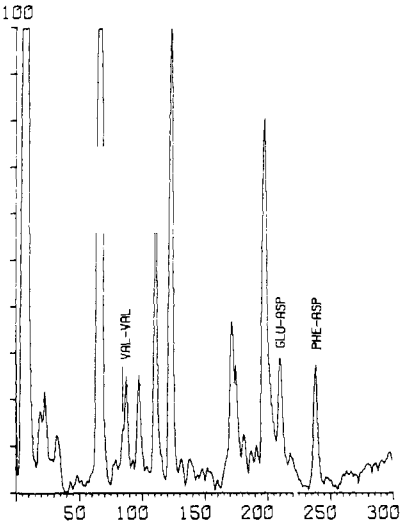


FIGURE 5: Total ion chromatogram from the GC/MS analysis of dipeptides released from the core peptide of spinach plastocyanin after digestion with dipeptidyl aminopeptidase I (see text for preparation of core peptide).

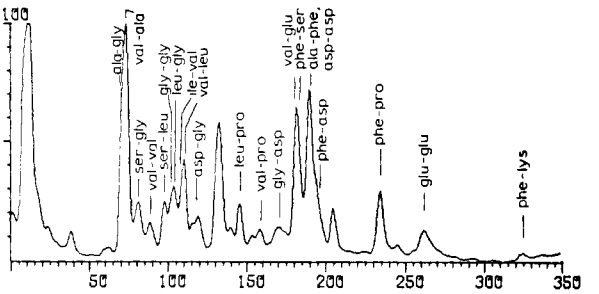
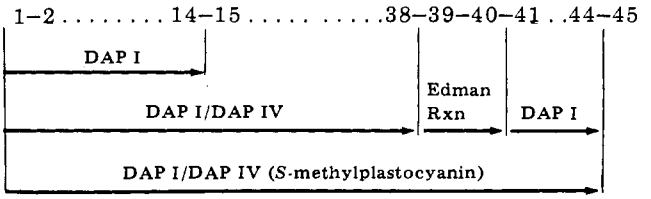


FIGURE 6: Total ion chromatogram from the GC/MS analysis of dipeptides released from spinach *S*-methylplastocyanin after digestion with the dipeptidyl aminopeptidase I/IV mixture.

DIAGRAM I



(M − 59) resulting from the loss of the carboxyl moiety. The two ions of greatest importance in terms of the identity of the dipeptide are formed from the fragmentation at bonds adjacent to the peptide bond, i.e., *m/e* 190 for the N-terminal alanine portion and *m/e* 162 for the C-terminal phenylalanine fragment. A more detailed account of the analysis of dipeptides with the techniques used here is given elsewhere (Caprioli and Seifert, 1975; Seifert et al., 1977). The dipeptides identified in this plastocyanin hydrolysis appeared to be in the same molar ratio (after correction for different response factors) indicating that 14 residues were removed prior to DAP I encountering a bond it could not hydrolyze. The other peaks shown in Figure 2 result from nonpeptide constituents of the buffer and organic impurities in the reagents and peptide samples. For example, the peaks at positions 15, 52, and 128 result from the oxidized β-mercaptoethanol dimer (R-S-S-R) and its oxygenated analogues.

A second sample of spinach plastocyanin was hydrolyzed with the DAP I/IV mixture. The total ion chromatogram is given in Figure 4. Nineteen dipeptides were identified in this mixture. Since it was not clear whether this was a limit digest, e.g., whether a prolyl residue was in position 3 of the remaining polypeptide chain, it was necessary to isolate the unhydrolyzed core by passing the reaction mixture through a Sephadex G-50 column. The two N-terminal residues were removed from the core by the Edman degradation. The resulting polypeptide was then hydrolyzed using DAP I. From this last analysis, only two new dipeptides were found to be present: Glu-Asp and Phe-Asp (see Figure 5). Small amounts of Val-Val and Phe-Pro were also identified. The large peaks not labeled in the chromatogram (i.e., at positions 8, 66, 111, 123, and 171) are nonpeptide constituents.

Spinach plastocyanin which had been modified by S-methylation was also hydrolyzed using the DAP I/IV mixture. This sample is of particular interest because the S-methylation was done in 6 M guanidine hydrochloride and therefore should have resulted in the formation of a random coil. The results of the hydrolysis are presented in Figure 6. Twenty-one dipeptides were identified in the hydrolysate.

The results obtained from the various experiments are summarized in the following scheme which shows the extent of hydrolysis on the first 44 residues of spinach plastocyanin. Each enzyme reaction was performed to obtain complete digestion under the specific hydrolysis conditions with either S-methylated or unmodified substrate. Comparisons of these lead to specific information on the primary structure of spinach plastocyanin and also on the effectiveness of the DAP enzymes toward their substrates.

Discussion

The twenty-one dipeptides identified in the DAP I/IV digest of spinach *S*-methylplastocyanin may be arranged by homology with other plastocyanins of known sequences, as shown in Table I. It can be seen that approximately two-thirds of the first 44 residues of spinach plastocyanin show a high degree of homology, with the remaining residues being conservative replacements. The data obtained from the hydrolysis of the native spinach plastocyanin is useful in reinforcing this alignment. The DAP I hydrolysis produced seven dipeptides, as expected from the proposed sequence, since the enzyme should not hydrolyze the peptide bond between residues 16 and 17 (all known sequences contain a prolyl residue at position 16). Six of the seven dipeptides were homologous, the fourth,

TABLE I: Alignment of Dipeptides from Spinach Plastocyanin with Plastocyanins of Known Sequence.[a]

Broad bean[b]		Val	Glu	Val	Leu	Leu	Gly	Ala	Ser	Asp	Gly	Gly	Leu	Ala	Phe	Val	Pro	Asn	Ser	Phe	Glu	Val	Ser
French bean[c]		Leu	Glu	Val	Leu	Leu	Gly	Ser	Gly	Asp	Gly	Ser	Leu	Val	Phe	Val Val	Pro	Ser Gly	Glu	Phe	Ser	Val	Pro
Elder[d]		Val	Glu	Ile	Leu	Leu	Gly	Gly	Glu	Asp	Gly	Ser	Leu	Ala	Phe	Ile	Pro	Ser	Asn	Phe	Ser	Val	Pro
Marrow[e]		Ile	Glu	Val	Leu	Leu	Gly	Gly	Asp	Asp	Gly	Ser	Leu	Ala	Phe	Ile	Pro	Asn	Asp	Phe	Ser	Val	Ala
Potato[f]		Leu	Asp	Val	Leu	Leu	Gly	Gly	Asp	Asp	Gly	Ser	Leu	Ala	Phe	Ile	Pro	Gly	Asn	Phe	Ser	Val	Ser
Ch. fusca[g]	Asp	Val	Thr	Val	Lys	Leu	Gly	Ala	Asp	Ser	Gly	Ala	Leu	Val	Phe	Gly	Pro	Ser	Ser	Val	Thr	Ile	Lys
Spinach[h]		Val	Glu	Val	Leu	Leu	Gly	Gly	Gly	Asp	Gly	Ser	Leu	Ala	Phe	(Leu)	Pro	Gly	Asp	Phe	(Ser)	Val	Ala
SPINACH		VAL	GLX	VAL	LEU	LEU	GLY	GLY	GLY	ASX	GLY	SER	LEU	ALA	PHE	LEU VAL	PRO PRO	GLY	ASX	PHE	SER	VAL VAL	ALA PRO
Broad bean		Ala	Gly	Asp	Thr	Ile	Val	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	Glu	Asp
French bean		Ser	Gly	Glu	Lys	Ile	Val	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	Glu	Asp
Elder		Ser	Gly	Glu	Lys	Ile	Thr	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	Glu	Asp
Marrow		Ala	Gly	Glu	Lys	Ile	Val	Phe	Lys	Asn	Asn	Ala	Glu	Phe	Pro	His	Asn	Val	Val	Phe	Asp	Glu	Asp
Potato		Ala	Gly	Glu	Lys	Ile	Thr	Phe	Lys	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Val	Val	Phe	Asp	Glu	Asp
Ch. fusca		Ala	Gly	Glu	Thr	Val	Thr	Trp	Val	Asn	Asn	Ala	Gly	Phe	Pro	His	Asn	Ile	Val	Phe	Asp	Glu	Asp
Spinach		(Ser)	Gly	Glu	Glu	Ile	Val	Phe	Lys	Asn	Asn	Ala	Gly	Phe	(Pro	His	Asn)	Val	Val	Phe	Asp	Glu	Asp
SPINACH		SER	GLY	GLX	GLX	ILE	VAL	PHE	LYS	ASX	ASX	ALA	GLY	PHE	PRO	HIS	(ASX)	VAL	VAL	PHE	ASX	GLX	ASX

[a] Residues in parentheses are uncertain. [b] Ramshaw et al. (1974a). [c] Milne et al. (1974). [d] Scawen et al. (1974). [e] Scawen and Boulter (1974). [f] Ramshaw et al. (1974b). [g] Kelly and Ambler (1974). [h] Scawen et al. (1975).

Gly-Gly, did not appear in the sequences of other plastocyanins. Since a glycyl residue appears in position 7 in some sequences and 8 in others, and Gly-Gly was the only unassigned dipeptide, there was little doubt that it appeared in that sequence. The DAP I/IV digest of the native protein produced nineteen dipeptides, although from homology, 22 dipeptides should have been released. This suggested that (i) a prolyl residue appears in the third position from the new N terminus, presumably in position 39, or (ii) a particular dipeptide or sequence, not previously described, has prevented further digestion, or (iii) the N terminus of the newly formed polypeptide is no longer accessible for further hydrolysis. The first two possibilities may be discounted on the basis of the results obtained from the hydrolysis of the *S*-methylplastocyanin which showed the DAP I/IV mixture to release dipeptides up to positions 43–44. It is not unreasonable to expect the native protein to undergo limited hydrolysis depending upon the availability of the N-terminal residues. In addition, the small amount of Val-Val found in the hydrolysate, which could only come from positions 39–40 in the proposed sequence and the appearance of some Phe-Pro in the digestion of the core peptide, suggested that the digestion was not stopped abruptly as it would be if a nonhydrolyzable prolyl bond was encountered. At this point, it appeared that the core peptide contained several polypeptide chains whose N terminals began with residues 35, 37, and 39. Finally, a double round of the Edman reaction will denature the remaining polypeptide, making the new N terminus accessible to hydrolysis. Thus, a DAP I digest of this polypeptide produced two new dipeptides: Phe-Asp and Glu-Asp, stopping presumably at the prolyl residue which appeared in position 47 of the original protein.

The only dipeptide not identified by GC/MS was His-Asx. The assignment of His and Asx to positions 38 and 39, respectively, was based on an amino acid analysis of the liberated dipeptides (after removal of the core polypeptide) obtained from the DAP I/IV digest of native plastocyanin. The analysis showed one histidyl residue to be present and at least one aspartyl residue unaccounted for. In the other six plastocyanin sequences, both these residues are invariant in those locations in the polypeptide. It was expected that this dipeptide would not be analyzed by GC/MS because extensive work with standards has shown that a dipeptide containing histidine and another polyfunctional residue is not amenable to analysis by gas chromatography, especially at the low sample levels used here.

The only dipeptide identified in the various digests which could not be readily assigned by reason of homology was Val-Pro (see Figures 5 and 6). Since it only appears in these two analyses, it must occur within positions 15–40. The most likely positions for this microheterogeneity would be either or both 15–16 and 21–22. It is relevant to note that in the sequence of elder plastocyanin, both Ile-Pro and Val-Pro were found to occur in positions 15–16.

The sequence of spinach plastocyanin obtained using classical methods is given in Table I. This sequence was in complete agreement with that obtained with the DAP-GC/MS method. The automated sequence, however, was not unambiguous; the identification of five residues in the N-terminal half of the molecule was uncertain as a result of excessive carry-over of the Pth-amino acids. These residues are noted in Table I by parentheses. The present work confirms the published sequence of spinach plastocyanin, especially in regard to the uncertain residues. This is easily seen in the dipeptide analysis given in Figure 6 where the uncertain residue is confirmed by a nearest neighbor present in the dipeptide.

The work presented here demonstrates the utility of the

DAP-GC/MS method in the determination of the primary sequence of proteins. Moreover, the method is rapid especially in that it requires very little fragment peptide purification and uses only very small amounts of sample. The total amount of native protein consumed in these analyses was approximately 85 nmol (~900 μg), a fraction of that which is routinely used by classical methods. Differential hydrolysis using DAP I and DAP I/IV can provide considerable information on the protein sequence because of their complementary specificities. The DAP-GC/MS method serves to provide the protein chemist not only with another tool with which to probe the molecular structure of proteins, but one having unique capabilities because its basic chemistry differs from that of more established techniques. This work supports the expectation that the methods described here based on the use of the DAP enzymes will become a valuable addition to sequence strategy in protein chemistry.

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