

β_2 -Inhibin contains the active core of human seminal plasma β -inhibin: synthesis and bioactivity

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The complete synthesis of the C-terminal 28 residues segment 67–94 of human seminal plasma β -Inhibin, called β_2 -Inhibin, is reported. The Inhibin-like activity of the native 94 amino acids β -Inhibin is compared to that of the synthetic replica of β_2 -Inhibin. In all assays used both peptides effectively suppress the FSH release induced by LHRH but have little effect on the LH release. In the mouse both peptides are equipotent on a mole basis. In the rat the synthetic β_2 -Inhibin is 3–10 times more potent than β -Inhibin. Both peptides are active in rat anterior pituitary primary culture assays where maximum suppression of FSH release induced by LHRH occurs around 300 pmol/ml of β_2 -Inhibin. In contrast, maximum suppression of FSH release in the mouse pituitary assay occurs at 10–15 pmol/ml of either Inhibin.

β_2 -Inhibin Chemical synthesis Human seminal plasma Pituitary FSH release

1. INTRODUCTION

In 1932, McCullagh [1] postulated that a testicular factor, later called 'Inhibin', causes the disappearance of 'castration cells' in the pituitary following its administration to the animal. This Inhibin-like activity, causing the selective suppression of pituitary follicle-stimulating hormone (FSH), was subsequently detected in various gonadal fluids from both sexes in a number of different species [2–4]. During the last 10 years, polypeptides exhibiting Inhibin-like activities were detected in human seminal plasma [5,6] and the amino acid sequence of a small basic peptide has been recently described [7,8]. The complete amino acid sequence of another human seminal plasma protein containing 94 amino acids has also been reported [9]. This polypeptide, named β -Inhibin, was shown to contain a very basic C-terminal segment and preliminary data on its Inhibin-like activity allowed the postulation that this C-terminal 28 residues segment contains the active core for

FSH inhibition [9]. Later, in an independent report, Johansson et al. [10] described the sequence of a peptide identical to β -Inhibin and observed that this structure is almost identical to that of a human sperm-coating antigen [11]. Based on this sequence identity, it was proposed that β -Inhibin functions might also include its ability to act as a sperm coating antigen.

In this report, we describe in detail the characterization of the Inhibin-like activity of a synthetic replica of the C-terminal 28 residues segment of β -Inhibin, called β_2 -Inhibin. Comparison of the activity of β - and β_2 -Inhibin was done in vitro using either the mouse/rat pituitary assay or the rat pituitary anterior lobe cell culture assay. Results show that both Inhibins are equipotent on a mole basis in the mouse pituitary assay. In contrast, in the rat, both in pituitary and cell culture assays, β_2 -Inhibin is 3–10-fold more potent than β -Inhibin. In all assays the levels of luteinizing hormone (LH) are not significantly affected by either Inhibin.

2. MATERIALS AND METHODS

The 94 amino acid β -Inhibin was purified as previously described [12]. The final purification of β -Inhibin by reverse-phase HPLC using a μ -Bondapak C-18 column (Waters) eluted with an acetonitrile/0.1% trifluoroacetic acid (TFA) gradient is similar to the procedure used for the purification of its reduced and carboxymethylated (CM) derivative shown in fig.1. Both β -Inhibin and CM- β -Inhibin tend to absorb to the C-18 column, unless the peptides are first dissolved in 6 M guanidine-HCl prior to injection onto the HPLC column. Recoveries under these conditions are of the order of 70–80%.

The chemical synthesis of β_2 -Inhibin with the proposed disulfide bridge was carried out by classical solid-phase peptide synthesis using Boc-isoleucine esterified to chloromethylated polystyrene crosslinked with 2% divinylbenzene as an anchor group. Chain elongation was performed on an automatic peptide synthesizer (Peptomat 82) using the classical TFA symmetrical anhydride cycle. A 40% concentration of TFA in dichloromethane containing 0.1% acetyltryptophan added as a *t*-butyl cation scavenger was used. Maximal side chain protection was used: Bzl ether for Ser and Thr; Bzl esters for Asp and Glu; dichloro-CBZ for Lys and Tyr; and acetamidomethyl for Cys. Each coupling step was monitored for completion with a ninhydrin test and couplings were repeated if necessary. If after 1 h at recoupling some free amine groups were present, the peptide was acetylated. Couplings after Lys 19 were performed in 20% DMF in dichloromethane, and all couplings after Ile 10 were performed in 50% DMF. After completion of the sequence, the side chain protecting groups (with the exception of Cys), were cleaved simultaneously together with the ester link to the support by liquid HF in the presence of 0.25% acetyltryptophan and 10% anisole. The product was finally subjected to gel filtration of LH20 and subsequently purified by two preparative HPLC (2×25 cm, 30 μ -C-18) runs using 10–40% acetonitrile gradient in 0.25 M NH_4OAc and 7.5% propanol at pH 8.0, and then at pH 6.0. Peptide fractions were collected and identified by both amino acid composition and sequence. Subsequently, 2.5 mg of the correct peptide was treated with mercury acetate to deprotect the Cys

residues, followed by gaseous hydrogen sulfide to precipitate the mercury, and finally by exposure to air in dilute NH_4OAc to allow cyclisation to occur.

2.1. Whole mouse/rat pituitary assays

In this assay, different concentrations of either β - or β_2 -Inhibins (calculated from quantitative amino acid analysis) were preincubated with whole pituitaries dissected from 20 day old animals. The preincubation medium contained 0.5 ml Dulbecco's Modified Eagles (DME) medium containing 0.1% BSA. Following a preincubation period of 1 h, 3 ng of synthetic LHRH in 0.5 ml of medium was added, giving a final incubation volume of 1 ml. Incubations were then continued for 3 h at 37°C in a 95% O_2 /5% CO_2 atmosphere. The amount of FSH and LH released into the medium was measured using a specific radio-receptor assay [13] employing highly purified ovine FSH and LH as standards.

2.2. Rat anterior pituitary cell culture assay

The cell cultures were obtained from the anterior pituitaries of 36-day-old male rats by a 25 min collagenase/DNase digestion. Cells were then washed twice with DME medium containing 0.1% BSA, resuspended in DME medium, and 0.5 ml of the suspension (containing 400 000–500 000 cells/0.5 ml) was used to coat each well. The cells were then cultured for 72 h at 37°C in a 95% O_2 /5% CO_2 atmosphere. Following this, the medium was replaced with a fresh one containing serum and the cells were incubated for 48 h with various concentrations of either Inhibin. Controls without Inhibin-like peptides were also made, in order to measure both the basal and non-inhibited LHRH stimulated release of LH and FSH. Following the 48 h incubation time, the cells were washed and incubated in a serum free medium containing LHRH (6 ng/well) for 5 h at 37°C. The levels of LH and FSH released into the medium were determined using a radioimmunoassay kit supplied by the NIH.

3. RESULTS

Trial experiments studying the susceptibility of β -Inhibin to trypsin digestion indicated that this molecule after purification by HPLC was resistant to low concentrations of enzyme. In fact, treatment of the reduced and carboxymethylated form

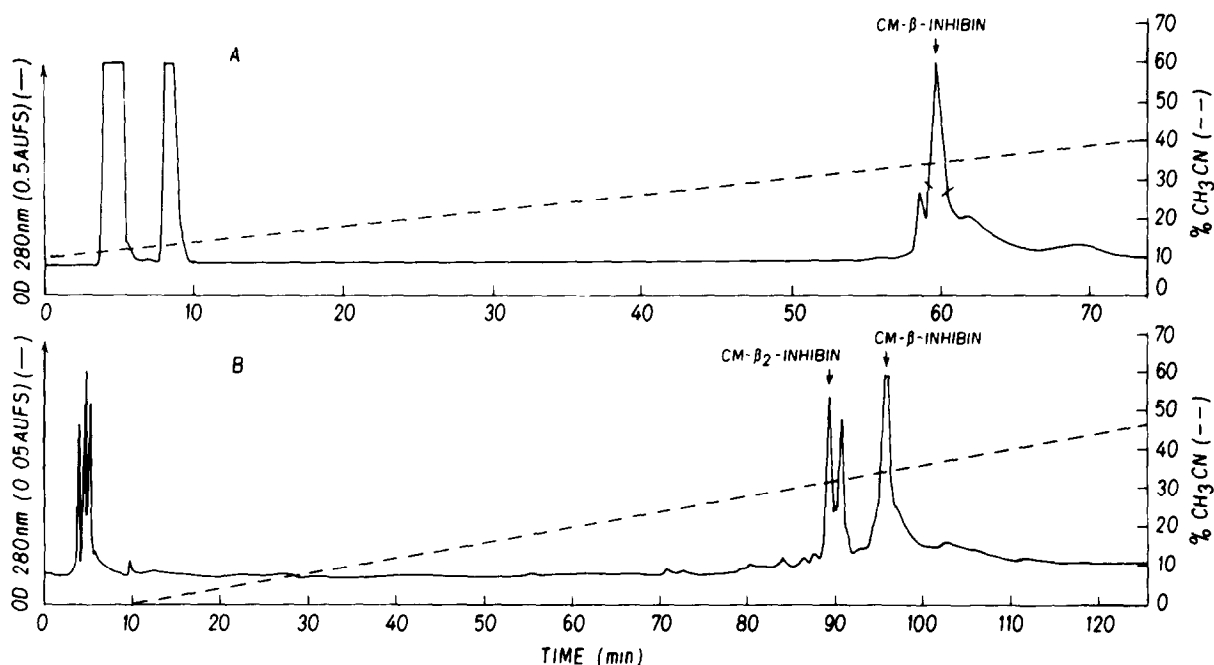


Fig.1. Reverse-phase HPLC purification of the reduced and carboxymethylated (CM) β -Inhibin (A) and the purification of peptides generated by a 2 h trypsin digestion of CM- β -Inhibin at an enzyme to substrate weight ratio of 1:50 in 0.1 M sodium phosphate buffer (pH 8.0), at 37°C (B). A μ -Bondapak C-18 column (0.38 $\times$ 30 cm) was used. Peptides were eluted at room temperature with a flow rate of 1 ml/min using 0.1% trifluoroacetic acid (TFA) and a gradient of acetonitrile in 0.1% TFA. The actual acetonitrile gradient used is indicated by a dashed line on each chromatogram. The elution position of CM- β_2 -Inhibin is shown in (B).

(CM) of β -Inhibin (fig.1A) with trypsin at an enzyme to substrate weight ratio of 1:50 for 2 h resulted in one cleavage at a single ARg occupying residue 66 in the β -Inhibin structure (fig.2B) [9]. The resultant products were the segments 1–66 and 67–94 of β -Inhibin. The C-terminal segment (β -Inhibin 67–94) has been named β_2 -Inhibin, and its sequence is shown in fig.2 [9].

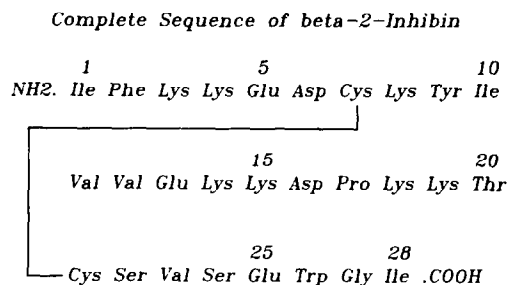
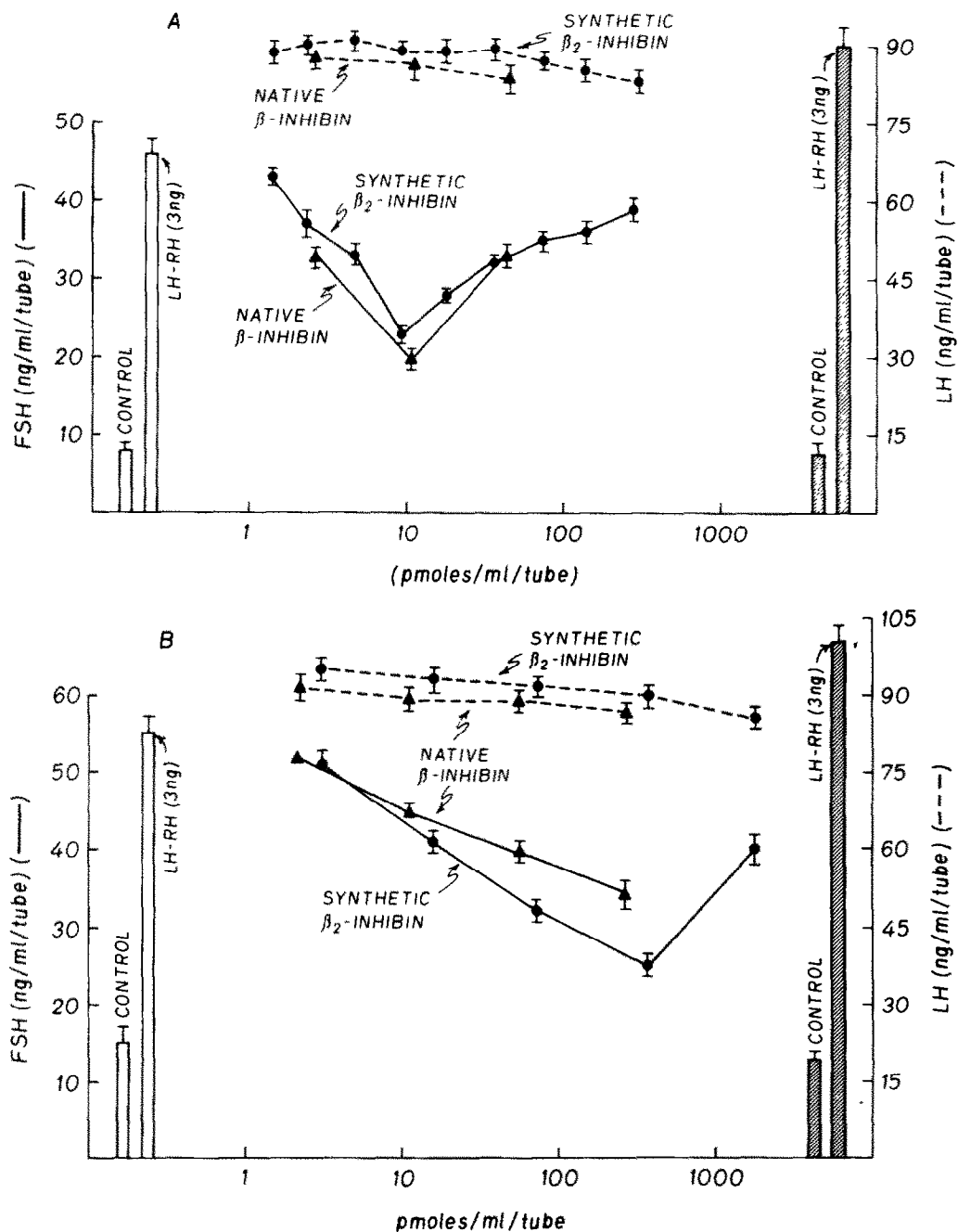


Fig.2. Complete amino acid sequence of β_2 -Inhibin with the proposed disulfide bridge [9].

The possibility that β_2 -Inhibin is the physiologically active component of β -Inhibin was reinforced by the fact that the first cleavage site by trypsin involved a post Gln-Arg in the stretch Lys-X-X-X-Gln-Arg of β -Inhibin [9], a site similar to that found in human pro-somatostatin to produce somatostatin-28 [9,14]. Since maturation of pro-hormones usually involved cleavage of C-terminal to basic residues [15], we reasoned that the β_2 -Inhibin segment of β -Inhibin could possess the required FSH suppression activity. The complete synthesis of β_2 -Inhibin was therefore made by a solid-phase approach. However, since the whole β -Inhibin structure contains 10 cysteine residues, potentially forming 5 disulfide bridges, it was not obvious 'a priori' whether the 2 Cys residues in β_2 -Inhibin would be linked by a disulfide bridge in the native molecule. These residues were nevertheless linked in the synthetic replica, and the Inhibin-like activity of the resulting molecule was tested.



The data on the suppression of the LHRH-induced FSH release by either the synthetic β_2 -inhibin or the native β -inhibin both in the mouse or rat are shown in fig.3. Both peptides were shown to inhibit FSH release at the pmol/ml level, but not to appreciably affect the LHRH in-

duced LH release at similar concentrations. In the mouse (fig.3A) both peptides are equipotent on a molar basis with the maximum suppression (55–60%) of FSH release observed at 10–15 pmol/ml. In the rat, both in the whole pituitary assay (fig.3B) or in the anterior pituitary

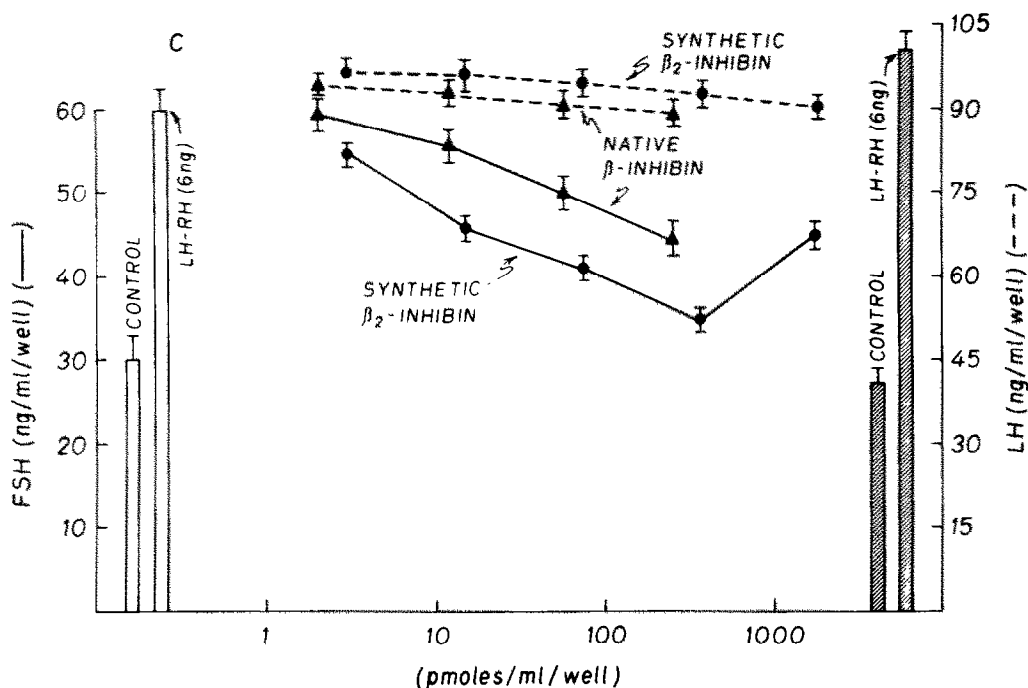


Fig.3. (A,B) The effect of human seminal plasma β -Inhibin and synthetic β_2 -Inhibin on FSH and LH release by LHRH stimulated mouse (A) and rat (B) whole pituitaries. The bar graphs represent the control incubation medium levels of FSH and LH, and the levels of the latter in the medium after the addition of 3 ng of LHRH. Each point on the graph represents the mean $\pm$ SE concentration of FSH and LH released into the medium obtained by duplicate analysis from at least 5 different pituitaries (= 5). Both the native β -Inhibin and the synthetic β_2 -Inhibin had no effect on the specific FSH and LH radio-receptor assays used. The concentrations of all Inhibin stock solutions were determined by quantitative amino acid composition. (C) The effect of human seminal plasma β -Inhibin and synthetic β_2 -Inhibin on the levels of FSH and LH released into the medium following stimulation by LHRH in rat anterior pituitary cell cultures. The levels of FSH and LH were determined using a radioimmunoassay kit supplied by the NIH. Each point on the graph represents the mean $\pm$ SE of FSH and LH concentrations in the medium obtained by duplicate measurements from at least 3 wells.

cell culture assay (fig.3C) 20–40 times higher concentrations of either peptide were needed to achieve 60–70% maximal FSH inhibition. In contrast to the equipotency observed in the mouse, β_2 -Inhibin was found to be more potent than β -Inhibin in the rat. The exact value is difficult to calculate in view of the non-parallelism of the inhibition curves, but it can vary between 3–10 times higher in potency (fig.3B,C). Within the sensitivity of the assay the effect of both peptides on the basal release of FSH could not be accurately estimated, but was found to be less than the one observed in the presence of LHRH in the incubation medium.

Interestingly, in all these in vitro assays, the effect of both peptides on FSH secretion is clearly

biphasic (fig.3A–C). In other words, at higher concentrations of either Inhibin, the suppression of FSH release is less effective than at lower concentrations. However, this effect has not been observed in vivo with β -Inhibin [9].

When mouse pituitaries were simultaneously exposed to either β -Inhibin (13 pmol/ml) or β_2 -Inhibin (10 pmol/ml) in the presence of 3 ng/ml of LHRH for different time intervals, the release of FSH, but not LH, was significantly lower than the LHRH control values at all times up to 12 h (not shown). In this experiment of cumulative release of FSH, both peptides were equipotent, in agreement with fig.3A.

4. DISCUSSION

The results presented in this work, indicate that the C-terminal 28 residues of β -Inhibin (β_2 -Inhibin), contain the active core for the suppression of pituitary FSH release induced by LHRH. Furthermore, based on this activity it is proposed that the Cys₇ and Cys₂₁ of β_2 -Inhibin (fig.2) are linked by a disulfide bridge in the native β -Inhibin. Rigorous proof of the latter will have to await complete disulfide bond assignment in the whole native β -Inhibin. In view of the presence of three pairs of Lys-Lys residues within the β_2 -Inhibin sequence (fig.2), and the known cleavage of pro-hormones at these sites [15], it would not be astonishing if further shortening of this peptide, either at the N- or C-terminus could still produce an active molecule. However, this would have to be confirmed by a systematic structure-activity study of this polypeptide. Nevertheless, the data presented here point out that the N-terminal 66 residues of β -Inhibin [9] are apparently not necessary for eliciting the observed Inhibin-like activity. A similar finding of an N-terminal extension not necessary for biological activity was observed previously for another pro-hormone, pronatriodilatin [16,17] containing within its C-terminus the active atrial natriuretic factor [16].

The potency of either β - or β_2 -Inhibin observed in this study, suggests that both human peptides when used in the rat pituitary cell culture assay are maximally active in the 1–2 μ g/ml concentration. Since the assays used involved the incubation of a human peptide with either mouse or rat pituitaries, the possibility of sequence variations of the active factor between species should be considered. Accordingly, the potency observed in our assays might not reflect the intrinsic Inhibin-like activity of this peptide in a homologous assay. The results presented here should permit a standardization of the assays with pure synthetic preparations in order to assess the real physiological involvement of either β - or β_2 -Inhibin *in vivo*.

Various reports have appeared in the literature showing that impure preparations of either bovine, porcine, or ovine follicular fluids cause a suppression of FSH release induced by LHRH in primary pituitary cell cultures in the mg/ml dose range [18–20]. Recent data on purified fractions of

either porcine [21] or bovine [22] follicular fluid show that 'Inhibin' migrate on SDS-PAGE with apparent M_r values of 140000 or 65000, respectively. The maximal activity of these ovarian Inhibin preparations is of the order of 60–70 ng/ml in the cell culture assay [21]. It is therefore possible that other Inhibin-like substances exist in gonadal extracts which could be more active than either β - or β_2 -Inhibin. However, in view of the variability between the assays used in various laboratories further work needs to be done for comparative activity purposes. Furthermore, the sensitivity of Inhibin preparations to organic solvents [23] is a factor to consider when reverse-phase HPLC is used as a final purification method.

The biphasic nature of the inhibition of FSH found in the *in vitro* assays used in this work is puzzling. This phenomenon was also observed in the same mouse or rat pituitary assays using a different 31 amino acid Inhibin-like peptide [8,24]. This biphasic phenomenon does not seem to be specific to Inhibin-like substances isolated from males, since a similar observation has been reported with porcine follicular fluid preparations [25]. The reason for the biphasic response, which is not always observed with all Inhibin-like preparations [26] is not well understood at the moment. It is possible that this phenomenon is related to the incubation conditions, since *in vivo* this effect is not observed with β -Inhibin [9].

The almost complete homology of the β -Inhibin structure [9] with that of a sperm-coating antigen [10,11], and the presence of this peptide in large quantities in either prostate [11] or gastric secretions [27] suggest that β - or β_2 -Inhibin might, in addition to causing FSH suppression, have a more general function. Future tests using oligonucleotide probes and 'in situ' hybridization would be helpful in defining the various sites of synthesis of β -Inhibin, and will be a step towards understanding the physiology of this novel series of human seminal plasma peptides.

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