Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4041–4044, June 1985
Biochemistry

Human seminal α inhibins: Isolation, characterization, and structure

(high-performance liquid chromatography/amino acid sequence/suppression of follitropin release/mouse pituitary *in vitro* assay/radioimmunoassay)

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Contributed by Choh Hao Li, February 25, 1985

ABSTRACT **Two additional peptides with inhibin-like activity have been isolated from human seminal plasma. One consists of 52 amino acids and the other, 92 amino acids. They are designated α-inhibin-52 and α-inhibin-92. Sequence analyses show that the NH_2-terminal 31 amino acids of α-inhibin-52 are identical to the structure of the inhibin-like peptide previously reported [ILP-(1–31), now designated α-inhibin-31], and the COOH-terminal 52 amino acids of α-inhibin-92 are identical to the structure of α-inhibin-52. The amino acid sequence of α-inhibin-92 is:**

H-Thr-Tyr-His-Val-Asp-Ala-Asn-Asp-His-Asp-Gln-Ser-Arg-Lys-Ser-Gln-Gln-Tyr-Asp-Leu-Asn-Ala-Leu-His-Lys-Thr-Thr-Lys-Ser-Gln-Arg-His-Leu-Gly-Gly-Ser-Gln-Gln-Leu-Leu-His-Asn-Lys-Gln-Glu-Gly-Arg-Asp-His-Asp-Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-Ile-His-His-Lys-Gly-Gly-Lys-Ala-His-Arg-Gly-Thr-Gln-Asn-Pro-Ser-Gln-Asp-Gln-Gly-Asn-Ser-Pro-Ser-Gly-Lys-Gly-Ile-Ser-Ser-Gln-Tyr-OH.

Bioassay data in mouse pituitaries *in vitro* show that α-inhibin-52 is 3.4 times more active and α-inhibin-92 is >40 times more active than α-inhibin-31 in suppressing follitropin-release. Radioimmunoassay data indicate that α-inhibin-52 and α-inhibin-92 have only 60% immunoreactivity.

Inhibins are gonadal peptides that inhibit the release of follitropin (FSH) from the anterior pituitary (1). We recently reported the isolation and synthesis of an inhibin-like peptide (ILP) with 31 amino acid residues (2, 3) from human seminal plasma (hSP). We now have isolated two additional peptides with activity in suppressing FSH release *in vitro*. The primary structures of these two new peptides contain the amino acid sequence of ILP. Because ILP is the first peptide with inhibin activity, it is now designated α-inhibin-31 (α-IB-31). The two additional peptides, which contain 52 and 92 amino acids, are referred to herein as α-IB-52 and α-IB-92.

MATERIALS AND METHODS

Fresh semen was obtained from men undergoing routine fertility examination at the Department of Urology in this campus. Sperm and other cells were removed by centrifugation (5 min at 15,600 × *g*). The supernatant (hSP) was acidified to 0.1 M HCl by the addition of 6 M HCl and stored at −20°C. Trypsin (L-1-tosylamido-2-phenylmethyl chloromethyl ketone-treated, 30A872) was obtained from Worthington, and carboxypeptidase Y was from Pierce.

Radioimmunoassay was performed with rabbit antiserum to α-IB-31. Amino acid analyses were performed in an automatic amino acid analyzer (model 119C, Beckman) as described (5). The NH_2-terminal residue was determined by the dansyl-Edman procedure (6). For COOH-terminal residue analysis, carboxypeptidase Y digests were carried out in 1 M pyridine acetate buffer (pH 5.5) for 4 hr at 37°C with an enzyme-to-substrate ratio of 1:50. Trypsin digestions were performed with an enzyme-to-substrate ratio of 1:50 in 0.05 M Tris/0.01 M Mg^{2+}, pH 8.5, at 37°C for 4 hr.

The dansyl-Edman method was used for manual sequence analysis as described (6). Automatic sequence analysis was performed under the direction of A. Smith of the Protein Structure Laboratory, University of California, Davis, using a Beckman 890M spinning-cup sequenator updated with a cold trap and microprocessor-based programmer. A dilute (0.1 M) Quadrol program (Beckman no. 050783) was used. Phenylthiohydantoin derivatives of amino acids were analyzed by GLC (7), TLC (8), and reversed-phase HPLC (RP-HPLC) (9).

Exclusion chromatography was carried out with a Sephadex G-50 (fine) column (2.3 × 75 cm) with 0.01 M NH_4OAc buffer (pH 4.6). Each fraction was assayed for immunoreactive (IR) α-IB-31 by RIA. Those fractions having immunoreactivity were pooled and lyophilized. Further purification was performed by RP-HPLC in a 4.5 × 250 mm column (Vydac 218TP104, Western Analytical Products, Temecula, CA) using a dual pump system from Laboratory Data Control (Riviera Beach, FL) with a variable-wavelength UV detector. Absorption was monitored at 210 or 225 nm. The solvents used were 0.1% CF_3COOH and 2-propanol.

Disc electrophoresis in polyacrylamide slab gels was performed as described (10). The gels were stained with Coomassie blue (R250). $NaDodSO_4$ gel electrophoresis was carried out as described (11). The inhibin activity was assayed by the *in vitro* mouse pituitary system (3, 12).

RESULTS AND DISCUSSION

Frozen acidified hSP (7 ml) was thawed, and 50 ml of cold ethanol was added. After centrifugation, the precipitate was dissolved in 10 ml of 50% HOAc and applied to two separate exclusion chromatography experiments on Sephadex G-50. Two immunoreactive fractions with V_e/V_o of 1.0 (designated EC-I) and 1.4 (designated EC-II) appeared (see Fig. 1). After lyophilization, yields of these fractions were 100 mg (EC-I) and 33 mg (EC-II).

α-IB-52. Two milligrams of EC-II were submitted to RP-HPLC, and one major IR-ILP peak was observed (Fig. 2). When the material in this peak was isolated (0.2 mg) by ly-

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Abbreviations: FSH, follitropin; ILP, inhibin-like peptide, now designated α-IB-31; RP-HPLC, reversed-phase high-performance liquid chromatography; hSP, human seminal plasma; IR, immunoreactive.

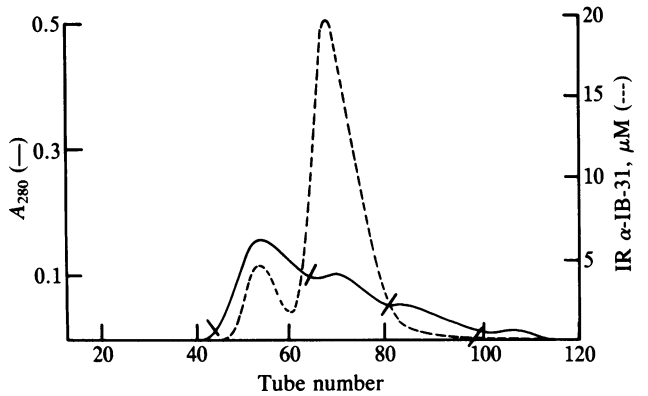

FIG. 1. Fractionation of the precipitate from hSP (3.5 ml) on Sephadex G-50 (fine) column in 0.01 M NH_4OAc (pH 4.6). Flow rate was 30 ml/hr; 3-ml fractions were collected per tube; and void volume was 145 ml. α-IB-31 was formerly designated ILP.

ophilization, it behaved as a single component in RP-HPLC. $NaDodSO_4$ gel and disc electrophoresis of the isolated material gave a single band. Histidine was found to be the sole NH_2-terminal residue, and tyrosine was at the COOH terminus. Amino acid analysis after a 22-hr hydrolysis in constant-boiling HCl gave (sequence values in parentheses): $Asp_{5.9(6)}$-$Thr_{1.1(1)}Ser_{5.5(6)}Glu_{6.3(6)}Pro_{2.1(2)}Gly_{7.9(8)}Ala_{1.1(1)}Val_{1.9(2)}$-$Ile_{1.8(2)}Tyr_{0.9(1)}Phe_{1.0(1)}His_{6.8(7)}Lys_{5.9(6)}Arg_{2.7(3)}$. Thus, the peptide consists of 52 amino-acid residues and is referred to herein as α-IB-52. From 10 ml of hSP, the average yield of α-IB-52 was 5 mg.

Tryptic digestion of α-IB-52 (30 nmol) was performed, and the digest was fractionated by RP-HPLC using a 2-propanol gradient of 7.5–25% in 0.1% CF_3COOH over 60 min. Six tryptic peptides were obtained. Two of these tryptic peptides differed from those obtained from the tryptic digest of α-IB-31. They were isolated from the fractions eluted at 14 min and 25 min and called T-14 and T-25. Amino acid composition of T-14 was: $Asp_{2.8(3)}Thr_{1.0(1)}Ser_{2.8(3)}Glu_{3.1(3)}Pro_{1.9(2)}$-$Gly_{3.0(3)}Lys_{0.9(1)}$. That of T-25 was: $Ser_{1.9(2)}Glu_{1.2(1)}Gly_{1.2(1)}$-$Ile_{1.0(1)}Tyr_{0.9(1)}$. Dansyl-Edman analysis of T-25 gave the sequence H-Gly-Ile-Ser-Ser-Gln-Tyr-OH. This was confirmed by automatic sequence analysis. Since T-25 does not contain lysine or arginine and since tyrosine is the COOH-terminal residue of α-IB-52, T-25 must be the COOH-terminal peptide. Peptide T-14 was submitted to automatic sequence analysis. Results are shown in Fig. 3. Since the analyses of the isolated tryptic peptides of both α-IB-31 and α-IB-52 were identical except for the forementioned two peptides, it

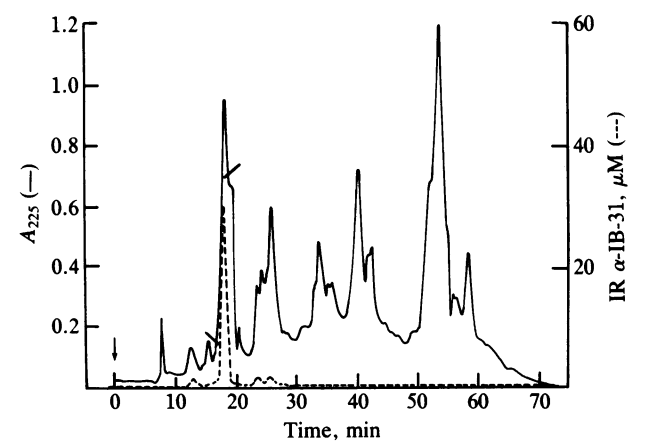

FIG. 2. Purification of EC-II (2 mg) on an RP-HPLC column with a linear 2-propanol gradient from 10% to 20% in 0.1% CF_3COOH in 60 min and a flow rate of 0.5 ml/min.

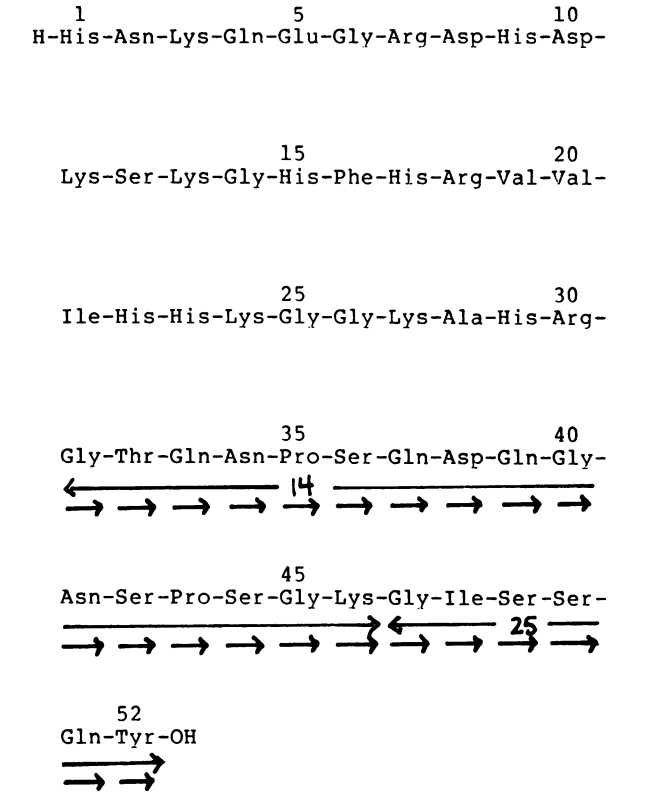

FIG. 3. Amino acid sequence of α-IB-52. Residues 1–31 constitute α-IB-31.

is evident that the first 31 amino acids of α-IB-52 are identical to the structure of α-IB-31.

α-IB-92. Three milligrams of EC-I were chromatographed by RP-HPLC, and several IR-ILP peaks were observed (Fig. 4). The main IR-ILP peak, eluted at 34 min, was isolated (0.05 mg) and shown to behave as a single component on RP-HPLC, $NaDodSO_4$ gel electrophoresis, and disc electrophoresis. Threonine was the only NH_2-terminal residue. Amino acid analyses of α-IB-92 gave the following values (sequence values in parentheses): $Asp_{12.3(12)}Thr_{4.1(4)}Ser_{9.6(10)}Glu_{12.0(12)}$-$Pro_{2.5(2)}Gly_{10.3(10)}Ala_{3.0(3)}Val_{3.0(3)}Ile_{1.8(2)}Leu_{5.0(5)}Tyr_{2.7(3)}$-$Phe_{1.0(1)}His_{10.4(11)}Lys_{9.2(9)}Arg_{5.2(5)}$. It consists of 92 amino acids and, thus, is designated α-IB-92. From 10 ml of hSP, an average yield of 2 mg of α-IB-92 may be obtained.

A tryptic digest of α-IB-92 (30 nmol) was submitted to RP-

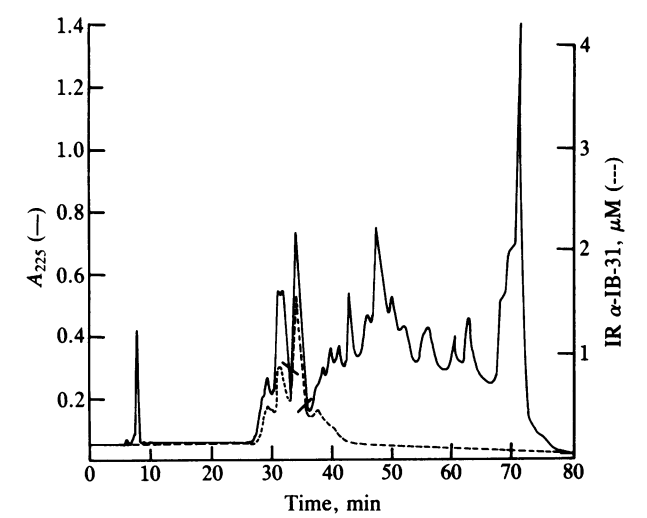

FIG. 4. Purification of EC-I (3 mg) on our RP-HPLC column; conditions were the same as for Fig. 2.

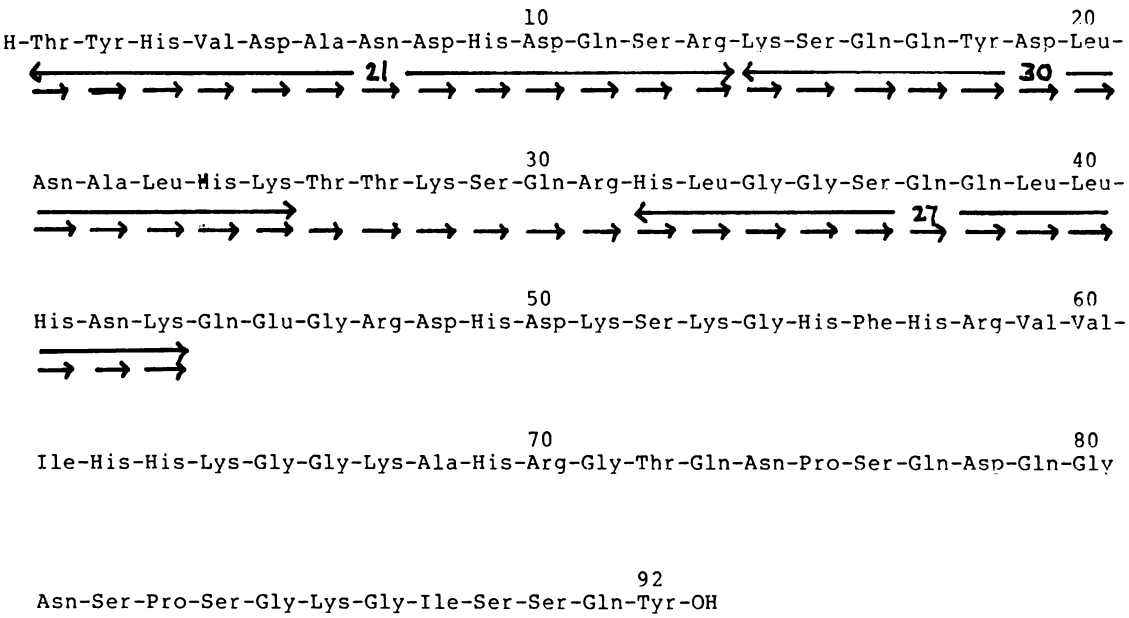

FIG. 5. Amino acid sequence of α-IB-92. Residues 41–92 constitute α-IB-52.

HPLC as described for α-IB-52. Nine tryptic peptides were eluted at 7, 9, 14, 15, 16, 21, 25, 27, and 30 min. Amino acid analyses of these peptides indicated that only three (T-21, T-27, and T-30) are different from those obtained from α-IB-52. Their compositions are as follows: T-21, $Asp_{4.3(4)}Thr_{0.9(1)}Ser_{0.8(1)}Glu_{0.9(1)}Ala_{1.0(1)}Val_{1.0(1)}Tyr_{0.7(1)}His_{1.8(2)}Arg_{0.9(1)}$; T-27, $Asp_{1.1(1)}Ser_{0.9(1)}Glu_{2.2(2)}Gly_{2.0(2)}Leu_{3.0(3)}His_{1.8(2)}Lys_{1.0(1)}$; and T-30, $Asp_{2.0(2)}Ser_{1.0(1)}Glu_{2.0(2)}Ala_{0.8(1)}Leu_{2.0(2)}Tyr_{0.6(1)}His_{1.0(1)}Lys_{1.8(2)}$. From these data and the definite occurrence of peptide T-25, it is evident that α-IB-52 is located at the COOH terminus of α-IB-92. In order to obtain the primary structure of α-IB-92, both T-27 and α-IB-92 were submitted to automatic sequence analyses. Results are shown in Fig. 5. It consists of 92 amino acids with three tyrosine residues in positions 2, 18, and 92 and a single phenylalanine residue in position 56. Cysteine and tryptophan are absent. The COOH-terminal sequence of residues 41–92 is that of α-IB-52. α-IB-92 is a very basic peptide with 5 arginine, 9 lysine, 10 histidine, 1 glutamic acid, and 6 aspartic acid residues. It has unusually high histidine content; >10% of the total residues are histidine residues. The NH_2-terminal 40-residue segment has only 12 charged groups and is the least hydrophilic part of the molecule.

Fig. 6 presents bioassay data for α-IB-31, α-IB-52, and α-IB-92 in suppressing the lutropin-releasing hormone (LHRH)-induced FSH secretion by using mouse pituitaries *in vitro*. α-IB-52 is 3.4 times and α-IB-92 is >40 times more active than α-IB-31 (Table 1). The addition of 40 amino acids at the NH_2 terminus of α-IB-52 greatly enhanced the inhibin activity.

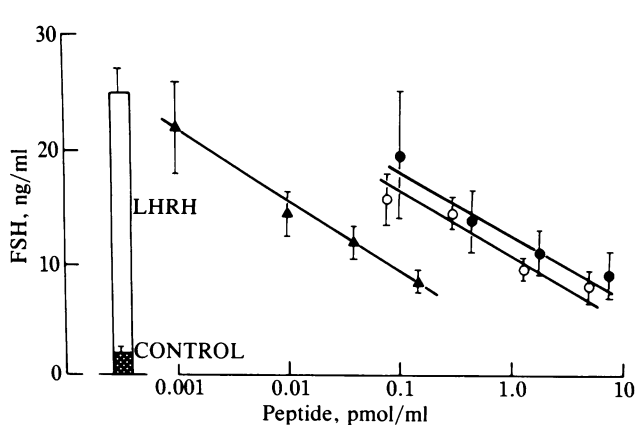

FIG. 6. Effect of α-IB-31, α-IB-52, and α-IB-92 on LHRH-induced FSH secretion by mouse pituitary. Means ± SEM (n = 5) are shown. ●, α-IB-31; ○, α-IB-52; ▲, α-IB-92.

The immunoreactivity of α-IB-52 and α-IB-92 is shown in Table 2. α-IB-52 and α-IB-92 cross-react fully to an antiserum raised against α-IB-31. They displaced labeled α-[Tyr^4]IB-31 with 60% of the potency of α-IB-31. If the antiserum to α-IB-31 had not recognized α-IB-52 or α-IB-92, it would have been difficult to discover these two inhibin peptides.

It is known that seminal fluids of various species contain inhibin-like activity in molecules with different sizes. Recently, Sheth and co-workers (4, 13) reported the isolation and complete amino acid sequence of hSP β inhibin. It consists of 94 amino acids with serine and isoleucine as NH_2- and COOH-terminal residues, respectively. β inhibin has 5 cystine and 2 tryptophan residues. It is obvious that α-IB-52 and α-IB-92 are different from β inhibin. It is possible that these ILPs are derived from a larger inhibin-like protein or inactive precursor molecule.

Note Added in Proof. When this paper was in press, Lilja and Jeppson reported the amino acid sequence of α-IB-52 (14). Total synthesis of α-IB-92 has been completed in our laboratory. The synthetic product is identical to the natural hormone.

Table 1. FSH-suppressing activity of α inhibins

Peptides	ED_{50},* pmol/ml	Slope	Relative potency
α-IB-31†	1.02 (0.34–2.9)	0.548	1.0
α-IB-52	0.30 (0.18–0.52)	0.414	3.4
α-IB-92	0.025 (0.01–0.049)	0.487	40.5

*95% confidence limits are in parentheses.
†Formerly called ILP.

Table 2. RIA of α inhibins using rabbit antiserum to α-IB-31

Peptide ligand	ED_{50},* fmol	Slope	Relative potency*
α-IB-31†	38 (29–50)	1.17	1.00
α-IB-52	65 (45–92)	0.99	0.58 (0.31–1.1)
α-IB-92	63 (42–94)	1.02	0.60 (0.31–1.2)

*95% confidence limits are in parentheses.
†Formerly called ILP.

We thank Dr. C. Cabrera for skillful assistance. This work was supported in part by grants from the National Institutes of Health (AM-6097 and GM-2907) and the Hormone Research Foundation.

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