Complete amino acid sequence of human seminal plasma β-inhibin

Prediction of post Gln-Arg cleavage as a maturation site

N.G. Seidah, N.J. Arbatti+,*, J. Rochemont, A.R. Sheth* and M. Chrétien

Laboratories of Biochemical and Molecular Neuroendocrinology, +Laboratory of Reproduction Research, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Canada H2W 1R7 and **Institute for Research in Reproduction (ICMR), Bombay, India*

Received 20 June 1984

The complete sequence of a 94 amino acid human seminal plasma polypeptide exhibiting inhibin-like activity is presented. This molecule, called β-inhibin, selectively and specifically suppresses the release of pituitary FSH in vivo as well as in vitro. It does not affect the secretion of LH. Such a novel acidic protein contains a very basic C-terminal segment which is easily cleaved by mild tryptic digestion. It is predicted that the FSH inhibiting activity may reside within this region of the molecule. This would imply a post Gln-Arg cleavage to release the basic C-terminal active moiety.

β-Inhibin *Pituitary FSH release* *Amino acid sequence* *Human seminal plasma*

1. INTRODUCTION

Many peptides can be found in extracts of seminal plasma and gonads. Among them is a rat 90 amino acid basic polypeptide, SVS-IV [1], whose secretion is stimulated by testosterone [2]. The complete amino acid sequence of another seminal plasma 109 amino acid protein, PDC-109, has been recently reported [3]. There is now ample evidence that the secretion of follicle stimulating hormone (FSH) by the anterior lobe of the pituitary gland is regulated by a non-steroidal hormone of gonadal origin, termed 'inhibin' [4–6]. Inhibin-like activity has been found to be associated with peptides of various sizes, depending on the purification methods used and sources of extraction from gonads and their secretions from different species [7–11]. Human seminal plasma is also found to contain polypeptides of varying molecular masses, exhibiting inhibin-like activity [12–14]. Recently, the structure [15] and activity [16] of a 31 amino acid human inhibin-like peptide and its synthetic replica have been described. The purification and partial N-terminal 30-residue sequence of yet another high molecular mass human seminal plasma polypeptide with inhibin-like activity has been reported [17].

We present the complete amino acid sequence of this latter human protein, termed β-inhibin, and report on its biological activity both in vitro and in vivo.

2. MATERIALS AND METHODS

2.1. *Purification of human β-inhibin*

The isolation and purification of the polypeptide sequences in this study have been reported earlier [17]. The final purification was achieved by reverse-phase high performance liquid chromatography (HPLC) using a μ-Bondapak C_{18} column (Waters) eluted with an acetonitrile/heptafluorobutyric acid (HFBA) gradient as reported elsewhere [18].

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

2.2. *Amino acid analysis and sequencing procedure*

Amino acid analyses of the reduced and carboxymethylated peptides were done in duplicate following hydrolysis in 5.7 M HCl in vacuo at 110°C for 24, 48 and 72 h.

Automatic amino-terminal Edman degradation of the reduced and carboxymethylated peptides was performed using 0.33 M Quadrol program on a Beckman 890C sequenator equipped with a Sequemat P6 autoconverter and a model SC-510 controller. Prior to sequencing, 3 precycles were run with 3 mg Polybrene (Aldrich) as a carrier. Modifications of the running order of cycles included a double coupling on cycle 1, and double cleavages at all suspected proline residues. Furthermore, to eliminate minor contaminating sequences and diminish background accumulation, *o*-phthalaldehyde blocking at certain proline residues was done [19]. Phenylthiohydantoin (PTH) amino acids were identified and quantitated by HPLC on a Varian LC5500/Vista 402 system as in [20,21].

2.3. *Tryptic, chymotryptic and CNBr digestion*

The reduced and carboxymethylated β-inhibin was digested at 37°C for 3 h with HPLC-purified trypsin [22], using an enzyme/substrate weight ratio of 1:20 in 0.1 M Na_2HPO_4, pH 8.5. The peptides were purified by HPLC as in [18]. Similarly, chymotrypsin digestion was performed with HPLC-purified enzyme. CNBr digestion was found to be more efficient on the native rather than the carboxymethylated β-inhibin. This was performed at room temperature, in the dark, in 70% formic acid for 18 h. The mole ratio of CNBr to methionine used was 500:1. Following lyophilization, the mixture was reduced and carboxymethylated, then desalted and purified by HPLC.

2.4. *Bioassays*

The biological activity of β-inhibin was measured by its specific suppression of FSH release, both in vitro [23], and in vivo [12].

2.4.1. In vitro mouse pituitary assay [23]

Whole pituitaries of 20-day old mice were preincubated for 30 min in Dulbecco's Modified Eagle's medium (DME) at pH 7.5, oxygenated with 95% O_2–5% CO_2 at 37°C. After the preincubation period, the medium was replaced by 500 μl of fresh DME in the absence (control) or presence of various doses of β-inhibin (calculated by quantitative amino acid composition). After 1 h of incubation, 500 μl of DME (control) or 500 μl of DME containing 3 ng of LH-RH was added to each tube. Incubation proceeded for another 3 h, after which the concentration of FSH and LH in the medium were quantitated by a radioreceptor assay [23].

2.4.2. In vivo castrated adult male rat assay [12]

Holtzman male rats (90-day-old) were bilaterally castrated, two weeks prior to assay. The animals were injected intramuscularly once daily for 3 days with either 0.1 ml saline (control) or 0.1 ml containing various doses of β-inhibin. 4 h after the last injection, the animals were bled and their sera collected. The plasma levels of FSH and LH were determined by RIA using NIAMDD-rat-FSH-RP-1 and NIAMDD-rat-LH-RP-1, respectively.

3. RESULTS

3.1. *Complete sequence determination*

The amino acid analysis of the purified β-inhibin is shown in table 1, together with that of two tryptic fragments obtained. It can be seen that the molecule seems to be composed of about 87–89 amino acids, excluding tryptophan. Noteworthy is the absence of alanine in this molecule.

The results of the N-terminal sequence of the first 81 amino acids are presented in fig.1A. The initial yield of this sequence was around 17 nmol, and the overall repetitive yield, obtained from the linear regression line, was calculated to be 95.07%. Although this peptide was purified at least 3 times on HPLC, it was found that 2 contaminating sequences were present. One of them was later identified as the segment 83–94 of the complete molecule (8% contamination). The other minor contaminant (3%) was of unknown sequence. Neither contaminant contained proline at position 6, and hence both were eliminated following the *o*-phthalaldehyde block. This sequence was repeated twice and it was found that unless a double coupling/cleavage was done in cycles 35 and 36, a major problem would occur causing a drastic increase in the carryover from cycle to cycle. This strategy was also used for cycles 62, 63, where the same pro-

Table 1

Amino acid	β-Inhibin			Tryptic segments				
	24 h	48 h	72 h	Sequence	67–94[b]	Sequence	86–94[c]	Sequence
Lys	10.8(11)	10.5(11)	10.8(11)	11	7.0(7)	7	1.0(1)	1
His	1.1(1)	1.1(1)	1.1(1)	1	–	–	–	–
Arg	2.1(2)	2.0(2)	2.1(2)	2	–	–	–	–
Asp	11.5(12)	11.5(12)	11.8(12)	12	1.9(2)	2	–	–
Thr	7.5(8)	7.1(7)	7.0(7)	8	1.0(1)	1	1.0(1)	1
Ser	5.7(6)	4.7(5)	5.0(5)	7	1.7(2)	2	2.1(2)	2
Glu	10.1(10)	9.8(10)	10.4(10)	10	2.9(3)	3	1.4(1)	1
Pro	5.2(5)	4.9(5)	5.1(5)	5	1.0(1)	1	–	–
Gly	4.3(4)	4.1(4)	4.7(5)	5	0.7(1)	1	1.1(1)	1
Ala	–	–	–	–	–	–	–	–
Cys[a]	9.2(9)	8.8(9)	9.4(9 – 10)	10	1.2(1)	2	0.6(1)	1
Val	6.4(6)	6.4(6)	6.1(6)	6	2.7(3)	3	0.7(1)	1
Met	0.8(1)	0.6(1)	0.8(1)	1	–	–	–	–
Ile	6.2(6)	6.1(6)	6.2(6)	6	3.1(3)	3	1.1(1)	1
Leu	2.3(2)	2.1(2)	2.2(2)	2	–	–	–	–
Tyr	4.0(4)	3.8(4)	3.5(4)	4	0.8(1)	1	–	–
Phe	2.1(2)	2.0(2)	2.1(2)	2	1.0(1)	1	–	–
Trp	N.D.	N.D.	N.D.	2	N.D.	1	N.D.	1
Total	89	87	89	94		28		10

[a] Determined as *S*-carboxymethylcysteine
[b] 48 h hydrolysis
[c] 24 h hydrolysis

N.D., not determined. Numbers in parentheses represent nearest integer. Sequence, the predicted values from the sequence of β-inhibin

blem recurred. Interestingly, both regions represent Asp–Asn–Cys bonds.

An identical sequence was also obtained following CNBr fragmentation, where the sequence starting at Asp 20 up to Ser 90, was determined. This allowed the extension of the sequence of the molecule to residue 90 (not shown). Similarly, one of the chymotryptic fragments purified was sequenced and umambiguously confirmed residues 53–66 of the molecule.

In order to obtain the complete sequence of this polypeptide, its reduced and carboxymethylated form was subjected to trypsin digestion. It was found that the earliest cleaved peptide represented the carboxyterminal sequence, showing that the peptide bond Arg_{66}–Ile_{67} was very sensitive to this enzyme. The complete sequence of this tryptic fragment is shown in fig.1B. This sequence was also confirmed at least 3 times. The initial and repetitive yields for this sequence were found to be 2.4 nmol and 96.39%. As can be seen from fig.1B, the PTH yields cannot be fitted to a single regression line. Rather, two other lines can be deduced following residues 13 and 25, both being Glu residues helping the anchorage of the residual polypeptide to the Polybrene carrier. Nevertheless this sequence confirms residues 67–81 and 67–90 obtained with the whole molecule and one of its CNBr fragments, and the whole chain to the carboxy-terminus of β-inhibin.

Carboxypeptidase *Y* digestion of β-inhibin shows the release of only one amino acid, namely Ile, even after 8 h reaction. This is taken as evidence that Ile_{94} represents the C-terminal residue of β-inhibin. Glycine is known to be resistant sometimes to the action of this exopeptidase, explaining the resistance to further cleavage following Ile_{94}. Furthermore, the amino acid com-

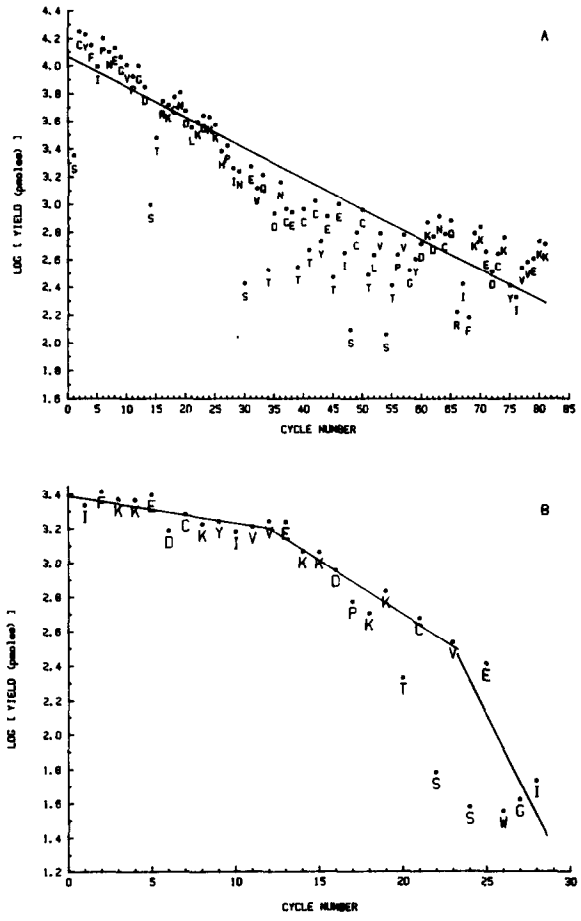

Fig.1. Phenylthiohydantoin (PTH) yield obtained during the sequence of the carboxymethylated β-inhibin (A) and its C-terminal tryptic fragment (B). The sequence of the whole β-inhibin allowed the unambiguous elucidation of the first 81 residues. The 28 residue C-terminal tryptic segment was sequenced to its carboxy-terminal Ile residue.

position of the C-terminal segment sequenced (table 1) shows a good fit with the deduced sequence, and the composition of another tryptic peptide (table 1) matches well the determined sequence of residues 86–94. Finally, the amino acid composition of the whole β-inhibin molecule fits well the proposed sequence shown in fig.2.

3.2. *Bioactivity of native β-inhibin*

Fig.3 shows the bioactivities of β-inhibin, both in vitro (fig.3A) and in vivo (fig.3B). In both cases,

Complete Sequence of Human beta-Inhibin

```
   1                5                10                15
NH2. Ser Cys Tyr Phe Ile Pro Asn Glu Gly Val Pro Gly Asp Ser Thr
                   20                25                30
     Arg Lys Cys Met Asp Leu Lys Gly Asn Lys His Pro Ile  Asn Ser
                   35                40                45
     Glu Trp Gln Thr Asp Asn Cys Glu Thr Cys Thr Cys Tyr Glu Thr
                   50                55                60
     Glu Ile  Ser Cys Cys Thr Leu Val Ser Thr Pro Val Gly Tyr Asp
                   65                70                75
    [Lys] Asp Asn Cys Gln Arg Ile Phe [Lys] Lys Glu Asp Cys Lys Tyr
                   80                85                90
     Ile [Val Val] Glu Lys Lys Asp Pro Lys [Lys] Thr Cys Ser Val Ser
                  94
     Glu Trp Gly Ile .COOH
```

Fig.2. Proposed complete amino acid sequence of human β-inhibin. Identical residues found at homologous positions upon alignment (starting at position 59) of the 31 amino acid sequence of the inhibin-like peptide [15,16] and of the human β-inhibin are enclosed in boxes. The proposed disulfide bridge linking Cys_{73} to Cys_{87} is also indicated by a solid line.

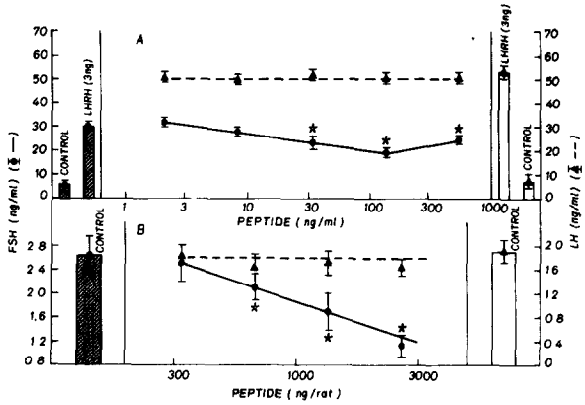

Fig.3. Bioactivity of human β-inhibin in vitro using the mouse pituitary assay (A) and in vivo using the castrated rat assay (B). It is seen that in both assays FSH release is inhibited in a dose-dependent manner by β-inhibin. The * means that the inhibition is statistically significant. In (A), each point represents FSH and LH duplicate measurements in the medium of at least 5 different mouse pituitaries for each dose, using a radioreceptor assay. In (B), each point represents duplicate measurements of plasma levels of FSH and LH in at least 6 different castrated rats per dose, using a specific RIA. In all cases, the concentration of β-inhibin was calculated based on its quantitative amino acid analysis and its calculated M_r of 10704.

this polypeptide selectively inhibits the release of FSH, but not LH. The potency in the in vitro assay is about 10–15-times higher than the in vivo one. For example, 143.4 ng (13.4 pmol) of β-inhibin cause a 45.6% suppression of FSH levels in the in vitro assay, whereas it takes 2.87 μg (268 pmol) to observe a 58.5% depression in the in vivo assay. Moreover, the peptide is active both in the mouse (in vitro) and the rat (in vivo). Lastly, it can be seen that the FSH inhibition observed in vitro, in the mouse pituitary assay, is biphasic. Namely, from 32–160 ng (3–15 pmol) of β-inhibin, a dose-dependent suppression of FSH is observed, whereas at a higher dose of 578 ng (54 pmol) the peptide is less effective (fig.3A).

4. DISCUSSION

From these data, it is proposed that β-inhibin is composed of 94 amino acids. This polypeptide, which is not glycosylated, migrates on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) with an apparent molecular mass of 14 kDa. The calculated molecular mass, based on its primary structure, is 10704 Da. This difference in molecular mass is probably due to the abnormal migration of this peptide on SDS–PAGE. Similar observations have been made previously for many other basic polypeptides, including rat SVS-IV protein [1], a non-glycosylated polypeptide of 90 amino acids which migrates on SDS–PAGE with an apparent molecular mass of 17 kDa [1].

The sequence of the first 30 residues of β-inhibin presented here agrees entirely with the one reported for that segment of the molecule by authors in [17]. However, the amino acid composition of the whole β-inhibin differs significantly. Since both papers are based on the same batch of β-inhibin, the interpretation of the difference can be attributed to contamination of the original batch by an Ala-containing peptide. Indeed, upon repurification of this batch by HPLC in HFBA/acetonitrile, we were able to eliminate most of this contamination, which represented up to 30% of the total. The so-purified β-inhibin is virtually devoid of alanine.

Examination of the sequence reveals a number of repetitive segments. First, the triad Asp-Asn-Cys appears twice, at residues 35–37 and 62–64. Next, the pairs Lys-Lys and Thr-Cys appear 3 times at residues 69–70, 80–81, 84–85 and 39–40, 41–42, 86–87, respectively. Other pairs also appear twice, including Lys-Asp, Ser-Cys, Ser-Glu, Ser-Thr, Glu-Trp, Glu-Thr, Cys-Tyr and Val-Ser. The meaning of such repetitive sequences is not clear at present.

A computer data bank search on the possible homology of the β-inhibin sequence to any known protein or segment thereof, using a mutation data matrix developed at the National Biomedical Research Foundation in Georgetown University, revealed no significant homologies (<25%). A comparison between the sequence of β-inhibin and the one reported for another basic human inhibin-like peptide [15,16] reveals that only 5 residues, as shown in fig.2, match out of 31. These include Lys 61, 69, 85 and Val-Val 77–78. Interestingly, a homology can be found between the sequences of human-β-inhibin and bovine seminal plasma PDC-109 [3]. Two stretches of 4 amino acids can be found in both molecules, namely Glu-Thr-Cys-Thr 38–41, and Tyr-Asp-Lys-Asp 59–62. In both cases, however, it can be concluded that the sequence presented for β-inhibin is a novel one.

Concerning the 4 pairs of basic residues found in this sequence, namely Arg-Lys 16–17 and Lys-Lys 69–70, 80–81 and 84–85, these could represent potential cleavage sites for a maturation enzyme [24]. Furthermore, it can be noted that Ile_{67} is preceded by a Lys-X-X-X-Gln-Arg- segment. An identical segment is also found in the sequence of residues 59–64 of the bovine seminal plasma protein PDC-109 [3]. In the case of human prosomatostatin, the cDNA sequence [25] predicts that in order to cleave it to somatostatin 28 [26–28], a cleavage following Gln-Arg in the stretch Arg-X-X-X-Gln-Arg- [25] is necessary. It is therefore quite conceivable that in the case of β-inhibin, one of its maturation products would be the 28 residue C-terminal peptide starting and ending in Ile_{67} and Ile_{94} respectively. This would generate, similar to somatostatin, a 28-residue peptide with one potential disulfide bridge. Indeed, preliminary data on the inhibin-like activity of a synthetic peptide comprising residues 67–94 with a closed disulfide bridge demonstrate the plausibility of this hypothesis. Our results [18] indicate that the inhibin-like activities of both β-inhibin 1–94 and

β-inhibin 67–94 are equipotent on a mole to mole basis. Based on these data, it is proposed that Cys_{73} is linked via a disulfide bond to Cys_{87} in the native β-inhibin. We have not yet defined the interlocking potential disulfide bridge pattern between the other 8 Cys residues predicted in the N-terminal sequence of β-inhibin (see fig.2). It is, therefore, tempting to speculate on the possibility that the segment following the Lys-X-X-X-Gln-Arg- sequence in PDC-109, namely residues 65–109 [10], might exhibit some inhibin-like activity. This possibility is further supported by a match of 7 residues between the sequences of β-inhibin 67–94 and the segment 65–109 of PDC-109. However, this hypothesis will have to be experimentally verified.

Finally, from the results presented, it can be concluded that β-inhibin is a very potent novel peptide which effectively and selectively inhibits the release of FSH, both in vitro and in vivo. In vitro, it is quite active at concentrations below 107 ng/ml (10 pmol/ml). Furthermore, the relation between the observed and reported specific inhibition of FSH release, both in vitro and in vivo, by the β-inhibin and by the inhibin-like peptide previously described [15,16] is intriguing. Although highly dissimilar in structure, it can be seen that both peptides are able to exhibit potent biological activities in the assays used. The biphasic nature of this inhibition, as observed with β-inhibin, is not yet adequately explained, but suffice to say that a similar phenomenon has been observed previously [29]. Much more work is necessary before the physiological potential of human β-inhibin can be fully assessed.

ACKNOWLEDGEMENTS

The authors thank Diane Marcil for typing this manuscript and Gilles De Serres for his technical help. We would like to thank Dr C. Lazure for his help in the HPLC purification of trypsin and chymotrypsin, and Dr M.R. Sairam for the generous supply of FHS and LH membrane receptors and iodinated hormones. This work was supported by a research training grant to N.J.A. from the World Health Organization, the Medical Research Council of Canada (PG-2) and the National Institutes of Health (MS16315-04).

REFERENCES

[1] Pan, Y.C.E., Silverberg, A.B., Harris, S.G. and Li, S.S.L. (1980) Int. J. Peptide Prot. Res. 16, 143–146.
[2] Ostrowski, M.C., Kistler, M.K. and Kistler, W.S. (1979) J. Biol. Chem. 254, 383–390.
[3] Esch, E.S., Ling, N.C., Bohlen, P., Ying, S.Y. and Guillemin, R. (1983) Biochem. Biophys. Res. Commun. 113, 861–867.
[4] McCullagh, D.R. (1932) Science 76, 19–20.
[5] De Jong, F.H. (1979) Mol. Cell. Endocrinol. 13, 91–102.
[6] Franchimont, P. and Channing, C.P. eds (1981) Intragonadal Regulation of Reproduction, Academic Press, New York.
[7] Chari, S., Duraiswami, S. and Franchimont, P. (1978) Acta Endocrinol. (Copen.) 87, 434–448.
[8] De Jong, F.H. and Sharpe, R.M. (1976) Nature 263, 71–72.
[9] Hopkinson, C.R.N., Sturm, G., Daume, E., Fritze, E. and Hirschhauser, C. (1975) Int. Res. Commun. Syst. 3, 588–592.
[10] Sheth, A.R., Joshi, L.R., Moodbidri, S.B. and Rao, S.S. (1979) J. Reprod. Fertil. Suppl. 26, 71–85.
[11] Schwartz, N.B. and Channing, C.P. (1977) Proc. Natl. Acad. Sci. USA 74, 5721–5724.
[12] Thakur, A.N., Vaze, A.Y., Dattatreyamurthy, B.D., Arbatti, N.J. and Sheth, A.R. (1978) Indian J. Exp. Biol. 16, 854–855.
[13] Franchimont, P., Demoulin, A., Proyard, J.V., Hazee-Hagelstein, M.T. and Turnbridge, W.M.G. (1979) J. Reprod. Fert. Suppl. 26, 123–133.
[14] Ramasharma, K. and Sairam, M.R. (1982) Ann. NY Acad. Sci. 383, 307–328.
[15] Seidah, N.G., Ramasharma, K., Sairam, M.R. and Chrétien, M. (1984) FEBS Lett. 167, 97–102.
[16] Ramasharma, K., Sairam, M.R., Seidah, N.G., Chrétien, M., Manjunath, P., Schiller, P.W., Yamashiro, D. and Li, C.H. (1984) Science 223, 1199–1202.
[17] Sheth, A.R., Arbatti, N.J., Carlquist, M. and Jornvall, H. (1984) FEBS Lett. 165, 11–15.
[18] Arbatti, N.J., Seidah, N.G., Rochemont, J., Escher, E., Sairam, M.R., Sheth, A.R. and Chrétien, M., submitted.
[19] Lazure, C., Seidah, N.G., Chrétien, M., Thibault, G., Garcia, R., Cantin, M. and Genest, J. (1984) FEBS Lett., in press.
[20] Lazure, C., Seidah, N.G., Chrétien, M., Lallier, R. and St-Pierre, S. (1983) Can. J. Biochem. 61, 287–292.
[21] Seidah, N.G., Rochemont, J., Hamelin, J., Lis, M. and Chrétien, M. (1981) J. Biol. Chem. 256, 7977–7984.

[22] Titani, K., Sasagawa, T., Resing, K. and Walsh, K.A. (1983) in: High Performance Liquid Chromatography of Proteins and Peptides (Hearn, M. et al. eds) pp.23–26, Academic Press, New York.
[23] Ramasharma, K., Sairam, M.R. and Ranganathan, M.R. (1981) Acta Endocrinologica 98, 496–505.
[24] Lazure, C., Seidah, N.G., Pélaprat, D. and Chrétien, M. (1983) Can. J. Biochem. Cell. Biol. 61, 501–515.
[25] Shen, L.P., Pictet, R.L. and Rutter, W.J. (1982) Proc. Natl. Acad. Sci. USA 79, 4575–4579.
[26] Pradayrol, L., Jornvall, H., Mutt, V. and Ribert, A. (1980) FEBS Lett. 109, 55–58.
[27] Schally, A.V., Huang, W.Y., Chang, R.C.C., Arimura, A., Redding, T.W., Millar, R.P., Hunkapiller, M.W. and Hood, L.E. (1980) Proc. Natl. Acad. Sci. USA 77, 4489–4493.
[28] Esch, F., Bohlen, P., Ling, N.C., Benoit, R., Brazeau, P. and Guillemin, R. (1980) Proc. Natl. Acad. Sci. USA 77, 6827–6831.
[29] Sairam, M.R., Ranganathan, M.R., Sharma, K.R. and Lamothe, P. (1981) Mol. Cell. Endocrinol. 22, 231–251.