Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 229-236

ISOLATION AND CHARACTERIZATION OF THE 1 to 49 AMINO-TERMINAL SEQUENCE OF PRO-OPIOMELANOCORTIN FROM BOVINE POSTERIOR PITUITARIES

H.P.J. Bennett

Endocrine Laboratory, 687 Pine Ave.West, Royal Victoria Hospital, Montreal, Quebec, Canada, H3A 1A1
and Department of Medicine, McGill University, Montreal, Quebec, Canada

Received October 9, 1984

An amino-terminal fragment of pro-opiomelanocortin (POMC) has been isolated from bovine intermediate pituitaries by reversed-phase high-performance liquid chromatography. Peptide mapping and amino acid analysis indicated that the primary sequence corresponds exactly to that predicted by cDNA techniques for the first 49 residues of POMC including the presence of four cysteine residues. This peptide is almost certainly generated together with the γ-melanotropins during the biosynthetic processing of the 16K amino-terminal fragment of bovine POMC. Careful analysis of the fragments resulting from V_8 protease and trypsin digestion has permitted assignment of cystine bridges between residues 2 and 24 and between residues 8 and 20.

Pro-opiomelanocortin (POMC) is a multi-hormone precursor which consists of various domains connected by pairs of basic amino acid residues (see 1 and 2 for reviews). The best understood of the biosynthetic products is adrenocorticotropin (ACTH) which lies in the central portion of POMC. β-Lipotropin (β-LPH) comprises the sequence immediately to the carboxyl-terminal side of ACTH, while the so-called 16K fragment constitutes the amino-terminal region. An acidic joining peptide links the 16K fragment to ACTH. It is now accepted that within the anterior pituitary, the major biosynthetic products are the 16K fragment, joining peptide, ACTH and β-LPH. In the intermediate pituitary biosynthetic processing is more complete. While processing of ACTH and β-LPH to their respective components is essentially complete, it is known that 16K fragment is only partially processed. Towards the carboxyl-terminus of 16K fragment there is a third melanotropin-like sequence (γ-MSH). Various

Abbreviations: POMC, pro-opiomelanocortin, ACTH, adrenocorticotropin; β-LPH, β-lipotropin; 16K fragment, 16,000 Dalton fragment of POMC; γ-MSH, γ-melanotropin; RP-HPLC, reversed-phase high performance liquid chromatography; ODS-silica, octadecylsilyl-silica; TFA, trifluoroacetic acid.

0006-291X/84 $1.50

molecular forms of γ-MSH have been identified. They all share the novel feature of retaining a lysine residue at their amino-termini (3,4,5). This constitutes one half of an original arginyl$_{49}$-lysine$_{50}$ sequence which is the point of cleavage of the 16K fragment. The remaining portion of the 16K fragment, presumably comprising the first 49 residues, has been detected in extracts of rat intermediate pituitaries (6,7) but has never been characterized by amino acid analysis and peptide mapping techniques. This paper reports the isolation of the 1-49 portion of the 16K fragment of POMC from bovine posterior pituitaries. Peptide mapping using reversed-phase high performance liquid chromatography (RP-HPLC) permitted the correct assignment of the cystine bridges.

MATERIALS AND METHODS

Tissue extraction and peptide purification:

Eighty lyophilysed bovine posterior pituitaries (5.3 g dry weight, Pel-Freez Biologicals, Rogers, Arkansas, U.S.A.) were defatted as described previously (8). The pituitary powder (4.2 g) was homogenized in 100 ml of an acidic extraction medium as described previously (9). Following centrifugation, the pellet was re-extracted with another 100 ml of extraction medium. The combined supernatants (i.e. 200 ml) were subjected to repeated batch reversed-phase extraction using a total of 10 octadecylsilyl-silica (ODS-silica) cartridges (C_{18} Sep-Pak cartridges, Waters Associates) as described previously. The eluates from the 10 ODS-silica cartridges were combined in a 100 ml conical flask (total volume 45 ml of 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA)) and the volume reduced to approximately 8.0 ml by removal of the acetonitrile. The pH of the aqueous residue was raised to approximately 5.5 by drop-wise addition of 10N sodium hydroxide. This initial peptide enriched extract (approximately 50 mM in terms of sodium ions) was further fractionated by batch anion-exchange extraction using a BIO-SIL TSK DEAE-3SW HPLC column as follows. The anion-exchange column was first equilibrated with 50 mM ammonium acetate (pH5.5) and then the extract was pumped directly onto the column using one of the HPLC pumps. The column was then washed with 50 ml of 50 mM ammonium acetate (pH5.5) and finally eluted with 20 ml of 1 M ammonium acetate (pH 5.5). The above operations were performed at a flow rate of 2 ml per minute. The pH of the anion-exchange eluate was reduced to approximately 2 by drop-wise addition of TFA and then applied to two C_{18} μBondapak RP-HPLC columns (Waters Associates) connected in series essentially as described previously (9). The column was eluted with a linear of gradient of aqueous acetonitrile containing 0.1% TFA throughout. Fractions containing the 16K fragment were subjected to sequential repurification steps using solvents containing 0.13% heptafluorobutyric acid, 0.01M triethylamine acetate (pH 5.5) and finally 0.1% TFA exactly as described previously for other pituitary peptides (10).

Characaterization of bovine 16K fragment (1-49):

Trypsin (Type XI, DPCC treated, Sigma), α-chymotrypsin (Type VII TLCK treated, Sigma) and V_8 protease (S.aureus, Miles Laboratories) digestions were performed at 37°C in 200 μl of 50mM ammonium bicarbonate buffer (pH 8.0) which

had previously been saturated with argon. Separations of peptide fragments were performed using a Waters C_{18} μBondapak column eluted with linear gradients of 1.6% to 48% acetonitrile containing 0.1% TFA throughout over 1 hour at a flow rate of 1.5 ml per minute. Amino acid analyses were done using a Beckman 6300 series autoanalyzer. Tryptophan was determined following chymotryptic digest of 1 nmole of 16K fragment (12 hours digest with enzyme to peptide ratio of 1:20 by weight). The digestion products were analyzed by RP-HPLC using a linear gradient of from 1.6% to 21.6% acetonitrile over 30 minutes at 1.5 ml per minute. Under these conditions tryptophan eluted at 18 minutes. Tryptophan released during enzymatic digestion was determined by comparing the peak area (214 nm) with that of the standard.

RESULTS

Following batch extraction of bovine posterior pituitaries by ODS-silica and DEAE-silica a fraction enriched in acidic peptides was obtained. The RP-HPLC of this material revealed a chromatogram dominated by neurophysin and acidic peptides derived from bovine POMC (Fig. 1). Experience with the HPLC fractionation of rat neurointermediate pituitaries (11) indicated that the 1-49 sequence of 16K fragment should have an elution position corresponding to approximately 37% acetonitrile. A large amount of material with this characteristic eluted between 108 and 117 minutes (Fig. 1) and was purified (see

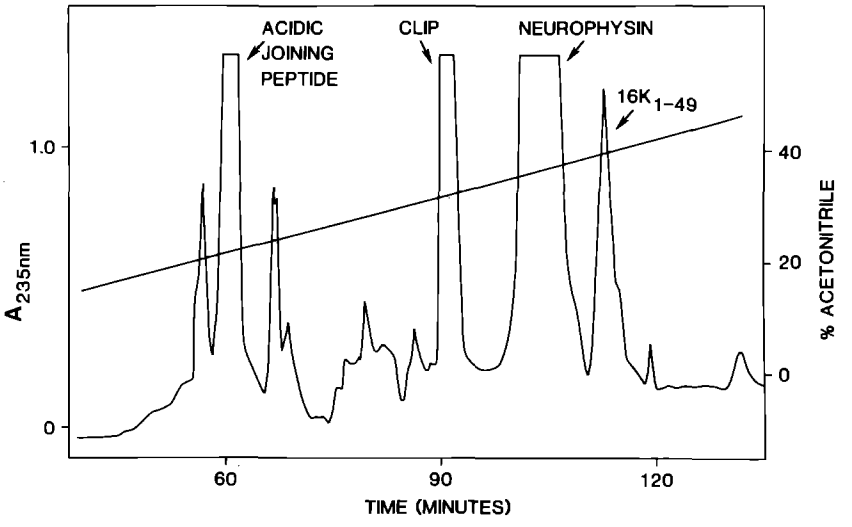

Figure 1: Initial reversed-phase HPLC of acidic peptides extracted from 80 bovine posterior pituitaries. Two C_{18} μBondapak columns connected in series were eluted over 3 hours with a gradient of 1.6% to 61.6% acetonitrile containing 0.1%TFA throughout at a flow rate of 1.5 ml per minute. Fragments of bovine POMC (12) were identified by amino acid analysis as acidic joining peptide, corticotropin-like intermediate lobe peptide (CLIP) and the 1-49 sequence of 16K fragment ($16K_{1-49}$). The 16K fragment was repurified as outlined in the text and subjected to V_8 protease/trypsin digestion as shown in Figure 2.

Table 1. Amino acid compositions* of bovine 16K fragment (1-49) and of its V_8 protease/trypsin (VT) peptides

Peptide	POMC 1-49	VT1 (5-14 +15-22)	VT2 (1-4 +23-49)	VT1A (5-14)	VT1B (15-22)	VT2A (1-4)	VT2B (1-4)	VT2C (23-49)	VT2D (23-49)
$CysO_3$	-	1.5(2)	1.7(2)	0.6(1)	0.7(1)	0.2(1)	0(0)	0.3(1)	0(0)
Asx	5.7(6)	1.7(2)	3.2(4)	0.9(1)	1.0(1)	0(0)	0(0)	3.9(4)	4.0(4)
Thr	3.7(4)	1.7(2)	1.3(2)	1.9(2)	0(0)	0(0)	0(0)	2.2(2)	2.0(2)
Ser	2.2(3)	0.9(3)	0.4(1)	1.0(2)	0.7(1)	0(0)	0(0)	0.6(1)	0.5(1)
Glx	8.6(8)	3.1(3)	5.0(5)	3.0(3)	0(0)	1.0(1)	1.0(1)	4.0(4)	4.2(4)
Pro	4.8(5)	0(0)	4.5(5)	0(0)	0(0)	0(0)	0(0)	5.0(5)	4.9(5)
Gly	1.8(2)	0(0)	1.2(2)	0(0)	0(0)	0(0)	0(0)	1.4(2)	1.6(2)
Ala	2.9(3)	0.8(1)	1.6(2)	0(0)	0.9(1)	0(0)	0(0)	1.6(2)	1.7(2)
½Cys	2.3(4)	-	-	-	-	0(0)	0.2(1)	0(0)	0.6(1)
Val	1.1(1)	0(0)	0.8(1)	0(0)	0(0)	0(0)	0(0)	0.9(1)	1.1(1)
Ile	1.0(1)	0.9(1)	0(0)	0(0)	0.8(1)	0(0)	0(0)	0(0)	0(0)
Leu	6.7(6)	3.2(3)	3.1(3)	0.9(1)	2.2(2)	1.0(1)	1.0(1)	2.0(2)	2.0(2)
Phe	1.0(1)	0(0)	0.8(1)	0(0)	0(0)	0(0)	0(0)	1.1(1)	0.8(1)
Lys	0.9(1)	0(0)	0.9(1)	0(0)	0(0)	0(0)	0(0)	0.8(1)	1.0(1)
Arg	1.9(2)	0.8(1)	0.9(1)	0(0)	0.9(1)	0(0)	0(0)	1.0(1)	0.9(1)
Trp	1.1**(1)	ND(0)	ND(1)	ND(0)	ND(0)	ND(1)	ND(1)	ND(0)	ND(0)

*Amino acid analyses were performed after hydrolysis in vacuo in constant boiling HCl for 48 hrs at 110°C. The expected values according to ref. 12 are shown in parenthesis. Values for methionine, tyrosine and histidine were zero throughout. Serine and threonine values have not been corrected for losses during hydrolysis.
**Tryptophan was estimated by reversed-phase HPLC as described in the text.
ND -not determined due to destruction during hydrolysis.

Methods). The amino acid composition closely resembled that predicted for the 1-49 sequence of bovine 16K fragment by cDNA techniques (12) (Table 1). The yield of this 16K fragment from 80 posterior pituitaries was approximately 150 nmoles or 800 μg. To determine the cystine bridge structure, this 16K fragment was digested with a mixture of V_8 protease and trypsin in order to cleave the peptide at three strategic points namely, $-Glu_4-Ser_5-$ and $-Glu_{14}-Ser_{15}-$ (both V_8 protease sites) and $-Arg_{22}-Ala_{23}-$ (a trypsin site). The resulting RP-HPLC peptide map is shown in Fig. 2. The amino acid analyses of the two major fragments VT1 and VT2 are shown in Table 1. It was clear from these results that the 5-14 sequence was linked to the 15-22 sequence (VT1) and the 1-4 sequence was linked to the 23-49 sequence (VT2). To confirm these findings the cystine bridge in each fragment was cleaved. VT1 was subjected to performic acid oxidation and then re-analyzed by reversed-phase HPLC (Fig. 3 top). Fragments VT1A and VT1B were observed whose amino acid compositions (Table 1) corresponded exactly to that expected for the sequences predicted for fragments 5 to 14 and 15 to 22 respectively (Fig. 4). Performic

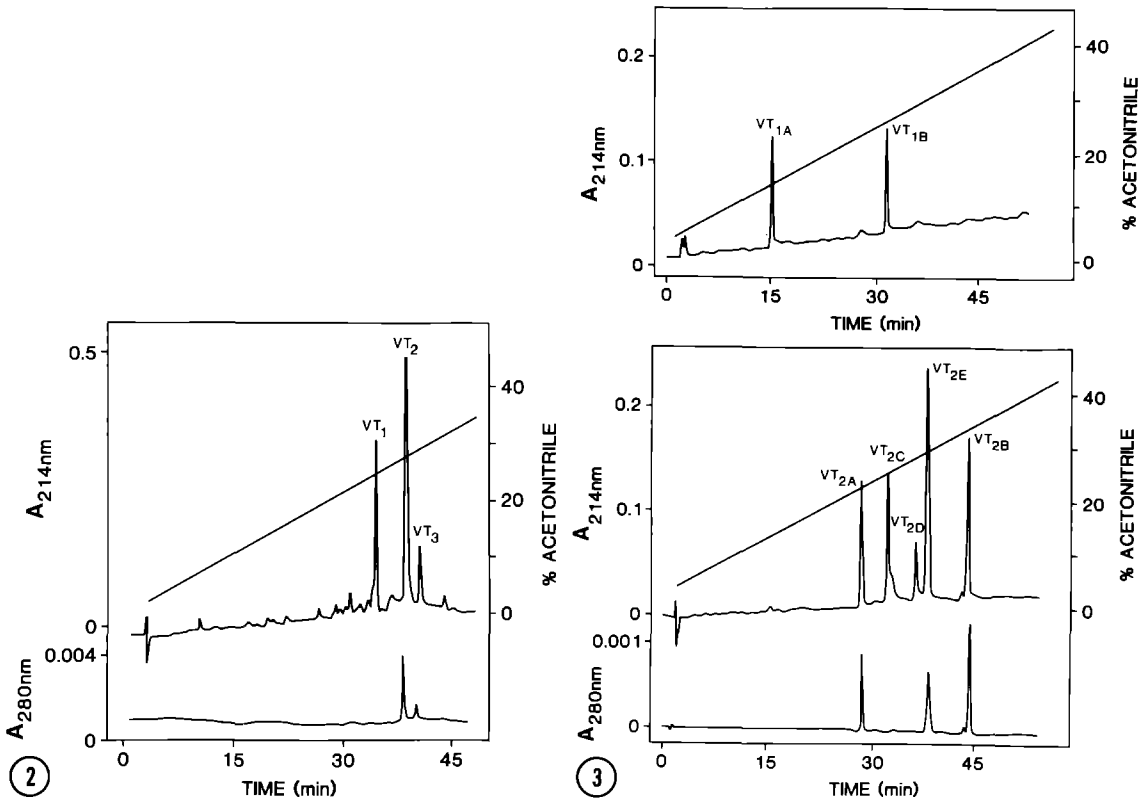

Figure 2: Separation of fragments derived from the 16K fragment by digestion with V_8 protease and trypsin. 30 nmoles (i.e. approximately 150 µg) of peptide was incubated with 50 µg V_8 protease and 25 µg of trypsin for two hours in 200 µl of 50 mM ammonium bicarbonate buffer (saturated with argon) at 37°C. Digestion products were separated by reversed-phase HPLC using a linear gradient of aqueous acetonitrile containing 0.1% TFA throughout. The amino acid compositions for fragments VT1 and VT2 are shown in Table 1. Fragment VT3 was identical in composistion to CT_2 but for the apparent absence of the carboxyl-terminal sequence 45-49. Thus VT3 is a minor chymotryptic cleavage product. All other peaks were present in an enzyme blank.

Figure 3 (top): Separation of the two peptides comprising fragment VT1 derived from a V_8 protease/trypsin digest of the purified 16K fragment (Figure 2). VT1 was subjected to performic acid oxidation and the products separated by reversed-phase HPLC using a linear gradient of aqueous acetonitrile containing 0.1% TFA throughout. The amino acid compositions for fragments VT1A and VT1B are shown in Table 1.

(bottom): Separation of the peptides comprising fragment VT_2 derived from a V_8 protease/trypsin digest of the purified 16K fragment (Figure 2). VT2 was incubated in 200 µl of 50 mM ammonium bicarbonate buffer for 14 hours at 37°C and the products separated by reversed-phase HPLC using a linear gradient of aqueous acetonitrile containing 0.1% TFA throughout. Fragments VT2 A to E were subjected amino acid analysis (Table 1).

acid oxidation was not suitable for VT2 since it contained tryptophan. VT2 was instead incubated in buffer saturated with oxygen since the cystine bridge tends to spontaneously cleave under these conditions generating a mixture of fragments containing either cysteine or cysteic acid. The fragments generated

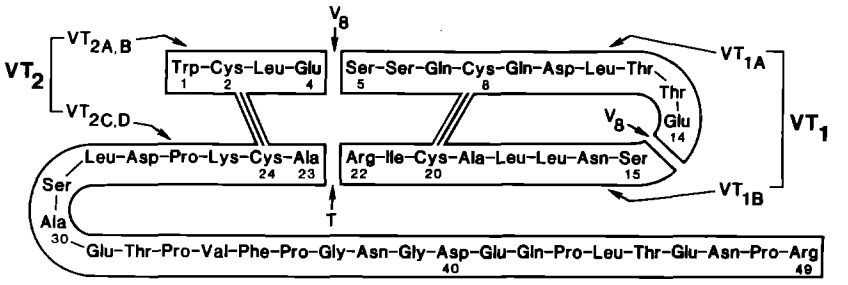

Figure 4: V_8 protease/trypsin peptide map of the purified 16K fragment. The amino acid compositions of the parent peptide and its various fragments are consistent with the 1 to 49 sequence of bovine POMC predicted by cDNA techniques (12). The results clearly assign cystine bridges between 2 and 24 and between 8 and 20. V_8,T: Cleavage sites of V_8 protease and trypsin respectively. ↓ ↓

from VT2 were analyzed by reversed-phase HPLC (Fig. 3 bottom). In addition to the parent peptide VT2E, four fragments were observed VT2A,B,C and D. Amino acid analysis (Table 1) revealed that VT2A and B corresponded to the oxidized and reduced forms of the sequence 1-4 respectively and VT2C and D corresponded to the oxidized and reduced form of the sequence 23-49 respectively.

DISCUSSION

The 16K fragment of POMC has been purified from human and porcine anterior pituitaries and characterized by peptide mapping and sequence analysis (13,14). In addition the 16K fragment from the mouse anterior pituitary cell line has been partially characterized using a micro-sequencing approach (15). In each instance the observed primary structure corresponded exactly to that predicted by cDNA techniques. Novel extended and shortened forms of the 16K fragment have recently been isolated from porcine anterior pituitary (16). Several forms of γ-MSH have been isolated from rat and bovine intermediate pituitaries (3,4,5). These findings indicate that a form of 16K fragment consisting of 49 residues should be produced during the biosynthesis of γ-MSH. Several studies, most notably those by Mains and Eipper (5) and Jackson et al (6) have indicated that such a peptide is to be found in rat intermediate pituitary tissue. This communication reports the successful isolation of such a peptide from bovine neurointermediate pituitaries. Amino acid analysis indicated that the amino-terminal tryptophan is not acetylated

and that the carboxyl-terminal arginine remains intact following cleavage of the γ-MSH sequence (Table 1). No evidence for amino-sugars was observed in the amino acid analysis of the purified peptide. For peptide mapping we incubated the purified 16K fragment with a mixture of V_8 protease and trypsin since only two fragments are generated each consisting of two peptides linked by a cystine bridge (Fig.4). This approach proved successful (Fig. 2). We were able to take the analysis one step further by cleaving the cystine bridges and separating the products by reversed-phase HPLC (Fig. 3). In the case of the second fragment generated during V_8 protease/trypsin digestion (i.e. VT2) we took advantage of the fact that cystine bridges can break spontaneously in ammonium bicarbonate buffer (Fig. 3 bottom). The evidence generated in this study suggests that the sulphur bridging in the 1-49 sequence of 16K fragment isolated from bovine posterior pituitaries is as illustrated in Figure 4 (i.e. linking cysteine residues 2 and 20 and cysteine residues 8 and 24).

The difference in cystine bridge orientation observed in this study of an intermediate lobe 16K fragment, and those of Seidah et al (12) who were studying an anterior lobe 16K fragment may reflect a true difference in the structures of these otherwise closely related peptides. However, Kawauchi et al (17) have found that an amino-terminal 16K fragment of POMC isolated from salmon pituitaries has a cystine bridge structure analagous to that found in this study. Our experience has suggested that ambiguous results can be generated by cleavage of cystine bridges in buffers used for peptidase digestion. It is important to clarify this issue especially since the 1-49 sequence of 16K fragment has been suggested to be an adrenal mitogen (18).

ACKNOWLEDGEMENTS

The author would like to thank Dr. S. Solomon for helpful discussions and support, Susan James and Isabel Lehmann for excellent technical assistance, Chris Passier for performing the amino acid analyses and Deborah Hartley and Sherville Walrond for preparing the manuscript. This work was supported by U.S. Public Health Grant HDO 4365, Medical Research Council of Canada grant MT 6733 and a scholarship from the Fonds de la recherche en santé du Québec.

REFERENCES

1. Eipper, B.A. and Mains, R.E. (1980) Endocrine Reviews, 1, 1-27.
2. Chrétien, M. and Seidah, N.G. (1981) Mol. Cell. Biochem., 34, 101-127.
3. Browne, C.A., Bennett, H.P.J. and Solomon, S. (1981), Biochem. Biophys. Res. Comm., 100, 336-343.
4. Böhlen, P., Esch, F., Shibasaki, T., Baird, A., Ling, N. and Guillemin, R. (1981) FEBS Letters, 128, 67-70.
5. Hammond, G.L., Chung, L. and Li, C.H. (1982), Biochem. Biophys. Res. Comm., 108, 118-123.
6. Mains, R.E.. and Eipper, B.A. (1979) J. Biol. Chem., 254, 7885-7894.
7. Jackson, S., Salacinski, P., Hope, J. and Lowry, P.J. (1983) Peptides, 4, 431-438.
8. Brubaker, P.L., Bennett, H.P.J., Baird, A.C. and Solomon, S. (1980) Biochem. Biophys. Res. Comm., 96, 1441-1448.
9. Bennett, H.P.J., Browne, C.A. and Solomon, S. (1981) Biochemistry, 20, 4530-4538.
10. Bennett, H.P.J. (1983) J. Chromatogr., 266, 501-510.
11. Browne, C.A., Bennett, H.P.J. and Solomon, S. (1982) Anal. Biochem., 124, 201-208.
12. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature, 278, 423-427.
13. Seidah, N.G., Rochemont, J., Hamelin, J., Lis. M. and Chrétien, M. (1981) J. Biol. Chem., 7977-7984.
14. Larivière, N., Seidah, N.G. and Chrétien, M. (1981) Int. J. Pept. Protein Res., 18, 487-491.
15. Bioleau, G., Larivière, N., Hsi, K.-L., Seidah, N.G. and Chrétien, M. (1982) Biochemistry, 21, 5341-5346.
16. Keutmann, H.T., Lampmann, G.W., Mains, R.E. and Eipper, B.A. (1981) Biochemistry, 20, 4148-4155.
17. Kawauchi, H., Takahashi, A. and Abe, K. (1981) Int. J. Pept. Protein Res., 18, 223-227.
18. Lowry, P.J., Silas, L., McLean, C., Linton, E.A. and Estivariz, F.E. (1983) Nature, 306, 70-73.