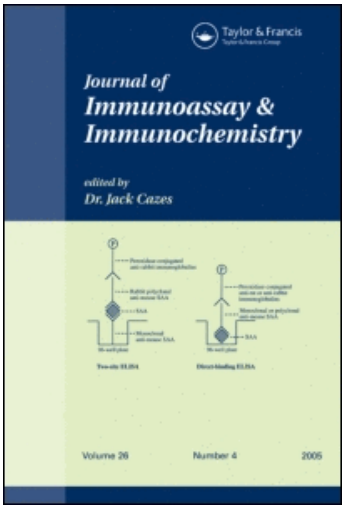

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Radioimmunoassay for an Inhibin-Like Peptide from Human Seminal Plasma

R. Glenn Hammonds Jr.[a]; Choh Hao Li[a]
[a] Donald Yamashiro, Claudia M. Cabrera, and Manfred Westphal Laboratory of Molecular Endocrinology, University of California, San Francisco, San Francisco, California

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RADIOIMMUNOASSAY FOR AN INHIBIN-LIKE PEPTIDE FROM HUMAN SEMINAL PLASMA

R. Glenn Hammonds Jr., Choh Hao Li,
Donald Yamashiro, Claudia M. Cabrera, and Manfred Westphal
Laboratory of Molecular Endocrinology
University of California, San Francisco
San Francisco California 94143

Abstract

Antisera raised in rabbits to a synthetic peptide consisting of 31 amino acids with a sequence identical to inhibin-like peptide (ILP) isolated from human seminal plasma afford a highly specific and sensitive radioimmunoassay. Synthetic ILP completely displaces antiserum binding of radioiodinated [Tyr^4]-ILP, with half maximal displacement at 36 fmoles ILP/tube. ILP, [Tyr^4]-ILP and ILP-(9-31) had essentially equal potency, while ILP-(1-25), ILP-(1-23) and ILP-(1-16) had reduced potency. No cross reactivity was found among a variety of peptide hormones and proteins. Human seminal plasma displaces 50% of [^{125}I-Tyr^4]-ILP at dilutions equivalent to 50-250 pl/tube, corresponding to immunoreactive ILP concentration of 0.5-2.5 mg/ml. (KEY WORDS: radioimmunoassay, inhibin, seminal plasma.]

INTRODUCTION

Inhibins are water soluble gonadal substances which exert a specific inhibition of follicle stimulating hormone release from the pituitary gland (1). The isolation, sequence determination and synthesis of a 31 residue inhibin-like peptide (ILP) from human seminal plasma gave the first structural information about this class of biologically active compounds (2,3). β-Inhibin, a peptide unlike ILP, has also been isolated on the basis of inhibin-like activity (4) and sequenced (5). We describe here the production of antisera specific for synthetic ILP (see Figure 1), the use of this antisera along with a radioiodinated ILP analog containing tyrosine in position 4 ([Tyr^4]-ILP) to develop a sensitive radioimmunoassay (RIA), and the use of the RIA to detect ILP immunoreactive material in human seminal plasma.

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                    5                   10
NH2-His-Asn-Lys-Gln-Glu-Gly-Arg-Asp-His-Asp-
                   15                   20
    Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-
                   25                   30
    Ile-His-His-Lys-Gly-Gly-Lys-Ala-His-Arg-Gly-COOH
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Figure 1: Amino acid sequence of human inhibin-like peptide.

MATERIALS

ILP, [Tyr^4]-ILP, ILP-(9-31) and ILP-(1-25) were assembled by solid phase methods and purified by a combination of partition chromatography and high performance liquid chromatography (HPLC) as described (3). ILP-(1-23) was isolated from chymotryptic digests of ILP by HPLC and its homogeneity confirmed by quantitative amino acid analysis. Peptide dry weights were measured on a Cahn electronic microbalance. ILP related peptides were dissolved in 0.1 N acetic acid at a concentration of 1 mg/ ml and stored in 5-20 µl aliquots at -70°. Aliquots were thawed once on the day of assay for use as standards and the remainder discarded. Other peptides and proteins were either commercial products, purified, or synthesized in this laboratory. ^{125}I-NaI was from New England Nuclear. Poly-L-lysine (MW=3800), limabean trypsin inhibitor, ß-mercaptoethylamine and chloramine-T were from Sigma. Bovine serum albumin was from Schwarz-Mann. Phenylmethylsulfonyl fluoride (PMSF) was from Calbiochem.

METHODS

Human Seminal Plasma

Semen samples from men undergoing routine fertility examination at the Department of Urology, University of California, San Francisco were freed from spermatozoa and other cells by centrifugation (5 min, 15600 xg) and the supernatant human seminal plasma (hSP) stored at -20°. Seminal plasma from 20 individuals was pooled, recentrifuged, and diluted 100 fold with hSP diluent. The resulting hSP standard (hSP-STD) was stored in 20 µl aliquots at -70°; each aliquot was thawed once on the day of assay for use as a standard and the remainder discarded.

Iodination

[Tyr^4]-ILP (5 µg), phosphate buffer (30 µl), ^{125}NaI (1 mCi), and chloramine-T (5 µl) were mixed in a polyproylene tube. The reaction was stopped

after 30 sec by the addition of 30 µl β-mercaptoethylamine. A 10 ml column of LH-20 (Pharmacia) was used to separate [^{125}I-Tyr^4]-ILP (elution volume 3.5-4.0 ml) from unincorporated ^{125}I-NaI (elution volume 9-10 ml). Over 98% of the radioactivity eluting in the void volume was retained on a reverse phase cartridge (Waters, sep-pak, C_{18}) developed with column buffer and could be eluted with 30% 2-PrOH in column buffer. The minimum specific activity calculated from observed incorporation assuming full recovery was 40 µCi/µg. After storage at 4° for 2 weeks 10-15% of the total radioactivity was not retained on the reversed phase cartridge.

Antisera

Synthetic ILP was coupled to BSA with a water soluble carbodiimide as described for another small basic peptide, corticotropin (6), and stored at 4° in sterile solution at 1 mg/ml. Adult male rabbits were injected intradermally at multiple sites on the back with the ILP-BSA conjugate. The injections were repeated at 2 week intervals. Sera containing antibodies were lyophilized and stored at -70°.

Radioimmunoassay

Polypropylene centrifuge tubes (Sarstedt, 1.5 ml) were used for the radioimmunoassay. All samples and solutions were kept on ice. Samples to be assayed were diluted appropriately in assay buffer and 50 µl added to 250 µl of assay buffer in each tube, followed by 100 µl of [^{125}I-Tyr^4]-ILP (15000 cpm),and 100 µl of antiserum diluted 1/10000. Charcoal adsorption of radioactivity was monitored by the inclusion of tubes with no antiserum (blanks). After overnight (16-24 hr) incubation at 4° charcoal (0.5 ml) was added, and after 10 min the tubes centrifuged for 5 min in a Brinkmann 5413 centrifuge. An aliquot of 0.75 ml was removed from each tube and counted for 1 min in a Beckman gamma spectrometer.

Data Analysis

Estimates of 50% effective dose (ED_{50}), slope factor, minimum binding (Bn), and maximum binding (Bo) were provided by nonlinear least squares fitting to the data using a 4 parameter logistic equation. Confidence intervals for the ED_{50} were calculated from the data by nonlinear minimization of the other parameters in the vicinity of the ED_{50} minimum. Comparison of slope factor between standard curves was by simultaneous fitting and F-test. Coefficients of variation (CV) were calculated as 100x(standard deviation/mean). Immunoreactive ILP (IR-ILP) content

of unknowns was interpolated from the standard curve or, calculated by potency ratios from displacement curves. Means are given along with standard deviations.

Solutions

Iodination reagents were: phosphate buffer, 0.5 M $NaPO_4$, pH 7.4; chloramine-T, 3.5 mg/ml in distilled water; β-mercaptoethylamine, 10% in distilled water; and carrier free ^{125}I-NaI, 1 mCi in 1-3 µl. The LH-20 column buffer was 0.5 M pyridine, 1.0 M acetic acid. The ILP-BSA conjugate was prepared for injection by mixing 1.2 ml of the conjugate with 1.2 ml Freunds complete adjuvant and 8 mg *Mycobacterium tuberculosis* protein; the mixture was emulisfied with a Brinkmann Polytron. Antisera were reconstituted in 0.001% sodium azide at a 1/10 dilution (8 mg dry weight/ml); this solution was stable for at least 1 month. The assay buffer, prepared daily, was 50 mM sodium phosphate, pH 7.4, containing 0.25% BSA, 0.005% sodium azide and 1 µg/ml poly-L-lysine. The diluent for the human seminal plasma standard was assay buffer plus 0.2 mg/ml lima bean trypsin inhibitor and 0.1 mM PMSF. The charcoal suspension,prepared daily, was a mixture of 10 mg/ml dextran (Pharmacia T-70), 5 mg/ml BSA, and 10 mg/ml charcoal (Norit-A) in 50 mM sodium phosphate, pH 7.4.

RESULTS AND DISCUSSION

At a final antiserum dilution of 1/50000 38% of the added label was bound, with a blank amounting to only 6% of the total (16% of bound). Synthetic ILP displaced binding completely (to within 2% of blank values). The mean ED_{50} from 10 standard curves was 36±10 fmoles/tube; the slope factor was 1.0±0.1. To estimate the detection limit, we measured the variation of the assay response in the absence of added ILP (Bo) and calculated the 95% confidence limits. The lower confidence limit corresponds to the response expected from the minimum detectable dose. The minimum detectable dose of ILP is 4 fmoles, and of hSP-STD is 5 pl. Intraassay variation was estimated by the CV of 25 replicates of a single dose of ILP equal to the ED_{50}. This determination was made in 3 assays. The mean and standard deviation of the CV was 8.0±1.5. Interassay variation was estimated from the CV of estimates of ILP content in hSP-STD on repeated assay. The hSP-STD was diluted to give 5,10,20,40,80,160, or 320 pl of hSP-STD/ 50 µl and assayed in triplicate in 4 separate assays. The mean and standard deviation of the CV for these dilutions

was 9±3. The assay reproducibility and precision is thus limited to around 10% of the measured value.

Recovery of ILP was measured by estimating the IR-ILP content of hSP-STD and a parallel sample of hSP-STD with a known quantity of ILP added. The IR-ILP content of the hSP-STD was 4.0 mg/ml, and 5.0 mg/ml ILP was added. Percent recovery was calculated as 100x(total IR-ILP - endogenous IR-ILP)/added ILP. The results were analyzed three ways. First, the hSP-STD and the hSP-STD+ILP were fit as standard curves, and the ED_{50} determined. The ILP content was estimated by the ratio of ED_{50} for ILP in fmoles to the ED_{50} of hSP-STD in pl. Second, IR-ILP content and percent recovery was calculated at each of several doses of hSP-STD and the recovery averaged. Finally, linear regression of IR-ILP vs pl hSP-STD gave a slope equal to the IR-ILP concentration. The percent recovery estimated by these methods was 108% (ED_{50}), 99% (individual points), and105% (linear regression). These results indicate that recovery is quantitative to within the precision of the assay.

No displacement of binding was observed with 1 μg of the following peptides and proteins: human serum albumin, human growth hormone, human corticotropin, human insulin, human β-lipotropin, human β-endorphin, ovine prolactin, bovine α-melanocyte stimulating hormone, bovine pancreatic trypsin inhibitor, Met-enkephalin, arginine vasopressin, oxytocin, insulin like growth factor (IGF) I, IGF II, gastrin, bombesin, substance P, growth hormone releasing factor, fibroblast growth factor (partially purified), epidermal growth factor, peptide E, calmodulin, and bovine myelin basic protein. The potency of ILP peptides in displacing [^{125}I-Tyr^{4}]-ILP from antiserum was compared (Figure 2). ILP, [Tyr^{4}]-ILP and ILP-(9-31) were found to be essentially equipotent. The first 8 residues do not contribute to recognition by this antiserum. ILP-(1-16) and ILP-(1-23) are not recognised at all, while ILP-(1-25) is poorly recognized, indicating that residues 24-31 are most important for antiserum recognition.

The level of immunoreactive material in human seminal plasma is quite high. The ED_{50} for samples from 9 individuals ranged from 50-250 pl (Table 1). This is equivalent to a concentration of 0.5-2.5 mg IR-ILP/ml. The IR-ILP content of hSP-STD, a batch of seminal plasma pooled from 20 individuals and treated with protease inhibitors, was 4.0 mg/ml. The high IR-ILP content of hSP and the sensitivity of the RIA combine to yield an assay for the presence of hSP of remarkable sensitivity, with a limit of detection of 5 pl of hSP. Two peptides

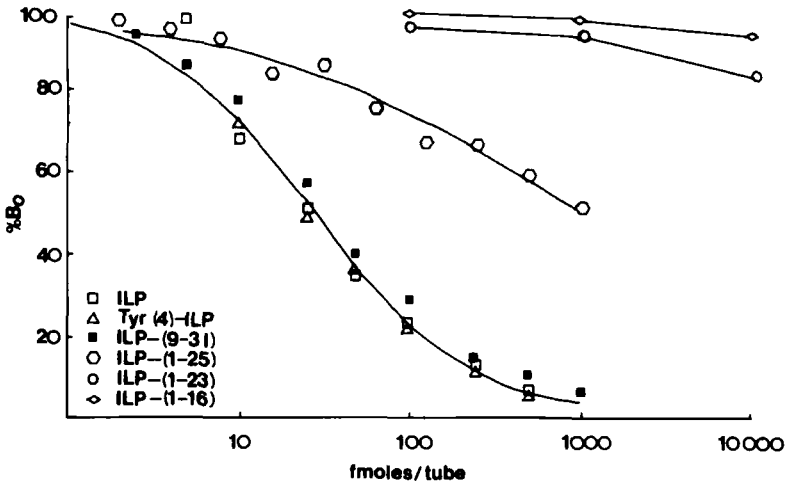

Figure 2: Cross reactivity of ILP peptides in an ILP specific RIA. ILP (□), [Tyr^4]-ILP (△), ILP-(9-31) (■), ILP-(1-25) (○), ILP-(1-23) (○), and ILP-(1-26) (◇) were compared in their ability to displace [^{125}I-Tyr^4]-ILP. By simultaneous fitting a common slope of 0.91 was found for ILP, [Tyr^4]-ILP, and ILP-(9-31), The ED_{50} values in fmoles/tube are: ILP, 26.5; [Tyr^4]-ILP, 28.2; ILP-(9-31), 36.2; ILP-(1-25), 960; ILP-(1-23), >10,000; ILP-(1-16), >10,000. The final dilution of the antiserum was 1/50,000.

Table 1 - Immunoreactive ILP in human seminal plasma.

Sample	ED_{50} pl/tube	IR-ILP[1] mM	mg/ml
1	116	0.3	1.1
2	103	0.35	1.2
3	195	0.18	0.6
4	196	0.18	0.6
5	133	0.27	0.9
6	47	0.77	2.7
7	81	0.44	1.6
8	267	0.13	0.5
9	238	0.15	0.5

[1]Immunoreactive ILP concentration (mM) was estimated as the ratio of the ED_{50} of the synthetic ILP, in fmoles, and the ED_{50} of seminal plasma in pl. The equivalent concentration of ILP was obtained using 3555 as the molecular weight for ILP.

structurally related to ILP have been isolated from human seminal plasma and characterized, based on identification by this RIA (7).

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