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Complete amino acid sequence of poplar plastocyanin *b*

Mitko I. Dimitrov*, Caesii A. Egorov+, Anthony A. Donchev* and Boris P. Atanasov*°

*Central Laboratory of Biophysics, Bulgarian Academy of Sciences, 1040 Sofia, Bulgaria, + Institute of General Genetics N.I. Vavilov, Academy of Sciences of the USSR, 117312 Moscow, USSR and ° Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1040 Sofia, Bulgaria

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A second type of plastocyanin, plastocyanin *b* (PC*b*) was isolated from leaves of poplar *Populus nigra* var. *Italica*. The complete amino acid sequence of 99 residues in the single polypeptide chain of plastocyanin *b* has been determined:

VDVLLGADDG SLAFVPSEFS VPAGEKIVFK NNAGFPHNVL FDEDAVPSGV
DVSKISMSEE DLLNAKGETF EVALSDKGEY TFYCSPHQGA GMVGKVIVN

The sequence obviously demonstrates, that PC*b*, in comparison with the known plastocyanin, plastocyanin *a* (PC*a*), has 12 amino acid replacements (underlined letters): Ile 1→Val, Ile 21-Ser 22-Pro 23→Val-Pro-Ala, Ile 39-Val 40→Val-Leu, Ser 45-Ile 46→ Ala-Val, Ala 52→Val, Asn 76→Asp, Ser 81→Thr and Thr 97→Ile. The replacements at positions 40 and 76 of PC*b* are probably essential for differences in its redox and electrochemical properties, respectively.

Primary structure; Plastocyanin *b*; Plastocyanin *a*; Molecular heterogeneity; (*Populus nigra*)

1. INTRODUCTION

The complete primary structures of plastocyanin from 14 higher plants, 4 green algae and 1 blue-green alga have been determined [1–5]. This type I Cu-protein, whose function is to transfer electrons from cytochrome *f* to the P700 system in chloroplasts, exhibits N-terminal group and amino acid sequence variability [5,6]. However, in the plastocyanin sequences examined from higher plants 45 residue positions are invariant. Some, His 37, Cys 84, His 87 and Met 92, are coordinated to the copper ion and are actually the residues crucial for the electron transferring properties of that protein. Some evidence reported previously [7,8] only implies the probable molecular heterogeneity in a single plant. Our previous investigations revealed that plastocyanin from leaves of poplar, *Populus nigra* var. *Italica*, is represented by two fractions [9]. The previously unknown plastocyanin, which we have designated PC*b*, in contrast to the known plastocyanin (designated PC*a*), exhibits different chromatographic and electrophoretic mobilities, p*I*, N-terminal and amino acid content [9]. We report here the results of the analysis and the complete primary structure of PC*b*.

Correspondence address: M.I. Dimitrov, Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Ul. Acad. G. Bontchev, Bl. 21, 1113, Sofia, Bulgaria

Abbreviations: T, tryptic peptides of PC*b*; ChT, chymotryptic peptides of PC*b*; CB, CNBr cleavage peptides of PC*b*

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

The material for sequencing, a highly purified PC*b*, was prepared from fresh leaves according to

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a modification [9] of the method in [10]. The procedure includes extraction and sedimentation of the proteins in a water-acetone solution followed by 3-fold chromatography of the soluble sediment on Whatman DE-52 anion-exchanger. Two fractions of plastocyanin, PC*a* and PC*b*, were obtained on the last column by isocratic elution with 0.1 M sodium phosphate buffer (pH 6.9) with considerable differences in their chromatographic mobilities (fig.1). The purity index A_{278}/A_{597} in all cases was found to be 1.0–1.2. The purity was additionally identified by isoelectrofocusing and quantitative N-terminal and amino acid analysis.

The enzymes trypsin and α-chymotrypsin were obtained from Merck and used without additional treatments. All solvents and reagents for high-performance liquid chromatography (HPLC) and sequencing were in a set purchased from Applied Biosystems. Double-distilled water, additionally filtered through a Milli-Q system (France), was used. All other chemicals and reagents were of a high degree of purity.

2.2. *Structural analysis*

The sequence of PC*b* was obtained after overlapping and comparison of three sets of peptide fragments, one derived by cleavage at methionyl bonds with CNBr, and the other two by digestion with trypsin and α-chymotrypsin, respectively. The fragments were also aligned using our own N-end partial sequential and compositional data on intact poplar PC*b* and the known primary structure of poplar plastocyanin [2].

Firstly, part of PC*b* was digested with trypsin, both directly [11] and after preliminary carboxymethylation at the Cys residue [12]. Further, CNBr cleavage of PC*b* was carried out [13] after blocking of the Cys SH group by 4-vinylpyridine [14]. Finally, chymotrypsin hydrolysis of the same Cys-vinylpyridylated PC*b* was carried out [15].

All peptides were separated by HPLC on reversed-phase columns, using an Altex HPLC system with a Beckman RP 256-06 column for tryptic peptides and Gilson HPLC system with a Pro-RPC Pharmacia column for CNBr and chymotryptic peptides.

The determination of the N-terminal groups of each of the peptides and the whole PC*b* was performed by manual dansyl-Edman degradation. The amino acid composition of the peptides and PC*b* was determined with a Durrum D-500 automatic amino acid analyzer after hydrolysis with 5.7 N HCl in evacuated tubes at 110°C for 24 h. All peptides and whole PC*b* were sequenced using an Applied Biosystems model 470 A gas-phase sequencer [16].

3. RESULTS

The procedure for preparation of poplar plastocyanins was performed several times during the May-August vegetation period. The ratio between the quantities of PC*a* and PC*b* obtained in the oxidized state varies from 1:1 to 4:1 from the beginning to the end of the indicated period (fig.1).

The N-terminal group determination confirmed [9] that Ile and Val are the N-terminal amino acid residues of PC*a* and PC*b*, respectively. The amino acid analysis also substantiated that PC*a* and PC*b* differ in composition [9] – PC*b* has a higher content of Val and a reduced content of Ile and Ser. Trypsin hydrolysis of PC*a*, resulting in the preparation of seven peptides and the N-terminal and amino acid analysis of these peptides (Dimitrov, M.I., unpublished), together with the results demonstrated above suggested that PC*a* is related to R. Ambler's previously reported plastocyanin structure [2].

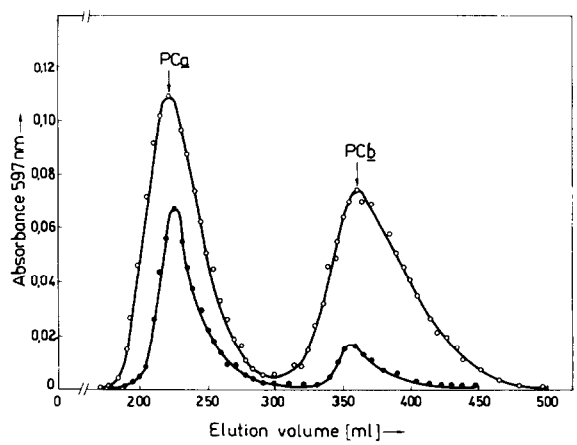

Fig.1. Separation of PC*a* and PC*b* by anion-exchange chromatography on Whatman DE-52 in 0.1 M sodium phosphate buffer (pH 6.9) by isocratic elution. Registration at 597 nm; optical length 0.2 cm. Elution profiles of the two plastocyanin fractions obtained in May (○—○) and June (●—●), respectively.

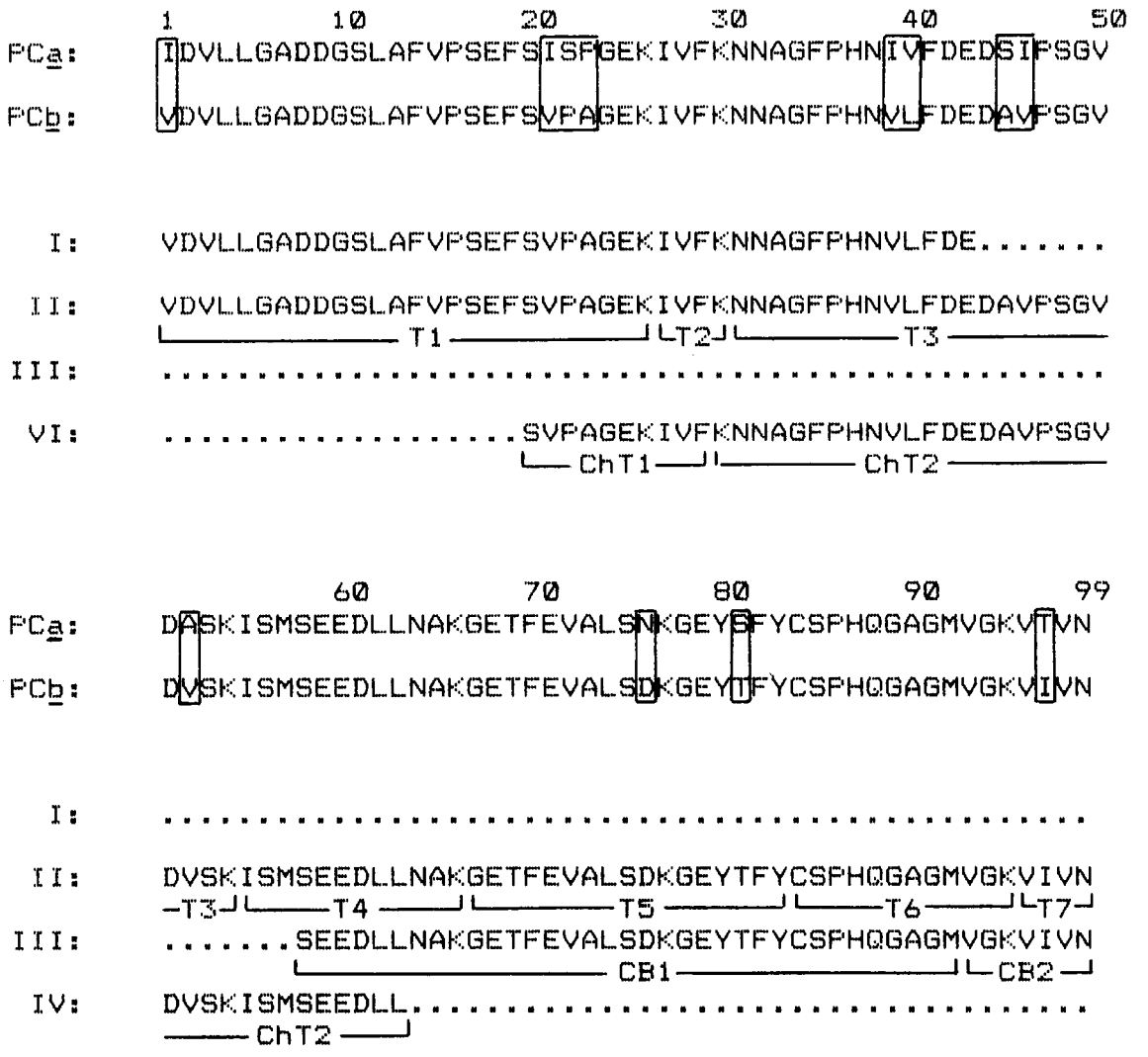

Fig.2. The primary structure of plastocyanin *b* (PC*b*) from poplar *Populus nigra* var. *Italica* and its peptide fragments (see the text) in comparison with the amino acid sequence of plastocyanin *a* (PC*a*) [2].

By total sequencing of the whole PC*b* the sequence of the first 43 amino acid residues was determined (fig.2,I). As a result substitutions at six positions were found on comparison with PC*a*: Ile in PC*a* is replaced by Val in PC*b*, the sequence Ile 21-Ser 22-Pro 23 by Val-Pro-Ala and Ile 39-Val 40 by Val-Leu (fig.2).

After trypsin hydrolysis of PC*b* five peptide fractions were obtained, designated here as T-1, T-2, T-3, T-4 and T-7 (fig.3). It was demonstrated by N-terminal and amino acid analysis that T-1 was in line with the N-terminal peptide Val 1–Lys 26 of PC*a*, T-2 with Ile 27–Lys 30, T-3 with Asn 31–Lys 54, T-4 corresponding to Ile 55–Lys 66 and T-7 being found to be C-terminal Val 96–Asn 99 (fig.2,II). The fragment corresponding to the Gly 67–Lys 95 peptide was not obtained. To find this fragment, carboxymethylation of the Cys-PC*b* residue was performed before trypsin treatment. After HPLC of the hydrolysate, together with the already known T-1, T-2, T-3, T-4 and T-7 peptides, two new peptides, T-5 and T-6, were obtained (fig.4). Their N-terminal and amino acid analysis revealed that they were in line with Gly

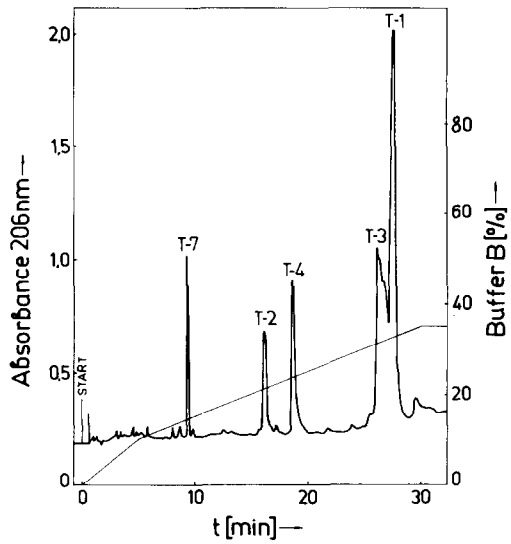

Fig.3. HPLC of trypsin hydrolysate of PC*b*. 0.3 mg were applied to a Beckman RP 256-06 reversed-phase column (ultrasphere ODS 5 μm, 4.6 × 150 mm) and eluted at room temperature with a linear gradient from 10 to 35% solvent B in 25 min. Flow rate, 1.5 ml/min; solvent A – 0.01 M CH_3COONH_4; solvent B – 0.01 M CH_3COONH_4 in 90% acetonitrile. The effluent was measured at 206 and 280 nm (not shown). Peaks were characterized by N-terminal and amino acid analysis.

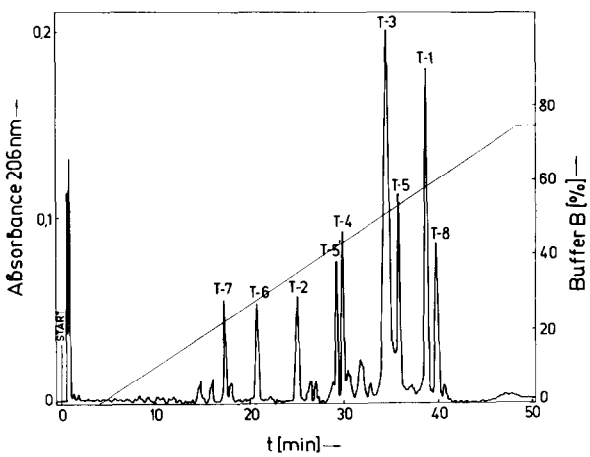

Fig.4. HPLC of trypsin hydrolysate of carboxymethylated PC*b*. A linear gradient from 0 to 75% solvent B in 48 min was used; solvent A – 0.1% trifluoroacetic acid (TFA); solvent B – 0.1% TFA in 70% acetonitrile; other conditions as in fig.3.

67–Tyr 83 and Cys 84–Lys 95 of PC*a*, respectively (fig.2,II). In contrast to the above results (fig.3), another two additional peaks T-5′ and T-8 were found (fig.4). As indicated by the results of the N-terminal and amino acid analysis, the T-5′ fraction was practically identical with the T-5 peptide; these two fractions contained the only two Tyr residues of PC*b*. The sequencing of T-3 revealed another three changes: Ser 45–Ile 46 in PC*a* was replaced by Ala-Val in PC*b* and Ala 52 by Val, respectively (fig.2). Further, there was no change in T-4 (55–66), but there were two changes in the T-5 sequence: Asn 76 in PC*a* was replaced by Asp in PC*b*, and Ser 81 by Thr (fig.2). The amino acid sequence analysis of T-6 did not reveal any changes, whereas the analysis of the last C-terminal peptide T-7 again exhibited a change in PC*b*: Thr 97 in PC*a* is replaced by Ile in PC*b* (fig.2).

In order to overlap the Lys regions, CNBr cleavage of Cys-vinylpyridylated PC*b* was performed (fig.5). Four principal CNBr fractions were obtained: an N-terminal peptide CB-3, a pep-

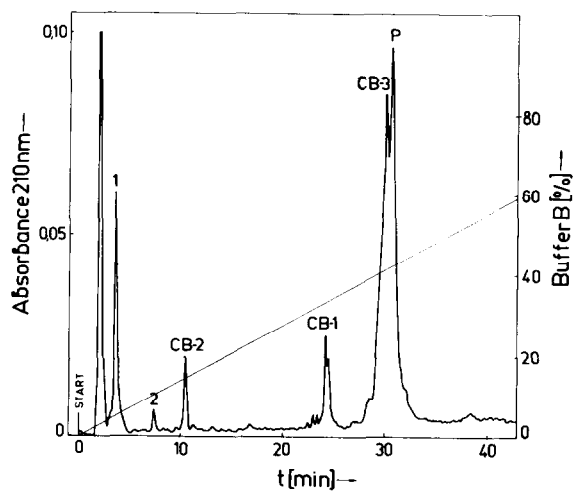

Fig.5. HPLC of CNBr cleavage of vinylpyridylated PC*b*. 0.2 mg were applied to a Pharmacia Pro-RPC reversed-phase column (5 × 100 mm) and eluted at room temperature with a linear gradient from 0 to 60% solvent B in 42 min. Flow rate, 1.5 ml/min; solvent A – 0.1% TFA; solvent B – 0.1% TFA in 85% acetonitrile. The effluent was measured at 210 and 254 nm (4-vinylpyridine absorption maximum, not shown) and characterized as described in fig.3.

tide CB-1 disposed between the only two Met residues of PC*b* and the C-terminal peptide CB-2 together with an uncleaved protein fraction P (fig.5). It was found that peaks 1 and 2 are of non-peptide nature. By sequencing of CB-1 and CB-2 (fig.2,III) overlapping of the Lys 66, Lys 77, Tyr 83 and Lys 95 positions was attained and the replacements established above by Asp 76, Thr 81 and Ile 97 were confirmed (fig.2).

In order to overlap the Lys 26, Lys 30 and Lys 54 positions a chymotrypsin hydrolysis of vinylpyridylated PC*b* was performed. It was found that the ChT-2 peptide corresponds to the Lys 30–Phe 70 amino acid sequence of PC*a* (fig.2,IV). By sequencing of this peptide to the 34th amino acid residue overlapping of the Lys 30 and Lys 54 positions was achieved and the replacements mentioned above Val 39-Leu 40, Ala 45-Val 46 and Val 52 were confirmed (fig.2). The sequence of the ChT-1 peptide (fig.2,IV) overlapped the Lys 26 position and confirmed the replacements Val 21-Pro 22-Ala 23.

4. DISCUSSION

The obviously different chromatographic properties on Whatman DE-52 of the two plastocyanin fractions were the first indication that two types of plastocyanin, PC*a* and PC*b*, are available in *P. nigra* [9]. The N-terminal and amino acid analysis indicated that PC*a* and PC*b* are chemically different [9]. On the other hand, additional investigations, including trypsin hydrolysis of PC*a* and N-terminal and amino acid analysis of the resulting peptides, suggested that PC*a* is related to the plastocyanin structure of R. Ambler.

It is not surprising that trypsin hydrolysis of uncarboxymethylated PC*b* does not yield the Gly 67–Lys 95 peptide (fig.2). Probably this is due to the formation of intrapeptide -S-S- derivatives which are usually strongly absorbed on the chromatographic column. This obstacle was overcome by carboxymethylation of the Cys-PC*b* residue before trypsin treatment, despite two extraordinary peptides, T-5′ and T-8, being found (fig.4). The T-5′ and T-5 peptides are practically identical in composition. The more hydrophilic properties of T-5′ are probably due to the secondary chemical reactions of Tyr iodination during the carboxymethylation of PC*b* by iodoacetic acid. T-8 was found to be a large polypeptide derivative probably due to incomplete trypsin hydrolysis of PC*b* because of the shorter period of enzymatic treatment. The question arises as to why T-5 does not end in Lys, as is logically the case in tryptic hydrolysis, but in Tyr (fig.2). Probably this is due to the negatively charged Asp 76 disposed together with Lys 77, in contrast to PC*a*, where position 76 is occupied by the uncharged Asn. Perhaps the charge of Asp is an obstacle to trypsin in giving rise to effective binding to the S_2 site of this peptide region and in hydrolyzing the peptide bond after Lys 77. A trace of a chymotrypsin admixture probably hydrolyzed the bond after Tyr 83.

The present data indicate that PC*b* is not identical with PC*a* [2], but differs at 12 amino acid positions (12%). The replacement Asn 76 ⟶ Asp is most essential for the electrochemical properties of PC*b*. Correspondingly, the p*I* values of the two proteins differ by 0.05 pH units as PC*b* is the more acidic fraction [9]. At four positions, 1, 21, 39 and 46, the bulkier Ile in PC*a* is replaced by Val in PC*b* and only in positions 52 and 97 are the smaller residues of Ala and Thr replaced by Val and Ile, respectively. The sequence Ser 22-Pro 23 in PC*a* is reversed with respect to the Pro residue in Pro 22-Ala 23 of PC*b*. This change will undoubtedly alter the conformation of the polypeptide chain in this Pro region. The replacements Ser 22 ⟶ Ala 23, Ser 45 ⟶ Ala, Thr 97 ⟶ Ile as well as the less nonpolar Ala 52 ⟶ Val and Ser 81 ⟶ Thr indicate that PC*a* contains more polar amino acid residues at these positions than PC*b*. The hydrophobic interactions in PC*b* are probably stronger and it is more stable than PC*a*. Although the majority of changes are 'neutral', the replacement Val 40 ⟶ Leu could be essential in the subtle alteration and/or local molecular dynamics of the closely located Cys 84 from the active site. It could be reflected as a change of the redox properties of PC*b*.

The functional role and biological significance of PC*a* and PC*b* with some different physicochemical properties are still unclear. Obviously the microheterogeneity of the primary structure is a common property for these electron carriers [7,8] and the presence of two plastocyanin types is probably functionally important [9]. Investigations to elucidate these relationships are now in progress.

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REFERENCES

[1] Boulter, D., Haslett, B.G., Peacock, D., Ramshaw, J.A.M. and Scawen, M.D. (1977) in: Plant Biochemistry II (Northcote, D.N. ed.) vol.13, pp.1040–1048, University Park Press, Baltimore.
[2] Guss, G.M and Freeman, H.C. (1983) J. Mol. Biol. 169, 521–563.
[3] Ramshaw, J.A.M. (1982) in: Nucleic Acids and Proteins in Plants I, Encyclopedia of Plant Physiology (Boulter, D. and Parthier, B. eds) vol.14A, pp.229–240, Springer, Berlin.
[4] Simpson, R.J., Moritz, R.L., Nice, E.C., Grego, B., Yoshizaki, F., Sugimura, Y., Freeman, H.C. and Murata, M. (1986) Eur. J. Biochem. 157, 497–506.
[5] Sykes, A.G. (1985) Chem. Soc. Rev. 14, 283–315.
[6] Lappin, G.A. (1981) Metal Ions Biol. Syst. 13, 15–71.
[7] Haslett, B.G., Gleaves, J.T. and Boulter, D. (1977) Phytochemistry 16, 363–365.
[8] Boulter, D., Gleaves, J.T., Haslett, B.G., Peacock, D. and Jensen, U. (1978) Phytochemistry 17, 1585–1589.
[9] Donchev, A.A., Dimitrov, M.I. and Atanasov, B.P. (1987) CR Acad. Bulg. Sci., in press.
[10] Plesničar, M. and Bendall, D.S. (1970) Biochim. Biophys. Acta 216, 192–199.
[11] Walsh, K.A. (1970) Methods Enzymol. 19, 41–63.
[12] Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem. 238, 622–627.
[13] Gross, E. and Witkop, B. (1961) J. Am. Soc. 83, 1510–1511.
[14] Friedman, M., Krull, L.H. and Cavins, J.F. (1970) J. Biol. Chem. 245, 3868–3871.
[15] Wilcox, P.E. (1970) Methods Enzymol. 19, 64–108.
[16] Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J. and Hood, L.E. (1983) Methods Enzymol. 91, 399–413.